

# Colonization of Gnotobiotic Mice with Human Gut Microflora at Birth Protects Against *Escherichia coli* Heat-Labile Enterotoxin-Mediated Abrogation of Oral Tolerance

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## ABSTRACT

Previous work has shown that the indigenous gut microflora in mice plays a protective role against *Escherichia coli* heat-labile enterotoxin (LT)-mediated abrogation of oral tolerance to an unrelated co-ingested protein. To assess potential protection by human gut microflora, we studied the effect of human gut microflora in a murine model. Oral tolerance was studied in adult gnotobiotic mice (*i.e.* ex-germ-free mice) colonized with the entire human fecal microflora and orally administered once with LT and ovalbumin. Systemic suppression of IgG, IgG1, IgG2a, and IgE antibody responses was assessed by ELISA. Both specific IgG subclasses and IgE hyporesponsiveness was induced in LT + ovalbumin-fed gnotobiotic mice, indicating that the human gut microflora can protect against the LT-mediated abrogation of oral tolerance. However, as confirmed with mouse gut microflora, this protective effect only occurs when the gut microflora is associated from birth on. Colonization of germ-free mice with a single bacterial strain, *E. coli*, predominant in the human and mouse gut microflora in the neonatal period, showed that this strain alone did not induce protection. These results

supported the hypothesis that the natural establishment of the gut microflora in neonates crucially influenced resistance to LT-mediated abrogation of oral tolerance by reinforcing suppression of both T helper type 1- and T helper type 2-controlled responses, and suggested that sequential bacterial colonization of the gut rather than a single bacterial species may be involved in this phenomenon. (*Pediatr Res* 54: 739–746, 2003)

## Abbreviations

**Ab**, antibody  
**Ag**, antigen  
**Bic**, bicarbonate  
**CV**, conventional  
**GALT**, gut-associated lymphoid tissue  
**GF**, germ-free  
**LT**, *Escherichia coli* heat-labile enterotoxin  
**OVA**, ovalbumin  
**Th**, T helper

The gut mucosal immune system is exposed to numerous environmental Ag, but it is important that it does not mount immune responses against harmless or potentially beneficial Ag such as food and commensal bacteria. The intestinal immune system has developed the ability to induce a profound systemic immunologic unresponsiveness to orally administered soluble Ag. Such unresponsiveness, termed “oral tolerance,” is believed to be of physiologic significance for food proteins (1, 2). Food-allergic diseases, most common in in-

fancy, may thus represent a breakdown or failure of oral tolerance induction or maintenance.

Among the environmental factors that affect the induction of oral tolerance, bacterial enterotoxins are major ones. It has been reported that in mice, the two related enterotoxins, cholera toxin and LT, responsible for numerous diarrheas in human infants and adults, abrogate oral tolerance induction to a simultaneously ingested protein Ag when estimated using the serum Ag-specific IgG and IgE Ab levels (3–7). This toxin-mediated abrogative effect (or adjuvant effect) may result from a direct effect on Ag-presenting cells and T cells through modulation of surface marker expression, such as B7.2 and/or CD40 ligand, and modulation of Th1- and Th2-type cytokines provided from mucosal and systemic immune compartments, which may affect subsequent development of Th1- or Th2-type

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immune responses (8–11). We observed that CV mice are less sensitive to LT-mediated abrogation of oral tolerance than GF mice. Whereas two oral doses of 10  $\mu\text{g}$  of LT administered simultaneously with 20 mg of OVA are required to abrogate anti-OVA IgG and IgE Ab suppression in CV mice, only one dose is effective in GF mice and may prime mice for subsequent Ab responses, thus suggesting that the indigenous gut microflora plays a protective role (6). Because of the possible consequences of enterotoxin on human hypersensitivity to dietary proteins, we tested whether the human gut microflora could protect against the LT-mediated effect on oral tolerance, without any relationship between infection of human newborns with *E. coli* producing LT and development of food allergies later in life being presently known.

Early life is thought to play a crucial role in the development of dietary hypersensitivities. A lack of exposure to bacteria and infections in childhood has been suggested to be one of the factors involved in the increased occurrence of allergies in children (12–15). In the present study, we investigated the influence of the neonatal period on the development of protection by testing the protective role of human gut microflora when it colonizes the gut either at birth or later, at the adult stage. Moreover, we investigated whether bacteria present in large amounts in the gut microflora in early life could prevent LT-mediated abrogation of oral tolerance. The experiments were performed using gnotobiotic mice, *i.e.* ex-GF mice, colonized with either the whole fecal microflora from a healthy adult human or a single bacterial strain predominant in neonates: *E. coli*. The influence of LT on oral tolerance to co-administered OVA was assessed by serum anti-OVA IgG and IgE Ab responses. To gain insight into the potential mechanisms by which the gut microflora might protect against LT-mediated abrogation of oral tolerance, we examined the Th1/Th2 balance as evaluated on the basis of the serum Th1- and Th2-associated isotypes IgG2a and IgG1, respectively (16).

Our data indicated that the human gut microflora prevented LT-mediated abrogation of oral tolerance only if the gut microflora was present from birth on, and protection may result from suppression of both Th1- and Th2-controlled responses. Moreover, natural sequential bacterial colonization of the gut as of the neonatal period may be more important to this phenomenon than the influence of particular bacterial species predominant in neonates.

## MATERIALS AND METHODS

**Mice.** Animal experiments were carried out with GF C3H/He mice bred at the breeding facility of INRA, in accordance with the regulations of the Animal Care Committee of INRA. Both GF and gnotobiotic mice were bred in a Trexler plastic isolator (La Cahlène, Vélizy, France) and fed *ad libitum* on a sterile OVA-free diet (6). All procedures were carried out in accordance with the European guidelines for the care and use of laboratory animals.

**Bacterial inoculum and infection of mice.** GF mice were colonized with a human fecal microflora, either from birth on (neonatal stage) or at 5 wk of age (adult stage). To obtain mice associated with human microflora at the neonatal stage, parent

GF mice were colonized with human microflora so that their offspring were naturally colonized with this microflora at birth. For colonization at the adult stage, mice were orally inoculated twice at a 24-h interval with 0.5 mL of a  $10^{-2}$  fresh human fecal homogenate prepared and kept for 24 h in an anaerobic glove box (17). Similarly, GF adult mice were colonized with a CV adult mouse fecal microflora.

In parallel, GF mice were monoassociated either at the neonatal or the adult stage with a strain of *E. coli* isolated from CV mouse fecal microflora. The strain of *E. coli* was isolated from a  $10^{-4}$  fecal dilution cultured selectively on a solid desoxycholate agar medium (Difco, BD Biosciences, Le Pont de Claix, France) incubated at 37°C for 2 d under aerobic conditions. Then, adult GF mice were inoculated with 0.5 mL of a 24-h culture of the strain in liquid LB broth (Difco) at 37°C under aerobic conditions. This strain was tested to ensure that it did not produce LT in comparison with the enterotoxigenic *E. coli* strain H10407, kindly provided by Dr. M Levine (University of Maryland, Baltimore, MD, U.S.A.). Briefly, as adult mice colonized with the enterotoxigenic *E. coli* did not develop diarrhea, LT synthesis *in vivo* was tested by assaying anti-LT IgG Ab in serum of gnotobiotic mice colonized with either the enterotoxigenic strain or our *E. coli* strain, by ELISA. Mice colonized with our strain did not develop any anti-LT response, in contrast to others, suggesting that our *E. coli* strain is not enterotoxigenic (data not shown).

**Bacterial analysis.** Characterization of the predominant human microflora (*i.e.*  $\geq 10^8$  bacteria/g of intestinal content) in both human fecal samples and feces of gnotobiotic mice colonized either from birth on or at the adult stage was performed using standard bacterial culture methods and morphologic examination of 100 isolated colonies representative of the cultivable predominant microflora (17–19). Briefly, the colonies were selected at random among colonies cultured 5 d at 37°C under anaerobic conditions on a nonselective pre-reduced solid brain heart infusion medium (BHI; Difco) supplemented with 0.5% yeast extract (Difco) and 5 mg/L of hemin (Sigma Chemical Co., St. Quentin Fallavier, France) (BHI-H), and inoculated with 0.1 mL of the  $10^{-8}$  fecal dilution. For further characterization, the 100 isolated colonies were subcultured on BHI-H Petri dishes. Their subsequent classification into bacterial groups was performed on the basis of cell morphology, motility, spore formation, and catalase production on BHI-H, Gram characteristics, and oxygen sensitivity (17, 18). Bacterial count for each genus identified was obtained by multiplying the total bacterial count on BHI-H by the frequency of each genus among the 100 colonies tested. In addition, in parallel with the initial cultivation on the nonselective BHI-H medium and to potentially enumerate subdominant microflora, some bacterial counts were performed on selective media. Enterobacteria were enumerated on a desoxycholate agar medium (Difco) and facultative anaerobic streptococci on BHI-H supplemented with 30 mg/L of sodium azide (Prolabo, Fontenay-sous-Bois, France), both grown at 37°C for 2 d under aerobic conditions. *Bifidobacterium* sp. and lactobacilli were enumerated on a solid lactobacilli MRS broth medium (pH 5.4, Difco) after incubation at 37°C for 5 d under

anaerobic conditions. Their further differentiation was performed by both morphology and colony examination.

**Induction and assessment of oral tolerance.** To study the LT-mediated effect on oral tolerance induction, gnotobiotic female mice were used at 8 wk of age. Three groups of mice (7–10 mice per group) were fed and immunized as previously described (6). Briefly, mice were fed once on d 0 either with 20 mg of OVA (grade V, Sigma Chemical Co.) (tolerant group), or 10  $\mu$ g of LT + 20 mg of OVA (LT from ICN Biochemicals, Orsay, France) (experimental group) or bicarbonate buffer only (Bic; Prolabo) (control group). Seven and 21 d later, all mice were immunized i.p. with OVA as follows: First with 10  $\mu$ g of OVA absorbed by 1 mg of alum (Pierce, Interchim, Montluçon, France) and then with 50  $\mu$ g of OVA in 4 mg of alum. Mice were bled on d 30 and sera were stored at  $-20^{\circ}\text{C}$  until use.

**Detection of serum antibodies.** Serum anti-OVA Ab responses were assayed by ELISA. The anti-OVA IgG and IgE responses were assayed as previously described (6). Briefly, microtiter plates were coated overnight at  $4^{\circ}\text{C}$  with OVA. Bound anti-OVA Ab were revealed with horseradish peroxidase-conjugated anti-mouse IgG or IgE, respectively (P.A.R.I.S, Compiègne, France). The specificity of the anti-mouse IgE Ab was tested by the reactivity with myeloma IgE proteins, but not with IgG1, IgG2a, IgG2b, IgG3, IgM, IgA, and IgD myeloma proteins in a solid-phase ELISA test (data not shown). Labeled Ab were revealed with *o*-phenylenediamine (Sigma Chemical Co.) or 3,3',5,5'-tetramethylbenzidine peroxidase substrate (Labsystem, Cergy-Pontoise, France), and absorbances were measured at 490 or 450 nm, respectively. IgG concentration in each sample was calculated with reference to purified polyclonal mouse anti-OVA IgG Ab, and anti-OVA IgE titers were expressed as (OD ratio of sample to standard at 1/40th dilution)  $\times$  100.

To detect both IgG2a and IgG1 Abs, the 96-well plates (Immunlon II, Labsystem) were coated overnight at  $4^{\circ}\text{C}$  with 100  $\mu\text{L}$  of a 10 or 5  $\mu\text{g}/\text{mL}$  solution of OVA (ImjectOVA, Pierce) in 0.1 M bicarbonate buffer, respectively. After washes with PBS-0.05% Tween 20 (PBS-Tw; Sigma Chemical Co.), nonspecific binding sites were blocked with 100  $\mu\text{L}/\text{well}$  of PBS-1% BSA (Sigma Chemical Co.) for 1 h at  $37^{\circ}\text{C}$ . The plates were then incubated first with 50  $\mu\text{L}$  aliquots of test sample in PBS-Tw for 2 h at  $37^{\circ}\text{C}$ . Secondly, peroxidase-conjugated rat anti-mouse IgG2a or IgG1 (both from BD PharMingen, San Diego, CA, U.S.A.) both diluted 1:2000 in PBS-Tw were added at 100  $\mu\text{L}/\text{well}$  and incubated for 2 h at  $37^{\circ}\text{C}$ . Finally, 100  $\mu\text{L}$  of 3,3',5,5'-tetramethylbenzidine peroxidase substrate (Labsystem) were added to each well. The reaction was stopped with 50  $\mu\text{L}/\text{well}$  of 4 M  $\text{H}_2\text{SO}_4$  and plates were read at 450 nm using an automatic Multiskan microplate reader (Labsystem).

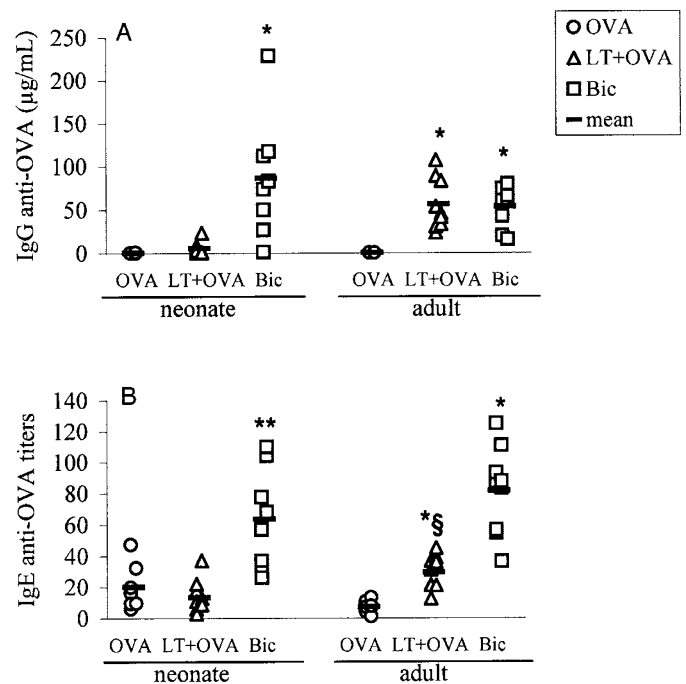
**Statistical analyses.** Data are expressed as individual values or means  $\pm$  SEM, for groups of 7–10 mice. Variances were homogenized by logarithmic transformation and statistical differences were analyzed using the two-tailed *t* test. Differences were considered statistically significant at *p* values of 0.05. Given that five independent experiments were carried out, *i.e.* human flora or *E. coli* at neonatal or adult stage and mouse

flora at adult stage, data were compared only within each experiment. Data are representative of two similar experiments.

## RESULTS

**The protective role of the human gut microflora depends on the stage at which mice are colonized.** To test the potential protective role of the human fecal microflora against LT-mediated abrogation of oral tolerance, induction of oral tolerance was first estimated in (LT + OVA)-fed gnotobiotic mice colonized with human gut microflora at the adult stage (*i.e.* 5 wk of age), by quantification of serum IgG and IgE anti-OVA Ab on d 30, *i.e.* 9 d after the second i.p. immunization with OVA. As shown in Figure 1A, there was no difference in the anti-OVA IgG levels between (LT + OVA)- and control Bic-fed groups, whereas levels for both were significantly higher than those of the tolerant OVA-fed group ( $p < 0.001$ ). When we assessed the anti-OVA IgE response in the (LT + OVA)-fed mice, they exhibited a partial abrogation of the response (Fig. 1B). The anti-OVA IgE titer was significantly higher than that in the OVA-fed mice ( $p < 0.001$ ) and significantly lower than that in the Bic-fed mice ( $p < 0.001$ ). This data thus reflected the LT-mediated abrogation of oral tolerance and suggested that the human gut microflora may not exert any protective effect.

As the protective effect of the mouse gut microflora has previously been observed in CV mice naturally colonized with



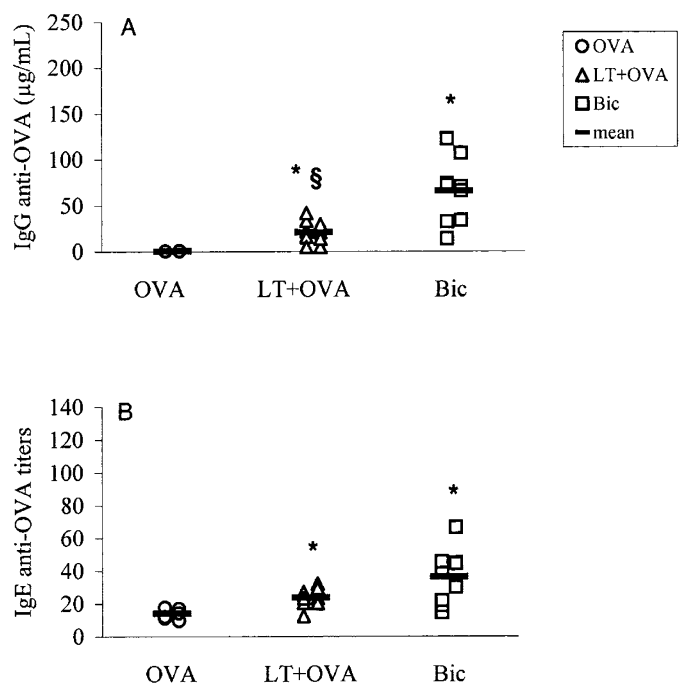
**Figure 1.** Systemic anti-OVA IgG (A) and IgE (B) responses in gnotobiotic mice colonized with human gut microflora at the adult stage (*adult*) or at the neonatal stage (*neonate*). Mice (7–10 per group) were fed once either with OVA, or LT + OVA or bicarbonate (*Bic*) buffer on d 0. Then, they were immunized i.p. with OVA twice, on d 7 and d 21. The anti-OVA IgG Ab levels ( $\mu\text{g}/\text{mL}$  of serum) and the IgE Ab titers were assayed by ELISA in serum collected on d 30. Individual data are represented and horizontal bars indicate the mean for each group. \* $p < 0.001$ , \*\* $p < 0.01$  compared with the respective OVA-fed group, and § $p < 0.001$  compared with the respective Bic-fed group.

gut microflora from birth on (6), we wanted to determine whether the presence of human gut microflora from the neonatal period on could influence its protective effect. Induction of oral tolerance was then assessed in (LT + OVA)-fed mice born of gnotobiotic parents and naturally colonized with human fecal microflora at the neonatal stage. Figure 1, *A* and *B*, shows that both anti-OVA IgG and IgE responses detected in the (LT + OVA)-fed group were significantly lower than those of the control Bic-fed group ( $p < 0.001$  and  $p < 0.01$ , respectively) and did not differ from those of the tolerant OVA-fed group. This reflected the induction of oral tolerance and a protective role of the human gut microflora under these experimental conditions.

In contrast, when the gut microflora from these adult gnotobiotic mice colonized with human gut microflora at the neonatal stage was inoculated to another group of GF adult mice (5 wk of age), no protective effect against the LT-mediated abrogation of oral tolerance to OVA was observed. Both IgG and IgE Ab responses in the (LT + OVA)-fed group were not significantly different from those of the Bic-fed group (data not shown). This data suggested the importance of the role of the gut microflora at the neonatal period rather than a difference in the bacterial composition of the gut microflora in adults for protection against the LT-mediated effect on oral tolerance.

**Mouse gut microflora inoculated in adults does not protect against LT-mediated abrogation of oral tolerance.** To confirm the key role of the establishment of the gut microflora at the neonatal period and to prove that the previous results were not related to the human gut microflora, experiments were performed in gnotobiotic mice colonized at an adult stage with the gut microflora from a CV adult mouse (Fig. 2). In (LT + OVA)-fed mice, both OVA-specific IgG and IgE responses were significantly higher than those of tolerant OVA-fed control mice ( $p < 0.001$ ). The OVA-specific IgG Ab response of (LT + OVA)-fed mice was also significantly lower than that of the Bic-fed control mice ( $p < 0.01$ ), whereas the IgE Ab responses were not significantly different between these two groups. These results indicated that oral tolerance to OVA was abrogated, at least partially, under these conditions and confirmed the data obtained with human gut microflora.

**Similar predominant microflora in feces from adult gnotobiotic mice colonized with human fecal microflora either from birth on or at the adult stage.** Because of the markedly different results between the two gnotobiotic models colonized with human gut microflora either at the neonatal or adult stage (*i.e.* 5 wk of age), we characterized the predominant microflora in feces of 10–12-wk-old gnotobiotic mice of both models. Moreover, to validate the use of gnotobiotic mice in studying the protective role of human gut microflora, we performed similar bacteriological studies on the human donor's fecal sample. As reported in Table 1, no major difference was observed in the predominant fecal microflora between adult gnotobiotic mice colonized with the human microflora either at the adult or at the neonatal stage. Similar levels of the main bacterial genera were observed. Strictly anaerobic bacteria dominated, whereas facultative anaerobes, *i.e.* enterobacteria and streptococci, were subdominant. Hence, these results sup-



**Figure 2.** Systemic anti-OVA IgG (*A*) and IgE (*B*) responses in gnotobiotic mice colonized with mouse gut microflora at the adult stage. Mice were treated and results are expressed as in Figure 1. \* $p < 0.001$  compared with the OVA-fed group and § $p < 0.01$  compared with the Bic-fed group.

ported the hypothesis that the time of bacterial association in the digestive tract is much more important for the protective effect of the human gut microflora against the LT-mediated abrogation of oral tolerance than the bacterial composition of the gut microflora in adults. In contrast, bacterial analyses performed before weaning in 15-d old gnotobiotic mice colonized with the human microflora from birth on showed that bacterial diversity substantially differed from that of adults (Table 1). The predominant microflora was essentially composed of facultative anaerobic enterobacteria (mainly *E. coli*), whereas most strictly anaerobic bacteria were absent or present in smaller numbers than in adults, except for the *Bacteroides* sp. These differences might contribute to the importance of the neonatal period.

Similar total bacterial counts were obtained between human stools and feces from gnotobiotic mice at about  $10^{10}$  bacteria/g of feces. The bacterial composition of the microflora in human samples and feces from adult gnotobiotic mice differed little, especially in enterobacteria and streptococci, which slightly decreased in gnotobiotic mice. Both bifidobacteria and lactobacilli were no longer detected in adult gnotobiotic mice. Nonetheless, the other bacterial groups were present in both samples.

***E. coli* does not protect against LT-mediated abrogation of oral tolerance irrespective of the time of colonization.** To further investigate the importance of the neonatal period, we tested whether a single bacterial species present in large numbers in this period could be involved in protecting against the LT-mediated effect on oral tolerance. As shown in Table 1, *E. coli* predominated in 15-d-old gnotobiotic mice colonized with human gut microflora from birth on. Therefore, we studied the

**Table 1.** Comparison of the microflora in adult human stools and feces from gnotobiotic mice colonized with human fecal microflora either at adult or neonatal stages

Bacterial genera	Human	No. of bacteria/g of feces		
		Gnotobiotic mice Adult stage	Gnotobiotic mice Neonatal stage <sup>a</sup>	
		Adult mice	Adult mice	15-d-old mice
<i>Bacteroides</i> sp. <sup>c</sup>	$1.5 \times 10^{10}$	$1.5 \times 10^{10}$	$1 \times 10^{10}$	$5 \times 10^9$
<i>Eubacterium</i> sp. <sup>c</sup>	$1 \times 10^{10}$	$6 \times 10^9$	$7 \times 10^9$	$1.8 \times 10^8$
<i>Clostridium</i> sp. <sup>c</sup>	$1 \times 10^9$	$3 \times 10^9$	$3 \times 10^9$	$1.8 \times 10^8$
<i>Peptostreptococcus</i> sp. <sup>c</sup>	$1 \times 10^{10}$	$1 \times 10^{10}$	$4 \times 10^9$	$<10^8$
<i>Bifidobacterium</i> sp. <sup>c</sup>	$2 \times 10^9$	$<10^3$	$<10^3$	$<10^3$
Enterobacteria <sup>b,d</sup>	$1.8 \times 10^8$	$4 \times 10^7$	$4 \times 10^6$	$1 \times 10^{10}$
<i>Streptococcus</i> sp. <sup>d</sup>	$2.9 \times 10^8$	$6 \times 10^7$	$3 \times 10^7$	$2 \times 10^9$
<i>Lactobacillus</i> sp. <sup>d</sup>	$1.2 \times 10^7$	$<10^3$	$<10^3$	$<10^3$
Total microflora	$3.8 \times 10^{10}$	$3.4 \times 10^{10}$	$2.4 \times 10^{10}$	$1.7 \times 10^{10}$

<sup>a</sup> Bacterial analyses were performed in both 15-d-old mice and adult mice (i.e., 10–12 wk old).

<sup>b</sup> Mainly *Escherichia coli* as enumerated on desoxycholate agar medium.

<sup>c,d</sup> Strictly anaerobic bacteria and facultative anaerobic bacteria, respectively.

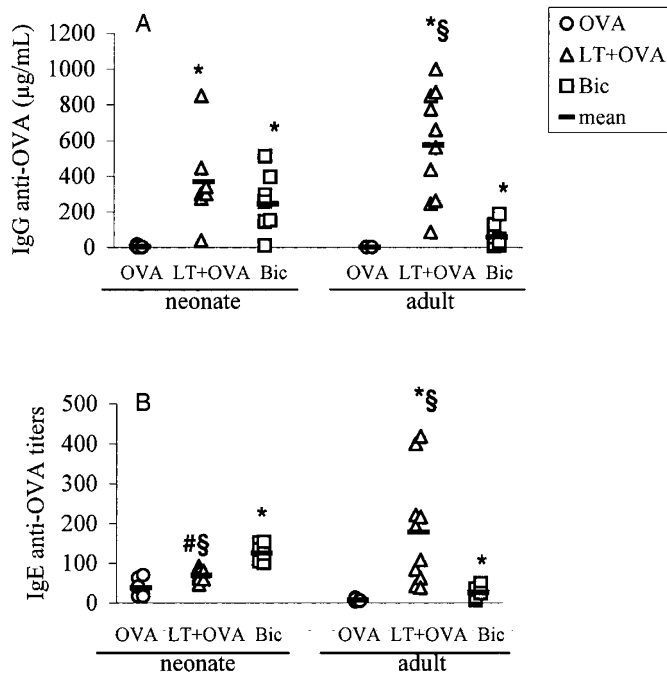
role of *E. coli* and assessed IgG and IgE Ab responses in gnotobiotic mice monoassociated with *E. coli* either at the neonatal or the adult stage. Bacterial counts were similar in the two gnotobiotic models at about  $10^{10}$  bacteria/g of feces. As shown in Figure 3, significantly higher anti-OVA IgG and IgE Ab responses were detected in mice fed with (LT + OVA) compared with tolerant OVA-fed mice ( $p < 0.001$  and  $p < 0.05$ , respectively) irrespective of the stage at which mice were monoassociated with *E. coli* (i.e. neonatal or adult stage). In *E. coli*-monoassociated mice colonized at the adult stage, (LT + OVA)-feeding induced a significantly higher level of both anti-OVA IgG and IgE Ab than Bic-feeding, as the Ab levels

were about 10 and 7 times greater than those of the Bic-fed group ( $p < 0.001$ ), suggesting hyperstimulation of the Ab responses, which was not observed when *E. coli* was associated from birth on. Thus, a single strain of *E. coli* associated from birth on or at the adult stage did not prevent the LT-mediated abrogation of oral tolerance. However, its association from birth on may prevent the hyperstimulation of both IgG and IgE responses observed when it was associated in adults, suggesting a role of *E. coli* in regulation of immune responses in early life.

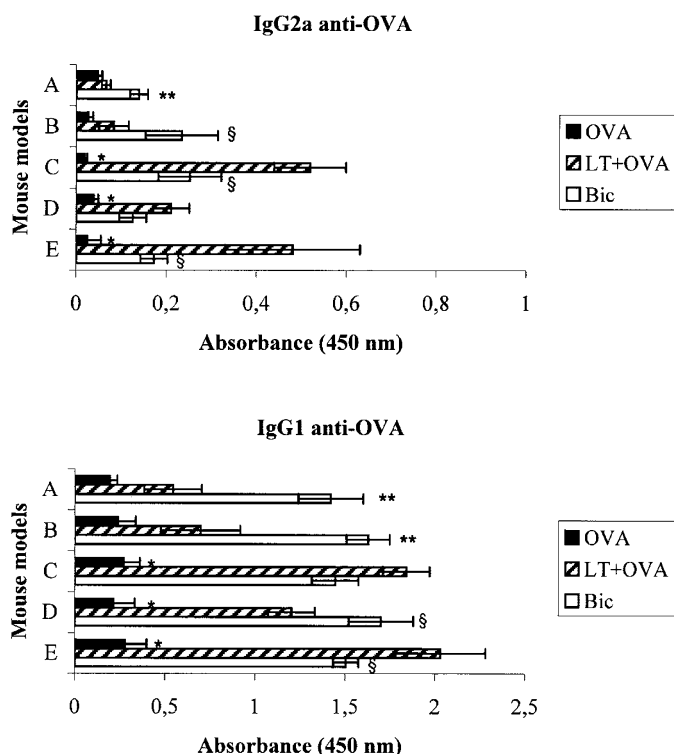
**Protective effect of the gut microflora is associated with hyporesponsiveness of both OVA-specific IgG1 and IgG2a.**

To further investigate the mechanisms of protection of the gut microflora and clarify whether its protective effect influences the Th1/Th2 balance, OVA-specific IgG1 and IgG2a Ab levels were compared between mouse models colonized with the human or mouse gut microflora, either at the neonatal stage (models A and B) or at an adult stage (models C and D). A GF model (model E) was included for comparison.

As shown in Figure 4, in all mouse models a single oral dose of 20 mg of OVA induced significant OVA-specific IgG2a and IgG1 hyporesponsiveness compared with the Bic-fed controls ( $p \leq 0.02$  and  $p < 0.001$ , respectively). Similarly, both anti-OVA IgG2a and IgG1 Ab levels in (LT + OVA)-fed mice colonized at the neonatal stage (models A and B) were significantly reduced compared with those in Bic-fed controls ( $p < 0.05$  and  $p < 0.01$ , respectively), and no different from those in the OVA-fed mice. In contrast, in mice colonized at an adult stage (models C and D), (LT + OVA)-feeding resulted in a significant increase in both the anti-OVA IgG2a and IgG1 Abs compared with OVA-feeding ( $p \leq 0.01$ ). This pattern was also observed in GF mice (model E). Moreover, especially in model C, such an increase was associated with IgG2a and IgG1 Ab levels, which tended to be higher than those in Bic-fed mice, as in GF mice (model E). Thus, a hyporesponsive state of both Th1- and Th2-controlled responses was maintained in presence of the gut microflora only when it was associated from birth on.



**Figure 3.** Systemic anti-OVA IgG (A) and IgE (B) responses in gnotobiotic mice monoassociated with *E. coli* at the neonatal stage (neonate) or at the adult stage (adult). Mice were treated and results are expressed as in Figure 1. \* $p < 0.001$ , # $p < 0.05$  compared with the respective OVA-fed group, and § $p < 0.001$  compared with the respective Bic-fed group.



**Figure 4.** Systemic anti-OVA IgG2a and IgG1 responses 30 d post feeding in CV mice (A), gnotobiotic mice colonized with human gut microflora either at neonatal (B) or adult stage (C), gnotobiotic mice colonized with mouse gut microflora at adult stage (D) and in GF mice (E). OVA-specific IgG2a and IgG1 were assayed by ELISA in serum from mice treated as in Figure 1. Data represent mean absorbances at 1:810 dilution  $\pm$  SEM from 7 to 10 mice per group. \* $p < 0.002$ , \*\* $p \leq 0.01$ , and § $p \leq 0.05$  compared with the (LT + OVA)-fed group.

## DISCUSSION

In the present study, using gnotobiotic mice colonized with human fecal flora, we showed that the human gut microflora can protect from the LT-mediated abrogation of oral tolerance to co-ingested OVA, as the suppression of serum specific IgG, especially IgG1 and IgG2a, and IgE Ab responses can still be observed. However, the protective role was only effective when the gut microflora was naturally associated from birth on, and it did not result from a single bacterial species predominant during the neonatal period.

We observed that the establishment of the gut microflora in early life was crucial for the protective effect against LT-mediated abrogation of oral tolerance to co-ingested OVA, and similar results were observed with human and mouse microflora. Indeed, when the gut microflora was associated in mice at 5 wk of age, its protective effect failed and results were similar to those in GF mice, as if the gut microflora still had no effect. It is known that gut microflora largely affects the full development and functions of the GALT (20), and several studies have indicated that the establishment of the gut microflora in the neonatal period could play a crucial role in determining immune regulation in the immune system (13, 21–25). Sudo *et al.* (21) have reported an age-dependent acquisition of full competence for GALT, inasmuch as the susceptibility of oral tolerance induction, especially the down-regulation of the

IgE Ab response after OVA feeding, was restored in GF mice colonized with *Bifidobacterium infantis* at the neonatal stage, but not at an older age. The concept of establishing, in early life, mechanisms involved in regulating oral tolerance by the gut microflora is also confirmed in the present study, inasmuch as the colonization of the gut by the whole microflora or by a single strain of *E. coli*, either from birth on or at an adult stage, led to considerable differences in susceptibility of the host's immune responses to LT-mediated effect. On the other hand, it has been reported that postnatal maturation of the immune system requires a Th1-polarized immune deviation and the endogenous gut microflora, present after birth, could contribute to this maturation (22–24). We believe that crucial events for the appropriate functioning of GALT occur in the neonatal period, because they cannot occur later, and that the gut microflora is an important environmental parameter for regulatory effects and endogenous stimuli, as also recently shown by Hooper *et al.* (26) through overall changes in intestinal gene expression after colonization. In addition, one cannot exclude that the protective role of the gut microflora against LT-mediated abrogation of oral tolerance might be due to effects on the parent immune system passed on to neonates, especially through maternal Ab, immunosuppressive factors, and/or maternal lymphocytes present in maternal milk, as suggested by the recent review of Kelly and Coutts (27). However, the mechanisms involved and the relationships between gut microflora, passively acquired maternal factors, and neonatal intestinal immunity are still poorly defined.

One of our hypotheses was to test whether some of the bacterial species predominant in the neonatal period are involved in the protective role of the gut microflora and whether a single strain may be sufficient for protection. From a bacteriological point of view, the neonatal period is characterized by the bacterial colonization of the digestive tract that occurs naturally as of birth and follows a sequence up to complex and diversified composition, largely influenced by dietary diversification. Similar complex events occur in humans and mice (28–32). However, early colonization of the gut is limited to very few bacterial species specifically present in large amounts in the digestive tract of mouse and/or human newborns. *E. coli* is one of the predominant genera in the gut of newborn infants and growing CV mice, and its level generally decreases at the adult stage (28, 30, 31, 33). For the first time, our data also showed that *E. coli* predominated in neonate gnotobiotic mice (*i.e.* preweaned 15-d-old mice) colonized with human gut microflora from birth on and decreased afterward. Previous studies in gnotobiotic mice on the role of the gut microflora on the immune system have shown that single bacterial species of intestinal origin can exert immunomodulatory effects and, depending on the immune responses studied, only these bacteria or the whole microflora are necessary to induce an immunomodulatory effect similar to that observed in CV mice (21, 34–36). Hence, it has been reported that strains of *E. coli* can maintain systemic anti-OVA IgG suppression induced after OVA feeding (36) or stimulate pro-inflammatory cytokine production by peritoneal macrophages (35), as does the whole mouse gut microflora. In these studies, bacterial colonization has been performed in adult mice. Moreover, as previously

observed, Sudo *et al.* (21) have shown that a single bacterial strain of *B. infantis* is able to restore the anti-OVA IgE suppression as exerted by the whole microflora. Our results showed that *E. coli* monoassociated in neonate or adult GF mice did not protect oral tolerance from LT-mediated abrogation, suggesting that this bacterial strain cannot provide full protection. Similar results were obtained with a single strain of *Lactobacillus* sp., predominant in newborn mice (data not shown). Therefore, our results in monoassociated mice showed that a single strain does not provide enough stimulation for protection. On the other hand, other studies have shown that a complex microflora is necessary for full stimulation of the intestinal IgA immune system (34, 37). In fact, it has been reported in gnotobiotic mice colonized with gut microflora isolated from 1- to 25-d-old newborn mice that only the colonization with the complex flora from a weaned 25-d-old mouse resulted in an IgA plasmocyte number similar to that obtained in CV mice. These results show that the whole sequence of bacterial colonization in the young can be necessary to fully stimulate development of immune parameters, thus suggesting that a similar situation could explain our failure to reproduce with only one bacterial strain the protective role observed with the whole gut microflora. This hypothesis was also supported by results in gnotobiotic mice colonized with the human gut microflora from birth on, inasmuch as the number of bifidobacteria and lactobacilli dropped dramatically in these gnotobiotic mice but did not impair the potential protective effect of the human gut microflora. This suggests that neither of these two genera are involved in the protection, although predominant in the gut of human infants and newborn mice, respectively. On the contrary, the absence of protection in gnotobiotic mice colonized at the adult stage with the whole adult fecal microflora might have been related to an anarchical and drastic bacterial colonization of the gut. However, we can discard this hypothesis as the bacterial profiles were similar between adult gnotobiotic mice, *i.e.* 10–12-wk-old mice, colonized with the human microflora from birth on or at 5 wk of age. Moreover, the composition of the gut microflora is stable at this particular stage of life, because it has been reported that the bacterial equilibrium of the gut microflora seems to be stabilized at around 6–7 wk of age in both CV and gnotobiotic mice (33). Similar results were obtained with human and mouse gut microflora, *i.e.* without heterogeneity between host and gut microflora origin, which strengthened our opinion that the natural sequential establishment of the gut microflora as of the neonatal period was more important for protection than the influence of specific bacterial species. This also underlined that gnotobiotic mouse models, which are good recipients of bacterial strains from heterologous species, especially human species (38), are appropriate models for our *in vivo* studies.

LT is described as a potent mucosal immunogen and adjuvant to orally co-administered Ag (as it abrogates oral tolerance to this specific Ag), which induces a mix of Th1/Th2 responses (4, 6–9, 11). As shown by both IgG1 and IgG2a responses, our results in the (LT + OVA)-fed gnotobiotic mice associated with gut microflora at an adult stage confirmed this dual Th1/Th2 response. It is interesting to note that this response was similar to that observed in GF mice, thus show-

ing that the gut microflora, especially the human flora, was unable to stimulate any protective effect against LT when associated after the postnatal period. In addition, the partial suppression of the anti-OVA IgE response in these mice might have resulted from a Th2-skewed immune deviation in adults, as recently described in absence of the gut microflora (24, 25). To some degree, this hypothesis may also be supported by the IgE responses in *E. coli* monoassociated mice, as no hyperstimulation of the Ab response was observed when the bacterial strain was associated from birth on. In mice colonized with the gut microflora from birth on, in contrast to others, the protective effect of the gut microflora was associated with the maintained suppression of IgG2a, IgG1, and IgE Ab responses. This implies that the influence of the sequential establishment of the gut microflora in early life induces a regulation of both Th1- and Th2-controlled immune responses, which leads to protection later in life. However, the molecular mechanisms involved and the relationships between gut microflora and T cell subpopulations need to be further investigated. On the other hand, the IgG isotype-responses in mice fed with a single high dose of 20 mg of OVA are consistent with studies indicating that both Th1 and Th2 subsets are equally susceptible to the induction of oral tolerance (21, 39, 40). Our data also supported the hypothesis that the gut microflora did not directly influence induction of Th1- and Th2-dependent tolerance as Ag-specific IgG2a and IgG1 hyporesponsiveness was observed in all the mice fed OVA, whatever the mouse models tested, *i.e.* CV, gnotobiotic, and especially GF.

IgE Ab have been described to play a well-defined role in numerous human food-hypersensitivity symptoms, especially in children (41), and oral tolerance is believed to play a physiologic role in the suppression of IgE Ab to dietary proteins (1, 2). The observed divergence between IgG and IgE abrogation in (LT + OVA)-fed gnotobiotic mice suggested that the IgE response was more tightly regulated and less easily abrogated than the IgG response, thus confirming its marked susceptibility to induction of oral tolerance and its different regulation from that of IgG (42, 43). In contrast, it might have been related to intrinsic properties of LT. Although LT induces both Th1- and Th2-type responses when used as a mucosal adjuvant, it did not induce high Ag-specific IgE response (8). Nonetheless, our data with the IgE response support accumulating evidence that early life and the presence of endogenous gut microflora in this period may reduce occurrence of food allergies (13–15, 24).

It has been recently recognized that multiple nonexclusive T cell-mediated mechanisms are involved in oral tolerance (44) and the gut microflora is an important factor in modulating oral tolerance (20). As previously observed, it can regulate mechanisms of induction of oral tolerance, and the present data supported that at least some of these regulatory events must be induced in the neonatal period. In contrast, some regulatory effects influence long-term maintenance of oral tolerance (6, 36). These differences indicate that the interactions between gut microflora and mechanisms involved in the induction and maintenance of oral tolerance are very complex and not yet fully understood.

## CONCLUSION

In conclusion, this work provides evidence that the natural establishment of the gut microflora in the neonatal period crucially influences the normal and life-long development of intestinal immune functions. These data suggest that maintaining both the spectrum and sequence of implantation of gut microflora is important to the avoidance of food allergy.

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