have recently shown that the inhibitory mechanism of another cross-class serpin, SCCA1, is unique among the serpin superfamily in that SCCA1 performs its inhibitory activity in two ways: contributing the suicide substrate-like mechanism without formation of a covalent complex and causing irreversible impairment of the catalytic activity of papain (32). The biochemical events occurring in SCCA2 and Der p 1 described above indicate that this mechanism would be common among cross-class serpins. It is speculated that the thiol-ester bond between a cross-class serpin and its target cysteine proteinase was unstable or that the distorted ester bond located not so far from catalytic partners, enabling the ester bond to hydrolyze. Concomitantly, the interaction may induce conformational change of the target proteinase, which irreversibly loses its catalytic activity. The reports that other cross-class serpins, CrmA and PI9, do not form SDS-resistant complexes with caspase proteins, although they do so with a serine proteinase, granzyme B (8–11), may indicate the same properties.

We also found that SCCA2 was resistant to cleavage by Der p 1 when compared with SCCA1 (Fig. 5). This is a unique property of SCCA2 contributing to the inhibition mechanism against Der p 1, different from the interaction of SCCA1 with papain. The analyses of substitution of each amino acid suggested that Leu-354 at SCCA2 would be critical for resistance against Der p 1 because SCCA2 mut4, in which Leu-354 was replaced with Ser, was susceptible to cleavage by Der p 1, diminishing the inhibitory activity against Der p 1 (Table II and data not shown). It is assumed that Leu-354 would block the nucleophilic reaction of Cys-34 at Der p 1 toward the P1 residue (Glu-353), based on the homology modeling of the interaction between SCCA2 and Der p 1.[2] In addition to the comparison between SCCA1 and SCCA2, by substituting each amino acid in the RSL of SCCA2, we unexpectedly succeeded in generating a very potent inhibitor (SCCA2 tm) when compared with native SCCA2 (Fig. 6). The analyses of amino acid replacement suggested that removal of ionic strength in Glu-353 would stabilize the interaction of the RSL and the cleft of Der p 1, leaving the SCCA molecule more resistant to the cleavage by Der p 1 (Table II and Fig. 5). Analysis of the comparison between SCCA2 and SCCA2 tm also supported the notion that enhancement of the resistance against cleavage by Der p 1 would lead to enhancement of the inhibitory activity of the SCCA2 molecule.

Although the precise role of the catalytic activity of Der p 1 in the pathogenesis of allergic diseases remains unclear, it is hoped that an inhibitor against its catalytic activity has the potential to be developed into a therapeutic reagent for allergic diseases arising from mite allergens. Actually, it has been reported that a peptide-based inhibitor, PTL11028, showed inhibitory effects for the catalytic activities of group I mite allergens and improved airway hyperreactivity, inflammation, and systemic sensitization induced by Der p 1 in rats (40, 41). Structural analyses of the interaction between SCCA/Der p 1 would give us a hint as to how to develop a novel low molecular weight compound to block the catalytic activity of group I mite allergens.

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Dietary Nucleic Acid and Intestinal Microbiota Synergistically Promote a Shift in the Th1/Th2 Balance toward Th1-Skewed Immunity

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Key Words
CpG motif · Food allergy · Germ free mice · Microbiota · Nucleic acids

Abstract
Background: Intestinal microbiota are known to play an important role in the establishment of oral tolerance, thereby protecting the organism from food allergies. Dietary intake of nucleic acid (NA) is also reported to have such an anti-allergic effect; however, one unsolved question is whether or not dietary NA would act through a process of toll-like receptor 9 signaling activated by DNA containing a CpG motif, a well-known sequence leading to immunostimulatory activity. In this study, we focused on the question of whether the addition of dietary NA lacking CpG motifs would allow continued modulation of the Th1/Th2 balance. ***Methods:*** Germ free (GF) and *Bifidobacterium-infantis*-monoassociated BALB/c mice were maintained on either an NA-free casein diet or on an NA-supplemented casein diet for 4 weeks. Thereafter, both the in vivo anti-casein antibody levels and in vitro splenocyte cytokine secretion pattern were evaluated. ***Results:*** Feeding with a casein diet elicited a substantial increase in the serum anti-casein-specific IgG1, IgG2a, and IgE levels of GF mice fed the NA free-diet. The in vitro cytokine production profile showed that enhanced IL-4 production in the GF mice fed the NA free-diet was markedly reduced by the supplementation with dietary NA in both the GF and *B.-infantis*-monoassociated mice. In addition, IFN-γ secretion increased in the *B.-infantis*-reconstituted mice fed the diet containing NA. ***Conclusions:*** These results suggest that dietary intake of NA devoid of CpG motifs may prevent the development of allergies via acceleration of Th1-dominant immunity.

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Introduction

Food allergy is the manifestation of an abnormal immune response to a food antigen delivered by the oral route. In order to avoid mounting such a response against harmless or potentially beneficial materials, gut mucosa is generally guarded by the following strong physical barriers: the presence of luminal enzymes that alter the nature of the antigen itself; the phenomenon of systemic unresponsiveness following oral presentation of the antigen, termed 'oral tolerance'; and the production of an

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antibody, secretory immunoglobulin A, that is highly suited to the hostile environment of the gut [1]. Together they protect against the development of allergic sensitization.

A number of factors are known to participate in the maturation of the mucosal immune system. As demonstrated by our previous work with germ free (GF) animals [2], intestinal microbiota, which colonize rapidly after birth, play a principal role in the postnatal maturation of the mammalian immune system. In addition, an optimal mucosal barrier function in the neonatal period is accomplished by an adequate supply of breast milk. In fact, breast milk contains a wide variety of immunomodulating and anti-inflammatory components, such as cytokines and prostaglandins [3]. One such breast-milk-derived factor, nucleic acid (NA), has also been shown to have the ability to promote a shift in the Th1/Th2 balance toward Th1-dominant immunity [4, 5]. In this study, considering recent topics regarding immunostimulatory DNA containing CpG motifs [6], we examined whether or not the addition of dietary NA lacking CpG motifs would still modulate the flora-induced maturation of the mucosal immune system.

Materials and Methods

Mice

GF BALB/c mice were originally obtained from Japan Clea (Tokyo, Japan) and were then bred in our facility. These GF mice were maintained in Trexler-type flexible-film plastic isolators with sterile food and water until just before death, as described previously [2]. To obtain *Bifidobacterium-infantis*-associated mice at the neonate stage, the parent GF mice were orally administered *B. infantis*, and then their offspring, which thus became infected with this bacterium at the neonate stage, were used in this study. Bacteriological examination of fecal samples revealed that *Bifidobacterium* colonization was completely accomplished by 3 weeks after birth. All experiments were approved by the Ethics Committee for Animal Experiments of the Kyushu University.

Study Protocol

GF and *B.-infantis*-monoassociated mice at 5 weeks of age were maintained on either an NA-free diet (casein 24.5%, cornstarch 45.5%, granulated sugar 10.0%, corn oil 6.0%, crystal cellulose 3.0%, cellulose powder 2.0%, α-starch 1.0%, vitamin mixture 1.0% and mineral mixture 7.0%) or an NA-supplemented diet (casein 24.5%, cornstarch 44.3%, granulated sugar 10.0%, corn oil 6.0%, crystal cellulose 3.0%, cellulose powder 2.0%, α-starch 1.0%, vitamin mixture 1.0%, mineral mixture 7.0% and purified salmon milt DNA 1.2%). On the 4th week of the diet, these mice were killed by cervical dislocation. Blood samples were obtained by cardiac puncture, and the sera were kept at -80°C until the measurement of serum antibody levels. Spleen was also removed for analysis of in vitro cytokine production.

Determination of Serum Antibody Level and Cytokine Measurement

The serum levels of anti-casein IgG1, IgG2a, and IgE were determined by an ELISA technique as described elsewhere [7]. Briefly, serum samples with the serial dilutions were placed on ELISA plates coated previously with casein. This was followed by the addition of goat anti-mouse IgG1, IgG2a, or IgE conjugated with peroxidase (Caltag Laboratories, Burlingame, Calif., USA). Thereafter, the plates were developed using a substrate solution containing 0.04% *o*-phenylenediamine dissolved in phosphate citrate buffer (pH 5.0).

Cytokine production by spleen cells in vitro was also quantified using a sandwich ELISA technique as previously described [4, 8].

Statistical Analysis

Statistical differences between the groups were determined by the post hoc Dunnett's test after factorial analysis of variance.

Results and Discussion

As summarized in table 1, the serum anti-casein-specific IgG1 level of the GF mice fed the NA-free diet was substantially higher than that of those fed the NA-supplemented diet. Similarly, the casein diet also induced a small but significant elevation in serum anti-casein-specific IgG2a and IgE levels in the GF mice fed the NA-free diet, indicating that oral tolerance to casein was not fully induced in the absence of dietary NA intake and gut microbes. No such apparent elevation in the serum antibody level was found in the *B.-infantis*-associated mice, regardless of whether or not the mice were supplemented with NA. However, the IgG1/IgG2a ratio, which was used as a marker of the Th1/Th2 balance in this study, was decreased by the addition of NA in both the GF and *B.-infantis*-monoassociated animals. These results were further supported by the in vitro cytokine production profile of the spleen cells in which the addition of casein to the cultures led to the detection of IL-4 at an appreciable level in the GF mice fed the NA-free diet. IFN-γ was not detected in this group (table 2). Supplementation with dietary NA markedly reduced in vitro IL-4 production in both groups of mice, while elevating IFN-γ production only in the mice monoassociated with *B. infantis*.

Recently, accumulating evidence has shown bacterial DNA acting as a strong inducer of Th1-skewing immunity. Immunostimulatory DNA sequences are unmethylated CpG dinucleotides within consensus sequences, such as CpG motifs. CpG motifs are common in certain bacterial and viral genomes and are underrepresented in mammalian genomes [6]. The possible involvement of CpG motifs in the present data, in which dietary NA accelerated Th1-skewing immunity in concert with intestinal

Table 1. The effect of dietary NA on anti-casein-specific antibody titers

Study groups		Casein-specific antibody titer, EU			IgG1/IgG2a ratio
		IgG1	IgG2a	IgE	
GF	NA (–)	106±12.8	44.8±6.1	20.5±3.8	2.5±0.2
	NA (+)	29.9±8.5***	20.3±3.9**	3.5±0.9***	1.7±0.3*
BI	NA (–)	13.2±1.8	15.5±3.4	3.2±1.2	1.0±0.1
	NA (+)	9.1±1.3	19.1±3.7	2.9±0.8	0.6±0.1*

GF and *B.-infantia* (BI)-monoassociated mice were fed the casein diet with or without dietary NA. The casein-specific antibody titer of the sample was calculated by comparing it with the internal standard. Standard serum was obtained by intraperitoneal sensitization of mice with casein plus aluminum hydroxide. The concentration of anti-casein IgG1, IgG2a, and IgE in the standard serum was arbitrarily assigned at 100 ELISA units (EU). All data are expressed as means ± SE (n=10/group). * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$, vs. the corresponding value of the NA (–) group.

Table 2. In vitro IL-4 and IFN-γ production by spleen cells

Study groups		IL-4, pg/ml	IFN-γ, pg/ml
GF	NA (–)	587±118	ND
	NA (+)	152±30.7**	ND
BI	NA (–)	115±23.8	ND
	NA (+)	45±10.4*	99.3±14.2

For a cytokine production assay, single cell suspensions from the spleen were suspended at a final concentration of 2.5 × 10^6/ml and cultured in 1-ml aliquots in 24-well plates in a medium with or without 100 μg/ml casein. Supernatants were collected either after 48-hour culture for IFN-γ or 72 h for IL-4, since at these times each cytokine production exhibited its peak level. Data shown are calculated by subtracting the value without casein from the value with casein, and are expressed as means ± SE (n = 5 per group). * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$, vs. the corresponding value of the NA (–) group. ND = Not detected.

microbes, seems unlikely because the NA used in this experiment was derived from purified salmon milt DNA, which is reportedly unable to mount a CpG-motif-related biological event [9] owing to the absence of the CpG sequence. On the other hand, as demonstrated in a more recent paper by Rachmilewitz et al. [10], toll-like receptor 9 signaling was essential for exerting the anti-inflammatory effects of probiotics in murine experimental colitis; hence, the Th1-promoting effect of *B. infantis*, with a genome that actually contains CpG motifs [11], may be mediated by its own DNA rather than by its ability to colonize the gut.

How can such dietary NA lacking CpG motifs exert its effect on the gut? In general, dietary NA is generally degraded by various types of enzymes such as ribonucleases and deoxyribonucleases, and is subsequently absorbed mainly in the form of nucleosides, which thus suggests a possible mechanism by which the effect of dietary NA may be exerted at the mucosal barrier, specifically through a sensing pathway that involves purinergic signaling. Indeed, purinergic receptors have recently been found in the membrane of virtually all gastrointestinal epithelia, and moreover, extracellular adenosine-5′-triphosphate and adenosine have established roles as potent stimulators of fluid and electrolyte secretion in colon, gallbladder, pancreatic duct, and bile duct models [12]. A new project in our laboratory is now in progress that applies a DNA microarray technique to the GF animal model to further clarify the precise molecular mechanism whereby adenosine modulates the epithelial barrier function of the gut.

Although almost all the common allergic diseases have a hereditary component, quantitative differences in environmental factors may also influence the development of IgE antibodies and the resultant allergic diseases [13]. Many studies have focused on the influence of dietary manipulation on the occurrence of allergies. Some of these studies have suggested that prolonged exclusive breast-feeding of high-risk infants may provide both short- and long-term benefits in reducing the incidence of atopic disorders in infancy and later in childhood [14], although such an allergy-preventing effect of breast-feeding remains controversial. Because cow's milk contains lower concentrations of some NAs than human milk and

certainly has a different profile of NA [15], the NA level and profile in breast milk may be involved in the anti-allergic effect of breast milk.

Most human diets are rich in NA; however, the few exceptions to this would include patients receiving NA-free total parenteral or enteral nutrition and infants receiving solely NA-free formulas. Together with the present data in which antibody response to casein was not fully tolerated in the absence of both dietary NA intake and gut microbes, these findings thus indicate that unfavorable intestinal flora, which are often found after antibiotic treatment, could enhance an aberrant sensitization to food antigens in infants fed formula devoid of NA.

In summary, dietary NA, in concert with intestinal microbiota, may act as an environmental factor against the development of allergic sensitization and Th2-skewed cytokine responses. Nonetheless, whether or not the effect of dietary NA is via a mucosal barrier function remains to be clarified. Clearly, further studies focusing on the cellular pathway are still needed to elucidate how dietary NA, particularly that lacking CpG motifs, interacts with the gut immune system. Such studies may extend existing options to effectively prevent high-risk infants from developing allergic diseases.

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INTERNATIONAL ARTICLE

Allergic Symptoms and Microflora in Schoolchildren

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We studied 867 junior high school children and administered a questionnaire documenting allergic symptoms and environmental variables, and measured Immunoglobulin E serum levels and the immunoglobulin G titers of serum antibody to microflora. A total of 716 subjects were ultimately used for statistics; those with at least two of the following allergic symptoms: asthma, rhinitis, eczema, or food allergy, showed significantly higher IgG titers to *Bactroides vulgatus* than other groups. This finding suggests that a species of the *Bacteroides* genus of the intestinal microflora tends to affect the gut issues, but further studies are needed to clarify this. © *Society for Adolescent Medicine, 2004*

KEY WORDS:
Allergy
Schoolchildren
Microflora

The prevalence of allergic diseases has been increasing in developed countries. However, many studies have demonstrated that the prevalence of these diseases is lower in the formerly socialist countries of Central and Eastern Europe than in Western European counties [1]. One factor associated with the rise of allergic diseases may be the decline of many infectious diseases in developed countries as the result of improved living standards and vaccinations [2]. In modern societies, it has been noted that allergies may be "mapped" according to gradients dictated by hygiene and by the individual degree of genetic predisposition to allergy [3]. These concepts are known as the "Hygiene hypothesis" [3–5]. It has been suggested that a reduced microbial stimulation during infancy and early childhood would result in slower postnatal maturation of the immune system and development of an optimal balance between Th1- and Th2-like immunities [6].

To clarify what and when bacterial species of commensal intestinal microbes are recognized as antigens that induce serum antibody responses in allergy patients, we studied the serum antibody responses to microflora in atopic school-aged children.

Methods

From a population of approximately 1000 12–13-year-old schoolchildren attending the 18 junior high schools in the country in 1995, we did a retrospective study of records of tuberculin responses in 867 children. They had been immunized with bacillus Calmette-Guerin (BCG) at 6 and 12 years of age and they showed negative tuberculin responses. Shirakawa et al showed the strong inverse association between delayed hypersensitivity to tuberculin and atopy [7]. The Institutional Review Board of RIKEN approved this study and 787 of all recruited subjects gave informed consent. We administered a questionnaire documenting atopic symptoms; the

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Table 1. The Relationship Between Allergy Symptoms and IgG Titers of Serum Antibody Microflora in School Children (Mean ± SE)

Microflora or IgE	Nonallergy (n = 433)	One allergy symptom (n = 200)	2 or more allergy symptoms (n = 83)	F	p
E. coli	3.04 ± 0.01	3.07 ± 0.02	3.04 ± 0.03	0.62	.54
B. vulgatus	2.94 ± 0.01	2.91 ± 0.01	2.97 ± 0.02	3.00	.05
E. faecalis	2.99 ± 0.01	2.99 ± 0.02	3.03 ± 0.03	1.11	.33
B. longum	3.02 ± 0.01	3.01 ± 0.02	3.06 ± 0.02	1.38	.25
IgE	2.19 ± 0.03	2.25 ± 0.04	2.41 ± 0.08	4.23	.02

All were analyzed by one-way analysis of variance. Each gut flora or IgE values were converted to $\log_{10}$ because they didn't show normal distribution. SE = means standard error; *E. coli* = *Escherichia coli*; *B. vulgatus* = *Bacteroides vulgatus*; *E. faecalis* = *Enterococcus faecalis*; *B. longum* = *Bfidobacterium longum*; IgE = Immunoglobulin E.

questions were: "Do you have asthma?," "Do you have rhinitis?," "Do you have eczema?," "Do you have food allergy?" Also included were environmental variables, family allergy history, tuberculin response, etc., and we also measured immunoglobulin E (IgE) serum levels (measured by Mitsubishi BCL, Inc., Kyoto, Japan) and the immunoglobulin G (IgG) titers of serum antibody to *Escherichia coli* (*E. coli*), *Bacteroides vulgatus* (*B. vulgatus*), *Enterococcus faecalis* (*E. faecalis*), and *Bifidobacterium longun* (*B. longun*). This assay was described in detail in a previous article [8]. The titers of IgG class antibodies reacting with bacteria in serum were measured by enzyme-linked immunosorbent assay, and the absorbance was read at 495 nm using a plate reader.

We avoided subjects who did not complete questions about atopic symptoms and whose serum sample could not be measured for technical reasons. Ultimately we used 716 subjects for statistics. The values of the IgG titers of serum antibody to microflora and the level of IgE were converted to $\log_{10}$. Differences in IgG titers to *E. coli, B. vulgatus, E. faecalis*, and *B. longun* between nonallergic schoolchildren with no allergic symptoms, and symptomatic schoolchildren with more than one allergic symptom among asthma, rhinitis, eczema, and food allergy, were analyzed by two-tailed Student's t-test. We also tested the relation in IgG titers by each symptom; asthma, rhinitis, eczema, and food allergy by two-tailed Student's t-test. Then we divided subjects into three groups; "nonallergy," who have no allergy symptoms; "one allergy," who have one allergic symptom among asthma, rhinitis, eczema, and food allergy; and "two or more allergy," who have at least two allergic symptoms among asthma, rhinitis, eczema, and food allergy. We examined the differences in IgG titers to *E. coli, B. vulgatus, E. faecalis*, and *B. longun* among the three groups by one-way analysis of variance. The statistical analysis for this study was done using SPSS 10.0J for Win.

Results

IgG titers to *E. coli, B. vulgatus, E. faecalis*, and *B. longun* showed no significant difference between nonallergic schoolchildren (n = 433) with no allergic symptoms, and symptomatic schoolchildren (n = 283) who have more than one allergic symptom among asthma, rhinitis, eczema, and food allergy. We also tested the relation by each symptom; asthma, rhinitis, eczema, and food allergy; there were, however, no differences between nonallergic children and children with one or more allergic symptoms. The subjects were divided into three groups; "nonallergy" (n = 433), who have no allergy symptoms; "one allergy" (n = 200), who have one allergic symptom among asthma, rhinitis, eczema, and food allergy; and "two or more allergy" (n = 83) who have at least two allergic symptoms among asthma, rhinitis, eczema, and food allergy. In the "two or more allergy" group, IgG titers to *B. vulgatus* were significantly higher than in the other two groups (Table 1) (F = 3.00 df = 2, p = .05). IgG titers to *E. coli, B. vulgatus*, and *E. faecalis* showed no significant difference among three groups. Also in the "two or more allergy" group, subjects showed the highest mean IgE levels (2.41 ± 0.08 vs. 2.19 ± 0.03, 2.25 ± 0.04; F = 4.23 df = 2, p = .02) among three groups (Table 1).

Discussion

The children with two or more of the allergic symptoms showed higher IgG titers to *B. vulgatus* than the groups of nonallergic or one-allergic-symptom schoolchildren. This finding suggests that a species of the *Bacteroides* genus of the intestinal microflora might be an organism that affects the gut issues and thus, induces inflammation accompanied by an elevation of serum antibodies to this bacterium [8], although the exact mechanism of this remains un-

clear. Some studies reported that there was a difference in the composition of intestinal microflora between allergic and nonallergic infants. They compared 1-year-old infants living in two countries with a low (Estonia) and a high (Sweden) prevalence of atopy, and found that *Lactobacilli* and *Eubacteria* were more frequently found in the intestinal microflora of Estonian infants, whereas *Clostridia* was more prevalent in Swedish infants [9]; 2-year-old allergic children were colonized less often by *Lactobacilli*, and harbored higher counts of aerobic bacteria (coliforms, *Staphylococcus aureus*) than nonallergic children [10]. These subjects were, however, all infants, and the number of samples was small. Kirjavainen et al recently showed that, in 4.2–10.5-year-old children, serum total IgE concentration correlated with bacteroides in heightened risk of subjects with atopic disorders, and those subjects also showed a greater number of lactobacilli/enterococci than those in the low-risk group [11]. Our data is partly consistent with this.

This is the first study to clarify the relationship between atopic symptoms and bacterial species of predominant commensal intestinal microflora in a large group of junior high school children. Further studies are needed to clarify when bacterial species of commensal microbes in the gut are recognized as the antigens that induce serum antibody responses in allergic subjects.

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Postnatal microbial colonization programs the hypothalamic–pituitary–adrenal system for stress response in mice

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Indigenous microbiota have several beneficial effects on host physiological functions; however, little is known about whether or not postnatal microbial colonization can affect the development of brain plasticity and a subsequent physiological system response. To test the idea that such microbes may affect the development of neural systems that govern the endocrine response to stress, we investigated hypothalamic–pituitary–adrenal (HPA) reaction to stress by comparing germfree (GF), specific pathogen free (SPF) and gnotobiotic mice. Plasma ACTH and corticosterone elevation in response to restraint stress was substantially higher in GF mice than in SPF mice, but not in response to stimulation with ether. Moreover, GF mice also exhibited reduced brain-derived neurotrophic factor expression levels in the cortex and hippocampus relative to SPF mice. The exaggerated HPA stress response by GF mice was reversed by reconstitution with *Bifidobacterium infantis*. In contrast, monoassociation with enteropathogenic *Escherichia coli*, but not with its mutant strain devoid of the translocated intimin receptor gene, enhanced the response to stress. Importantly, the enhanced HPA response of GF mice was partly corrected by reconstitution with SPF faeces at an early stage, but not by any reconstitution exerted at a later stage, which therefore indicates that exposure to microbes at an early developmental stage is required for the HPA system to become fully susceptible to inhibitory neural regulation. These results suggest that commensal microbiota can affect the postnatal development of the HPA stress response in mice.

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Early postnatal life represents a period of bacterial colonization, a time when a previously sterile milieu is inhabited by microorganisms that are likely to remain as residents throughout the life of the animal. The human intestine is more densely populated with microbes than any other organ, and 10^{14} bacteria inhabit the gastro-intestinal tract of adult humans, which exceeds the number of eukaryotic cells (10^{13}) of which the human body is constituted (Tannock, 1999; Borrielo, 2002). Therefore, it seems natural that such colonizing bacteria would play a principal role in the postnatal maturation of the mammalian immune system (Sudo *et al.* 1997; Hooper & Gordon, 2001). In addition, these bacteria aid in the digestion and absorption of macromolecules and act as a barrier to gut pathogens by blocking attachment to gut binding sites, which is the first step of bacterial pathogenicity (Finlay & Falkow, 1990). Thus, there is no doubt that most of our bacterial symbionts have several beneficial effects on host physiological functions; however, little is known about whether or not such microbes can affect the development of brain plasticity and a subsequent physiological system response.

The hypothalamic–pituitary–adrenal (HPA) axis is a neuroendocrine system that is subjected to programming by early life events. For example, as adults, neonatally handled animals exhibit dampened HPA responses to stress compared with non-handled animals (Meaney *et al.*

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1988). In contrast, adult animals exposed to repeated periods of prolonged maternal deprivation as neonates display increased HPA response to stress (Schmidt *et al.* 2002). These effects persist throughout the life of the animal and the resulting differences in HPA activity are associated with the incidence of age-related neuropathology (Meaney *et al.* 1988). Because of the close, bidirectional communication between the neural and immune systems (Turnbull & Rivier, 1999) early in life, a time when the central nervous system (CNS) is especially susceptible to environmental influences, we speculated that such microbial colonization and subsequent immune reaction during early life might alter the development of HPA responsiveness.

To test this hypothesis, we investigated the HPA response to stress by comparing genetically identical mice that had no exposure to microorganisms (germfree; GF), mice raised with a normal functional microbiota but not with specific pathogens (specific pathogen free; SPF) and mice raised with a selected group of organisms (gnotobiotic).

Methods

Animals

GF and SPF BALB/c mice (male, 9 weeks old) were maintained in Trexler-type flexible-film plastic isolators with sterile food and water (Sudo *et al.* 1997). Surveillance for bacterial contamination was done by a periodic bacteriological examination of faeces. To obtain *Bifidobacterium infantis*-, rabbit-derived enteropathogenic *Escherichia coli* (EPEC)-, or EPEC mutant strain (ΔTir)-monoassociated mice whose flora were composed of a single strain of bacterium, the parent GF mice were administered a bacterium orally, and their offspring thus became infected with this bacterium at the neonate stage. These mice were used for the experiment at 9 weeks of age. To produce SPF flora-reconstituted mice, the GF mice were inoculated with 0.5 ml of a 1×10^{-2} dilution of fresh SPF mouse faeces at either 1 or 3 weeks before the commencement of the stress protocol. Such reconstituted mice were subjected to the stress regimen at 9 or 17 weeks of age. All experiments were approved by the Ethics Committee for Animal Experiments of Kyushu University.

Stress protocol

Acute restraint stress was applied by placing the animals in a 50 ml conical tube (Nukina *et al.* 1998, 2001). Mice were killed by cervical dislocation before (basal), and immediately, 30, 60, or 120 min after being subjected to 1 h of restraint stress. This procedure was performed according to our Institutional Guidelines for Animal Experiments. For ether stress (Diorio *et al.* 1993), animals were maintained for 2.5 min in a glass container lined with absorbent paper soaked with ether, then killed by cervical dislocation before (basal), and immediately, 30, 60, or 120 min after ether exposure. Blood samples for ACTH and corticosterone measurements were obtained by cardiac puncture and stored at −80°C before assay. To avoid fluctuations in the biological measurements resulting from differences in circadian rhythm, all samples were collected at the same time of day (between 9.00 and 11.00 h).

Maternal behaviour

As described previously (Anisman *et al.* 1998; Francis *et al.* 1999), maternal behaviour was scored for three 60 min observation periods daily (starting at 09.00, 13.00 and 17.00 h) on the second and third days postpartum. The behaviour of each mother ($n = 8$) was scored every 4 min (total of 360 samplings per group). The data were analysed as the percentage of observations in which animals engaged in the target behaviour. Individuals unaware of the origin of the animals checked the following behaviours: mother off pups, mother licking/grooming any pup, mother nursing pups in an arched-back posture, nursing in a 'blanket' posture in which the mother lays over the pups, or nursing in a passive posture in which the mother is lying either on her back or side while the pups nurse. Mean ($\pm$ S.D.) frequency of the behaviours is expressed as a percentage of the total observation.

Determination of ACTH and corticosterone levels

The plasma level of ACTH was measured by an immunoradiometric assay using the Allégro HS-ACTH kit (Nihon Medi-Physics Co., Ltd, Nishinomiya Japan; Zahradnik *et al.* 1989). In this assay, the concentration of ACTH is linearly related to the amount of radioactivity bound to the beads over a wide analytical range (1–1500 pg ml^{-1}). This assay system has been demonstrated to have a reliable sensitivity of 1 pg ml^{-1}.

The plasma level of corticosterone was measured using a commercially available radioimmunoassay kit (ICN Biomedicals, Costa Mesa, CA, USA). The concentration of corticosterone in the serum samples was calculated from a standard curve and expressed in nanograms per millilitre. The detection limit of the assay was about 1 ng ml^{-1}.

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Determination of faecal bacterial flora

The faecal bacterial flora was determined according to methods previously described (Mitsuoka *et al.* 1965; Benno & Mitsuoka, 1992; Sudo *et al.* 2000). Briefly, approximately 1 g (wet weight) was suspended in an anaerobic diluent, and then serial tenfold dilutions from 10^{-1} to 10^{-8} were prepared. From the appropriate dilution, a 0.05 ml aliquot was then spread on three non-selective agar plates (trypticase soy blood agar, glucose blood liver agar, Eggerth-Gagnon agar) and selective agar plates to allow the incubation of *Streptococci*, *Enterobacteria*, *Staphylococci*, yeasts, *Pseudomonas*, *Bacteroides*, *Bifidobacteria*, *Eubacteria*, *Veillonella*, *Lactobacilli* and *Clostridia* (Eiken, Tokyo, Japan; OXOID, Basingstoke, UK). Staphylococcus medium no. 110 supplemented with 2.5% egg yolk and 5 μg ml^{-1} methicillin (Nissui, Tokyo, Japan) was used for methicillin-resistant *Staphylococcus aureus* and methicillin-sensitive *Staphylococcus aureus*. After incubation for 2 days (aerobes) or 3 days (anaerobes), 13 bacterial groups and yeasts were identified by colonial and cellular morphology, Gram staining, spore formation, and aerobic and anaerobic growth.

Semi-quantitative RT-PCR methods

Gene expression levels of corticotropin-releasing factor (CRH), glucocorticoid receptor (GR) and NMDA receptor subunits (NR-1 and NR-2a) were analysed by a semiquantitative RT-PCR method. The animals were killed by cervical dislocation and each brain was removed immediately. Total RNA was extracted from the cortex, hippocampus and hypothalamus of the GF and SPF mice using a commercially available kit (Sepasol-RNA II, Nacalai Tesque, Kyoto, Japan), according to the manufacturer's instructions. Each brain section was identified according to a stereotaxic atlas (Paxinos & Franklin, 2001). To normalize the signals from different RNA samples, glyceraldehydes-3-phosphate dehydrogenase (GAPDH) mRNA was co-amplified as an internal standard and then the relative values of each transcript to GAPDH mRNA were calculated. In preliminary experiments, optimal reaction parameters were adjusted to obtain a linear relationship between the number of PCR cycles and RT-PCR products and between the initial amount of RNA and RT-PCR products. Additionally, to verify the linearity of GAPDH signals in each experiment, RT-PCR products were collected after 26, 28, 30, 32 and 34 cycles in each sample and then checked to see if the GAPDH signals would be within the linear range of product accumulation. The following primers were designed according to the method described in the indicated literature, and their validity was checked in our laboratory: GAPDH (Nukina *et al.* 2001), sense primer 5′-TCCTGCACCACCAACTGCTTAG-3′, antisense primer 5′-TCTTACTCCTTGGAGGCCATGT-3′; c-Fos (Arrieta *et al.* 2000), sense primer 5′-CCCCTGTCAACACACAGGAC-3′, antisense primer 5′-CCGATGCTCTGCGCTCTGC-3′; CRH (Glasgow *et al.* 1999), sense primer 5′-AACTCAGAGCCCAAGTACGTTGAG-3′, antisense primer 5′-TCACCCATGCGGATCAGAATC-3′; GR (Kizaki *et al.* 1996), sense primer 5′-GCATGGAGAATTATGACCAC-3′, antisense primer 5′-ATCAGATCAGGAGCAAAGCA-3′; NR-1 (Cai & Rhodes, 2001), sense primer 5′-CTCCCACCAGTCCAGCGTCT-3′, antisense primer 5′-GTCATGTTCAGCATTGCGGC-3′; NR-2a (Cai & Rhodes, 2001), sense primer 5′-GGCTGTCAGCACTGAATCCAAAGG-3′, antisense primer 5′-CGAAAGGCAGCTTCTGCAATGTGTG-3′. PCR products were separated by electrophoresis on 1.5% agarose gel and visualized by ethidium bromide staining and UV light. Densitometric analysis was then done to quantify the mRNA levels using public domain NIH image software.

Measurement of neurotrophin and CRF protein levels

Sections of cortex, hippocampus and hypothalamus were quickly removed after the animals were killed and samples homogenized in a lysis buffer (137 mM NaCl, 20 mM Tris, 1% NP-40, 10% glycerol, 1 mM PMSF, 10 μg ml^{-1} aprotinin, 1 μg ml^{-1} leupeptin and 0.5 mM sodium vanadate). Homogenates were centrifuged and the supernatants used as enzyme-linked immunosorbent assay (ELISA) samples. Neurotrophin protein concentrations were determined by the method of Bradford, using bovine serum albumin as a standard. ELISAs were performed using the brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), or nerve growth factor (NGF) Emax ImmunoAssay System kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. CRF protein levels in the hypothalamus were measured using a commercially available radioimmunoassay kit (Phoenix Pharmaceuticals Inc., Belmont, CA, USA).

Assays for cytokine levels in plasma

The plasma bioactivity of interleukin (IL)-6 was determined by measuring the proliferation of the B9 cell line, an IL-6-dependent B cell hybridoma, as previously described (Nukina *et al.* 1998, 2001). The B9 cell line

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was kindly provided by Dr L. Aarden, the Netherlands Red Cross Blood Transfusion Service, Amsterdam, the Netherlands (Aarden, 1987). Briefly, B9 cells (5×10^3 per 100 μl) were cultured in 96-well microtiter plates with serial dilutions of the plasma samples. After 72 h of incubation at 37°C with 5% CO_2, 20 μl of MTT tetrazolium (5 mg ml^{-1}; Research Organics Inc., Cleveland, OH, USA) was added to determine the proliferation. After an additional 4 h of incubation the supernatant was removed and 100 μl of 10% SDS with 0.01 N HCl was added to dissolve the crystals. The level of cell proliferation was determined using a microplate ELISA reader at 570 nm. The IL-6 activity in the plasma samples was calculated based on a purified recombinant mouse IL-6 standard (Boehringer Mannheim Corp., Mannheim, Germany) run in the same assay. The sensitivity of the assay was about 1 pg ml^{-1} and the specificity of the assay was confirmed by using IL-6 neutralizing antibody, which can antagonize 95% of the B9 cell proliferation induced by the mouse plasma.

The IL-1β level of the plasma samples was assayed using a commercially available ELISA kit (BioSource International, Camarillo, CA, USA).

Anti-IL-6 treatment of mice

In some experiments, pre-treatment with anti-IL-6 antibody was done to neutralize the plasma IL-6 activity upon exposure to *Bifidobacterium infantis*. Hybridoma producing rat monoclonal antibody to mouse rIL-6, MP5-20F3 clone (Starnes *et al.* 1990) was obtained from the American Type Culture Collection (Rockville, MD, USA) by courtesy of the DNAX Research Institute of Molecular and Cellular Biology (Palo Alto, CA, USA). Purified monoclonal antibodies from the ascitic fluid from athymic nude mice were then used in the experiment. The proper dosage of anti-IL-6 antibody, that which sufficiently neutralizes plasma IL-6 response, was determined according to previous reports (Neta *et al.* 1992; Nukina *et al.* 1998). Chromatographically purified rat IgG (Seikagaku Corp., Tokyo, Japan) was also used as a control antibody.

Statistical analysis

All data are expressed as the means ± s.d. The data were analysed by Dunnett's *post hoc* test after the factorial analysis of variance. In some experiments, statistical analysis was done using the Mann–Whitney *U* test. A value of $P < 0.05$ was considered to indicate significant difference.

Results

Plasma ACTH and corticosterone responses of GF mice were more susceptible to restraint stress than those of SPF mice

To investigate the difference in HPA response to stress stimuli between the GF and SPF mice, both groups of mice were subjected to either 1 h of restraint stress or ether exposure. Plasma ACTH and corticosterone elevation in response to restraint stress was substantially higher in GF mice than in SPF mice (Fig. 1*A*). When the mice were exposed to ether stimulus, no significant difference in plasma ACTH or corticosterone response was found between the groups of animals (Fig. 1*B*).

GF mice revealed reduced expression levels of cortical GR transcript

Morphological examination of the adrenal and pituitary glands by HE staining failed to reveal any obvious difference of structure or cell type between the GF and SPF mice. The average adrenal cortical thicknesses were 310 ± 35.4 μm in GF mice ($n = 5$) and 306.6 ± 28.8 μm in SPF mice ($n = 6$). Furthermore, when pituitary sections were stained with anti-ACTH antibodies, no quantitative difference was noted in the corticotrophs between the groups of animals. However, in the hypothalamus, the mRNA expression level of CRF transcript, a main stimulator of ACTH secretion from the pituitary gland, was significantly higher in GF mice than in SPF mice (relative values of CRF to GAPDH mRNA: GF 1.36 ± 0.10 *versus* SPF 0.85 ± 0.13, $n = 5$ per group, $P < 0.05$ by Mann–Whitney *U* test). Such elevated CRF mRNA levels in the hypothalamus of GF mice were also confirmed by CRF protein concentrations (GF 9.8 ± 3.4 ng mg^{-1} protein *versus* SPF 5.3 ± 2.7 ng mg^{-1} protein, $n = 6$ per group, $P < 0.05$). The mRNA expression level of GR, which negatively regulates HPA axis activation by inhibiting hypothalamic CRF gene expression, was significantly lower in the cortex, but not the hypothalamus or hippocampus, of GF mice than in SPF mice (relative values of GR to GAPDH mRNA: cortex, GF 0.94 ± 0.69 *versus* SPF 1.82 ± 0.62; hypothalamus, GF 0.49 ± 0.16 *versus* SPF 0.48 ± 0.34; hippocampus, GF 3.21 ± 0.72 *versus* SPF 3.92 ± 0.77; $n = 4$–7 per group, $P < 0.05$).

The GF condition failed to affect maternal behaviour

In view of previous data indicating that interaction of the dam with her litter can program HPA development (Liu *et al.* 1997, 2000), maternal behaviour was scored for three

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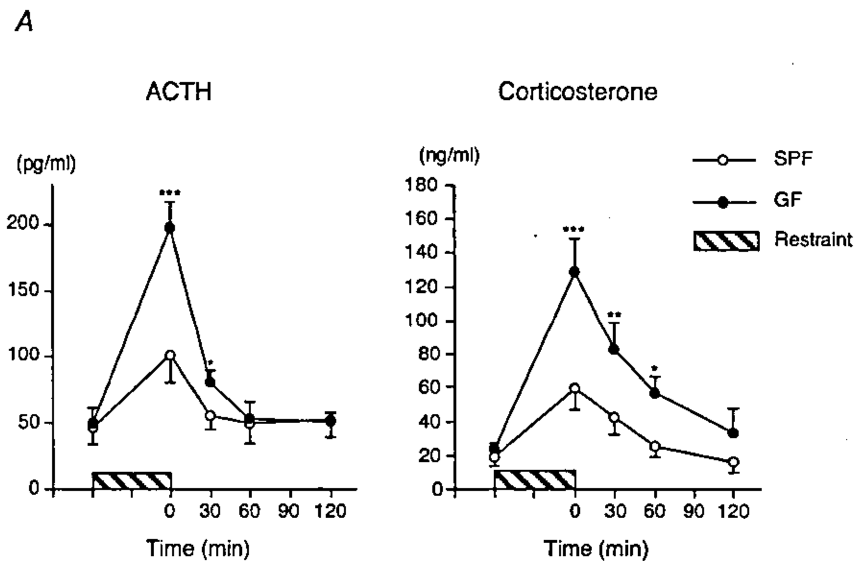
60 min observation periods daily on the second and third days postpartum. No difference in maternal behaviour was observed, with GF and SPF dams equally arch-backed-nursing or grooming/licking their pups (arch-backed nursing: GF 13.0 ± 2.9% *versus* SPF 11.8 ± 3.9%; grooming licking: GF 5.7 ± 1.3% *versus* SPF 5.2 ± 1.7%), suggesting that the enhanced HPA stress response of the GF mice was unlikely to have been due to reduced maternal contact.

The GF mice showed reduced expression levels of cortical and hippocampal BDNF

Previous reports demonstrated that early life events that take place during brain maturation can modulate the expression of neurotrophins of cellular plasticity within selected brain regions (Liu *et al.* 2000; Roceri *et al.* 2002). We therefore compared the expression levels of neurotrophins and their related receptors in the various brain areas of the GF mice with those of the SPF mice. A semiquantitative RT-PCR analysis of NR subunits, neurotransmitters that regulate the expression of BDNF, showed decreased gene expression of cortical NR-1 and NR-2a and hippocampal NR-2a subunits in GF mice compared with SPF mice (Fig. 2*A–C*). Consistent with these results, the BDNF protein level in the cortex and hippocampus was significantly lower in GF mice than in SPF mice (Fig. 2*D*), whereas other neurotrophins, NT-3 and NGF, in these areas of GF mice were identical to those of SPF mice (NT-3: cortex, GF 66.8 ± 13.2 *versus*



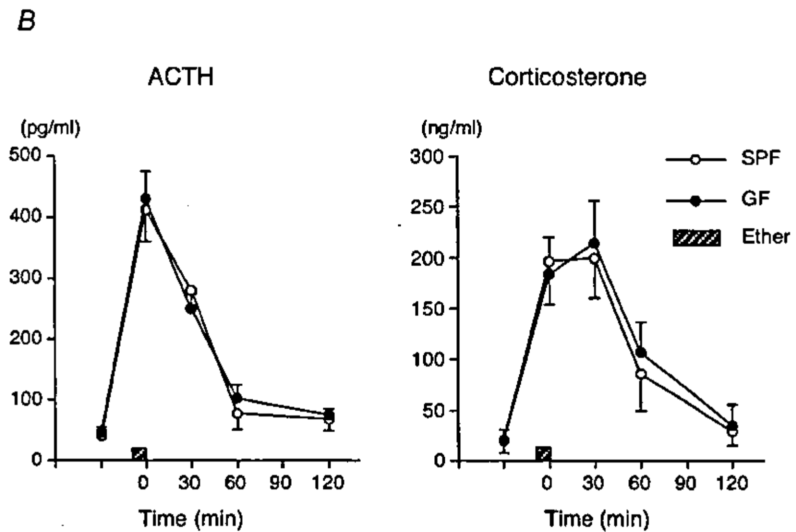


Figure 1. Increased plasma ACTH and corticosterone response to restraint stress, but not to ether exposure in GF mice
A, mice were subjected to a 1 h period of restraint stress (GF, $n = 6$–11 for each time point, total of 52 animals; SPF, $n = 6$–11 for each time point, total of 50 animals). The baseline data were obtained by cardiac puncture from mice that were killed by cervical dislocation before stress exposure. The baseline ACTH and corticosterone levels in the GF and SPF mice were 49 ± 12 pg ml^{-1} and 23 ± 4.2 ng ml^{-1}, in the GF mice, 46 ± 13 pg ml^{-1} and 19 ± 5.6 ng ml^{-1}, respectively. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ in Dunnett's *post hoc* test between GF and SPF. *B*, GF and SPF mice failed to show any difference in HPA response to ether exposure ($n = 6$ for each time point, total of 30 animals per group).

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SPF 65.8 ± 10.0; hippocampus, GF 126.5 ± 37.8 *versus* SPF 124.0 ± 14.1; NGF: cortex, GF 169.0 ± 87.0 *versus* SPF 188.7 ± 82.8; hippocampus, GF 136.4 ± 58.1 *versus* SPF 159.0 ± 87.5 pg (mg protein)$^{-1}$).

HPA response to stress in the gnotobiotic mice

To further elucidate the involvement of gut microbiota in the HPA stress response, gnotobiotic mice whose flora were composed of a single strain of bacterium at the neonate stage were tested for their susceptibility to restraint stress at 9 weeks of age. Monoassociation with *Bifidobacterium infantis*, which is a representative inhabitant of the neonate gut, dampened the HPA stress response to the SPF (Fig. 3). In accordance with previous reports that neonatal endotoxin treatment increases the HPA stress response later in life (Shanks *et al.* 1995, 2000), the hormonal stress response in rabbit-derived enteropathogenic *Escherichia*

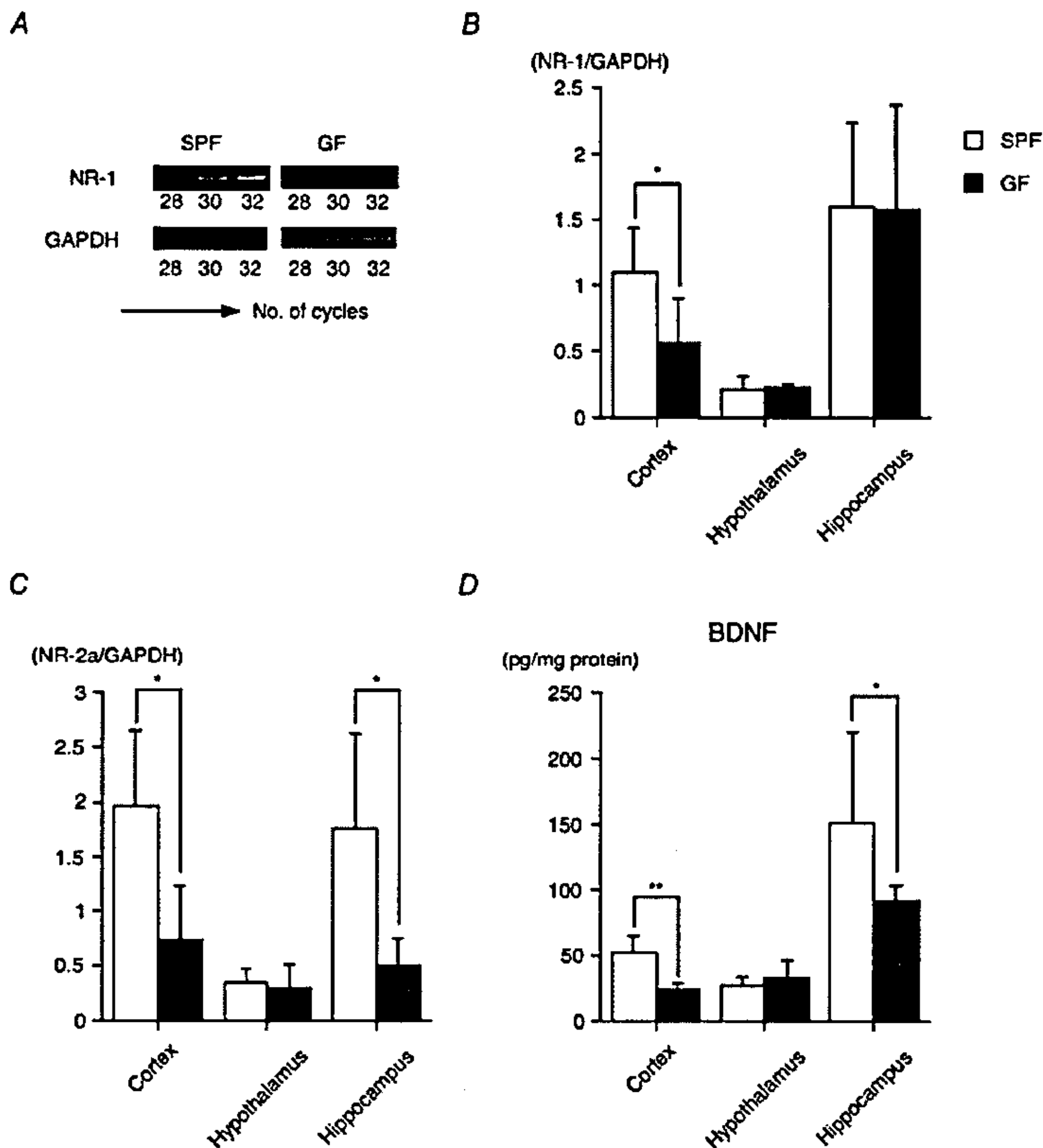

Figure 2. NR subunit gene expression and protein BDNF concentration
A, a typical RT-PCR product with NR-1 specific primers revealed PCR products with the predicted 333 bp length of NR-1 mRNA. Total RNA was extracted from a single cerebral cortex of GF and SPF mice. NR-1 (*B*) and NR-2a transcripts (*C*) were detected by RT-PCR in the cortex, hypothalamus or hippocampus of GF and SPF mice (9 weeks old). Histograms show the relative band intensities on densitometric analysis as ratios of NR subunit and GAPDH mRNA after 30 cycles of amplification ($n = 4$–7 per group). *D*, BDNF protein concentration was measured by ELISA ($n = 6$–10 per group). *$P < 0.05$, **$P < 0.01$ by Mann–Whitney U test.

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coli (EPEC)-monoassociated mice was substantially higher than that in GF mice, although no such exaggerated response was found in the mice reconstituted with an EPEC mutant strain, ΔTir (Kenny *et al.* 1997), which is not internalized owing to defects in the translocated intimin receptor. Since there was no difference in the number of intestinal bacteria between the EPEC- and ΔTir-associated mice, these results indicate that bacterial internalization to the intestinal epithelial layer is an indispensable condition that enables the EPEC strain to influence the regulatory system of the HPA response.

Colonization by bacterium induced increases in the c-Fos mRNA levels in the paraventricular nucleus and in the corticosterone and cytokine concentrations in the plasma

To further clarify the mechanism involved in the different sensitivity of each gnotobiotic mouse to stress, GF mice were orally inoculated with EPEC, ΔTir or *Bifidobacterium infantis*. Then either the IL-1β and IL-6 levels in the plasma or the c-Fos expression level in the paraventricular nucleus, a marker for neuronal activity, was measured before and 6, 12 and 24 h after the inoculation with each bacterium. IL-1β and IL-6 levels in the plasma substantially increased and reached a peak at 12 h after the inoculation with EPEC (Fig. 4*A* and *B*). Administration of ΔTir or *Bifidobacterium infantis* also triggered a small but significant increase in plasma IL-6 levels at 12 h after the inoculation without elevating the plasma IL-1β levels. Interestingly, the c-Fos mRNA level in the paraventricular nucleus was already elevated at 6 h after the inoculation regardless of which of the bacterial strains was used (Fig. 4*C* and *D*). This c-Fos response was accompanied by a concomitant increase in plasma corticosterone levels on exposure to microbes (control: basal 19 ± 10 ng ml^{-1}, 6 h 25 ± 12 ng ml^{-1}, 12 h 26 ± 16 ng ml^{-1}, 24 h 17 ± 9 ng ml^{-1}; *Bifidobacterium*: basal 20 ± 11 ng ml^{-1}, 6 h $142 \pm 36^{***}$ ng ml^{-1}, 12 h 35 ± 26 ng ml^{-1}, 24 h 30 ± 19 ng ml^{-1}; EPEC: basal 19 ± 8 ng ml^{-1}, 6 h $175 \pm 41^{***}$ ng ml^{-1}, 12 h $120 \pm 29^{***}$ ng ml^{-1}, 24 h $86 \pm 24^{**}$ ng ml^{-1}; ΔTir: basal 22 ± 14 ng ml^{-1}, 6 h $116 \pm 32^{***}$ ng ml^{-1}, 12 h 60 ± 41 ng ml^{-1}, 24 h 51 ± 32 ng ml^{-1}; $n = 5$–7 for each time point; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *versus* each corresponding basal value). Moreover, pretreatment with anti-IL-6 antibody failed to affect the elevated c-Fos response in the paraventricular nucleus (Fig. 5*A* and *B*) and corticosterone response in the plasma (Fig. 5*C*) on exposure to *Bifidobacterium infantis*. These results taken together indicate that visceral information derived from bacterial colonization can be transmitted to the brain at least partly through a humoral cytokine-independent pathway, probably via a neural route.

A complete SPF flora partly reversed the HPA response to stress only when the flora was introduced at an early stage of development

Finally, the effects of a complete SPF flora on the hormonal response to stress were examined. The enhanced HPA stress response of GF mice was partly corrected 3 weeks after reconstitution of SPF faeces at an early stage of development (Fig. 6*A*), while such correction was not found following any reconstitution exerted at a later stage (Fig. 6*B*). Bacterial examination of faecal samples revealed

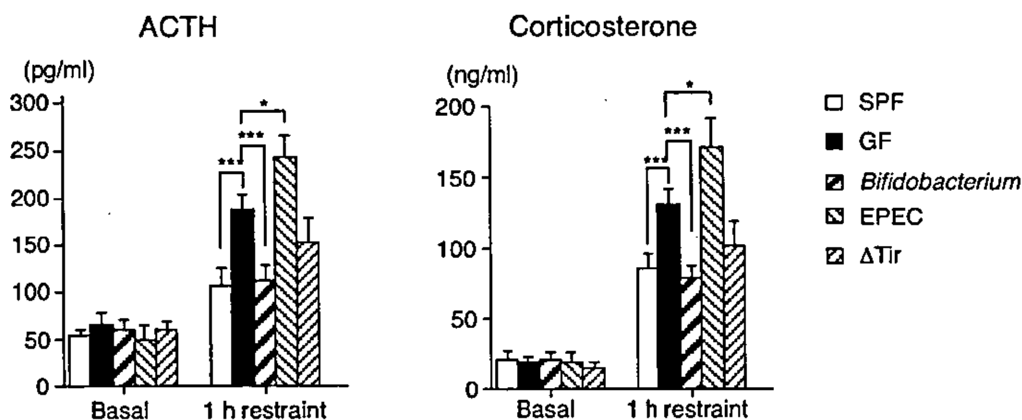

Figure 3. Effects of restraint stress on plasma ACTH and corticosterone levels in gnotobiotic mice
Plasma ACTH and corticosterone levels were measured before or immediately after 1 h restraint in GF ($n = 20$), SPF ($n = 18$) and monoassociated mice ($n = 18$–24 per group) at 9 weeks of age. $^{*}P < 0.05$, $^{***}P < 0.001$ by Dunnett's test.

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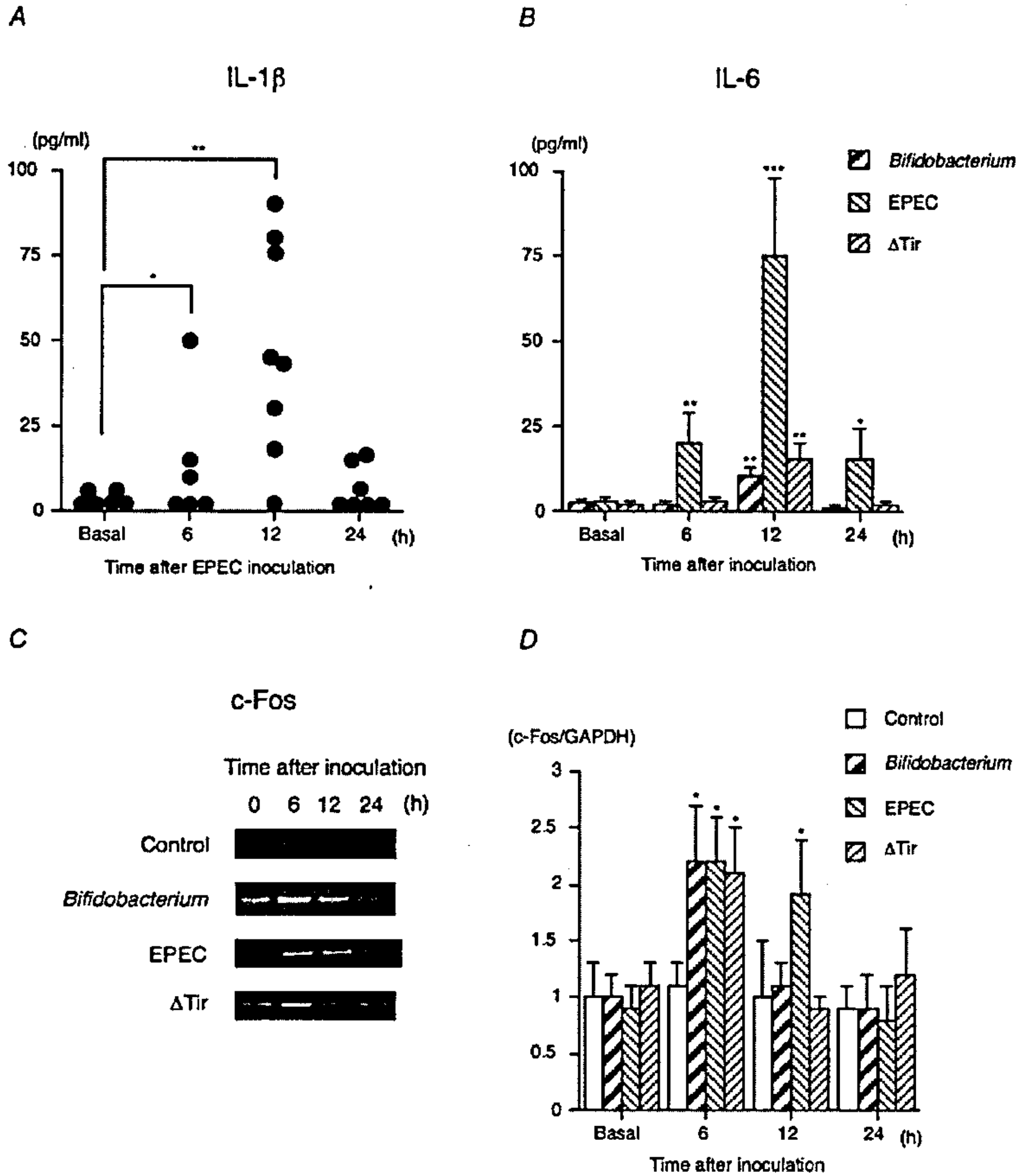

Figure 4. Kinetics of cytokine concentration in the plasma and c-Fos gene expression in the paraventricular nucleus upon exposure to *Bifidobacterium infantis*, EPEC or ΔTir

GF mice at 5 weeks of age received a gavage of either 0.5 ml skimmed milk containing one of the bacterial strains (1×10^9 CFU; *Bifidobacterium infantis*, EPEC or ΔTir) or skimmed milk alone (control), after which plasma and brain samples were collected before (basal) and 6, 12 or 24 h after inoculation with each bacterium. *A*, the plasma IL-1β level of the mice exposed to EPEC was measured by ELISA ($n = 6$–8 for each time point). No significant IL-1β elevation in the plasma was found after inoculation with *Bifidobacterium infantis*, ΔTir or skimmed milk alone (control). *B*, plasma IL-6 levels were analysed by the B9 cell bioassay as described in the Methods ($n = 6$–8 for each time point). *C*, a typical RT-PCR product with c-Fos specific primers revealed PCR products with the predicted 247 bp length of c-Fos mRNA. *D*, histogram shows the relative band intensities on densitometric analysis as ratios of c-Fos and GAPDH mRNA after 30 cycles of amplification ($n = 4$ for each time point). $^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ were considered to be significantly different from the corresponding basal values.

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that colonization was almost completely accomplished 3 weeks after the administration of the SPF faeces, whereas aerobes, such as *Escherichia coli*, were more prominent than anaerobes 1 week after the administration of the SPF faeces. Collectively, these findings suggest that exposure to indigenous microbiota at an early developmental stage, when brain plasticity may still be preserved, is required for the HPA system to become fully susceptible to inhibitory neural regulation.

Discussion

Accumulating evidence has demonstrated a bidirectional communication between the brain and the gut. Researchers in this field preferentially call this cross-talk the 'brain–gut axis' (Aziz & Thompson, 1998). Indeed, it has been shown that a stressful experience can lead to altered gastrointestinal motility, secretions and blood flow; while, in turn, such alteration in gastrointestinal function is transmitted to the brain and can ultimately bring about the perception of visceral events such as nausea, satiety and pain (Drossman, 1998). Interestingly, a number of research papers reported that physical and psychological stress can affect the composition of intestinal microbiota in rodents (Porter & Rettger, 1940; Tannock & Savage, 1974; Suzuki *et al.* 1983) and primates (Holdeman *et al.* 1976; Bailey & Coe, 1999). The present results, in which colonizing microbes altered the HPA response to restraint stress, indicate that the interaction of gut bacteria with the brain is also bidirectional, just like the brain–gut axis. To our knowledge, this is the first report that shows commensal microbes affecting the neural network responsible for controlling stress responsiveness.

In this study, the HPA response of the GF mice was more sensitive to restraint stress than that of the SPF mice, while both groups of mice failed to show any difference in the sensitivity to ether stress. Additionally, GF mice also exhibited reduced BDNF expression levels in the cortex and hippocampus relative to SPF mice. Because the HPA response to restraint stress is affected by the limbic system, including the prefrontal cortex, hippocampus and amygdala, and requires assembly and processing of signals from multiple sensory modalities before initiation of a stress response, whereas ether stress does not (Herman & Cullian, 1997), these results indicate that cognitive processing in the limbic system may be involved in an exaggerated HPA response by GF mice. Nonetheless, it should be noted that we cannot rule out the possibility that the sensitivity of our methods is too low to detect a minimal change in the GR and NR subunit mRNA levels in the hypothalamus. More quantitative methods, such as real-time PCR and *in situ* hybridization, are needed to definitely conclude region-specific changes in the GR or NR subunit gene expression.

To date, we cannot clearly explain the exact mechanism by which the visceral signals originating from bacterial colonization are transmitted to the brain; however, the following pathways have been suggested to be involved: one is through a cytokine-mediated humoral route and the other is via a neural pathway. As summarized in a recent review by Turnbull & Rivier (1999), there is now overwhelming evidence that several cytokine families, especially interleukin-1, increase the secretory activity of the HPA axis; hence, it is reasonable to suppose that endotoxin and/or peptidoglycan, components of the bacterial cell wall, stimulate immune cells within the gut or elsewhere to release these cytokines, which consequently influence the parts of the CNS involved in the regulation of the HPA axis response. In fact, a previous report by Dahlgren *et al.* (1995) and our results showing that plasma IL-1 and IL-6 levels substantially increase upon exposure to EPEC also support the significant role of this humoral route. In this respect, since it was demonstrated that Gram-negative bacteria, especially bacteria such as *Escherichia coli*, translocated in large numbers to the mesenteric lymph node, whereas obligately anaerobic bacteria did so at only very low levels (Steffen *et al.* 1988), such pronounced cytokine response after exposure to EPEC may be due to the enhanced translocation rates of this strain.

Alternatively, c-Fos activation in the paraventricular nucleus was rapidly induced at 6 h after the inoculation of *Bifidobacterium infantis* or ΔTir, when plasma cytokine levels had not yet elevated. Together with results showing the inability of anti-IL-6 antibody pretreatment to block such an increase in the brain c-Fos expression and plasma corticosterone elevation upon exposure to *Bifidobacterium infantis*, these results thus suggest the importance of another pathway, probably via a neural-mediated pathway. In accord with this speculation, Wang *et al.* (2002) reported that subdiaphragmatic vagotomy attenuates c-Fos expression in the paraventricular nucleus and supraoptic nucleus in rats inoculated with *Salmonella Typhimurium*. Clearly, further studies, especially using molecular techniques, will be necessary to clarify how and to what extent neural- and cytokine-mediated pathways can contribute to the flora-mediated modulation of the HPA response. However, our experimental system using gnotobiotic animals proved to be a useful animal model for this clarification.

Recently, Rescigno and colleagues (Rescigno *et al.* 2001) have reported a new mechanism for bacterial uptake in the mucosal tissues that is mediated by dendritic cells, which

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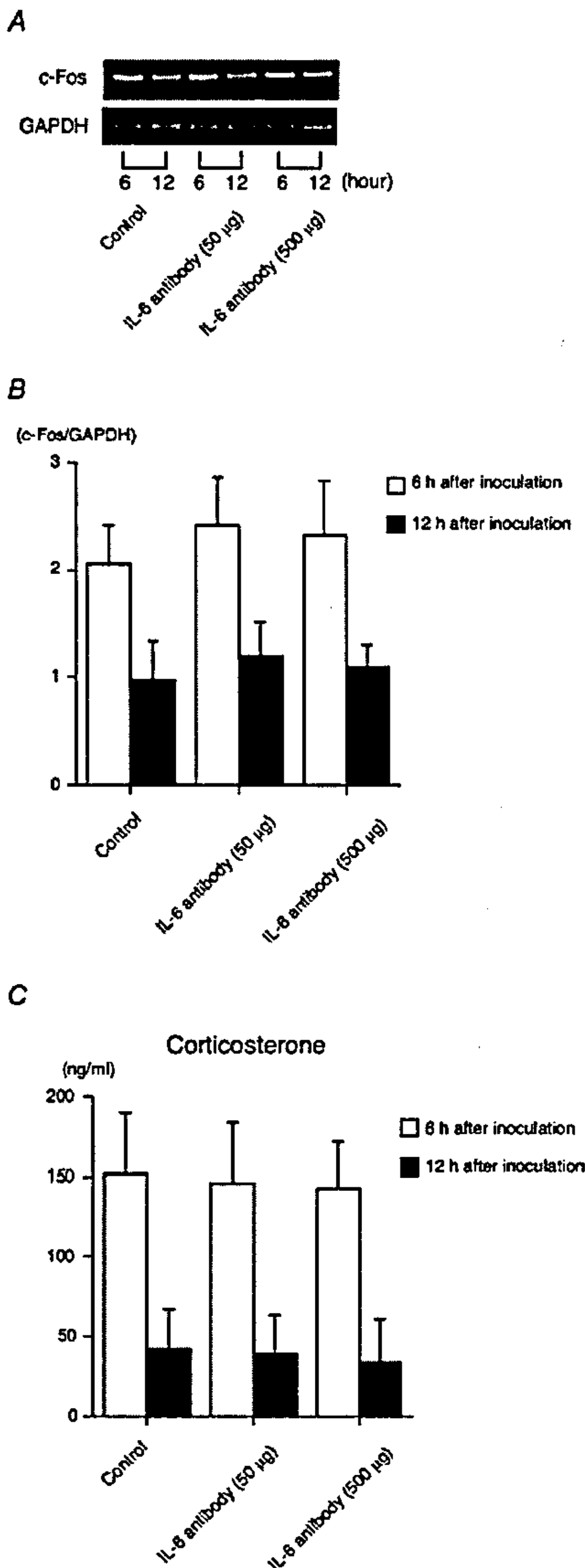

open the tight junctions between epithelial cells, send dendrites outside the epithelium and directly sample both pathogenic and non-pathogenic bacteria that are unable to induce their own phagocytosis through the M cells. In addition, a recent *in vitro* study (Dunzendorfer *et al.* 2001) showed a variety of neuropeptides, including calcitonin gene-related peptide, vasoactive intestinal polypeptide and the neurotransmitter secretin, to induce chemotaxis of immature dendritic cells. Since there is a close anatomical association between the neuropeptide-containing nerves and dendritic cells (Bellinger *et al.* 2001), these findings taken together indicate the potentially important role of dendritic cells in signal transmission from the gut lumen to the CNS. A new project in our laboratory is now in progress, in which a DNA microarray technique is being applied to the gnotobiotic animal model to further clarify the precise molecular mechanism whereby dendritic cells interact with intestinal bacteria.

The series of experiments performed by Husebye *et al.* (1994, 2001) has shown different bacterial strains to differentially affect the motility of the gastrointestinal tract. Therefore, it should be noted that such a change in the gut motility after bacterial colonization is perceived as new visceral information and may then indirectly modulate the microbiota-induced alteration in the endocrine stress response.

Although the glucocorticoid response to stress is essential for survival, prolonged glucocorticoid elevation can present serious health risks, including diabetes, hypertension, hyperlipidaemia, hypercholesterolaemia, arterial disease, amenorrhoea, impairment of growth and tissue repair and immunosuppression (McEwen, 1998). In an elegant study by Shanks *et al.* (2000), it was clearly demonstrated that exposure of neonatal rats to a low dose of endotoxin ($0.05\ \mathrm{mg\ kg^{-1}}$) resulted in long-term changes in HPA axis activity, with elevated mean plasma corticosterone concentrations that resulted from increased

Figure 5. Effects of anti-IL-6 treatment on the c-Fos expression in the paraventricular nucleus and the corticosterone response in the plasma

GF mice at 5 weeks of age were injected intraperitoneally with either anti-IL-6 antibody (MP5-20F3; 50 or 500 μg) or control rat IgG antibody (control) 1 h before being inoculated with *Bifidobacterium infantis*. The analysis of c-Fos mRNA expression levels in the paraventricular nucleus was done at 6 or 12 h after the inoculation. *A*, the results shown are representative of 4 independent experiments. *B*, histogram shows the relative band intensities on densitometric analysis as ratios of c-Fos and GAPDH mRNA after 30 cycles of amplification ($n = 4$ for each time point). *C*, determination of the plasma corticosterone levels was carried out according to the protocol described in the Methods ($n = 6$ for each time point).

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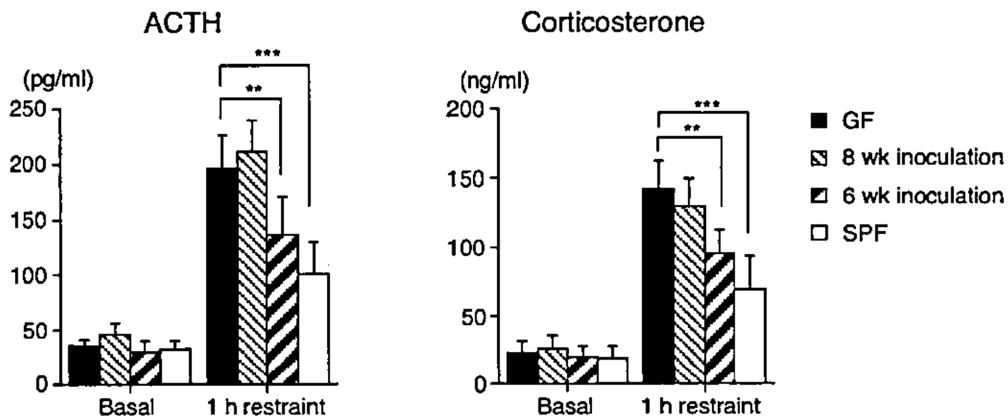
corticosterone pulse frequency and pulse amplitude. In addition, they also showed neonatal endotoxin exposure to have long-lasting effects on immune regulation, including increased sensitivity of lymphocytes to stress-induced suppression of proliferation and a remarkable protection from adjuvant-induced arthritis. Such a protective effect of pretreatment with endotoxin in adults on the development of arthritis was also confirmed in a later study by Harbuz *et al.* (2002). Together with the present results that the EPEC strain enhanced the HPA response to stress, these findings lead to the clinically important possibility that neonatal infection with pathological bacteria may alter the development of neural systems that govern the endocrine response to stress and may thereby predispose the individuals to stress-related pathology later in life. Alternatively, the enhanced HPA response to stress in GF



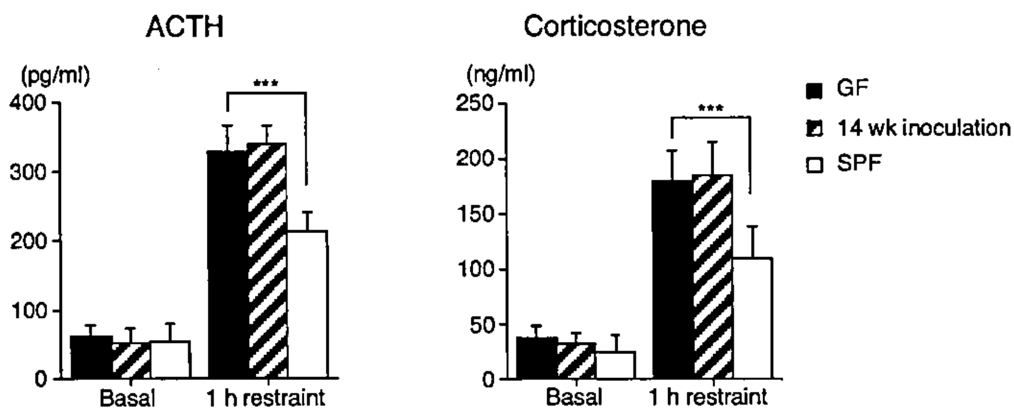


Figure 6. Effects of restraint stress on plasma ACTH and corticosterone levels in mice reconstituted with SPF faeces

SPF flora-reconstituted mice were established by orally introducing fresh SPF mouse faeces into the GF mice at either 1 or 3 weeks before they were subjected to the stress protocol. Restraint stress was applied to these reconstituted mice at 9 (*A*) and 17 weeks of age (*B*) (n = 18–24 per group). $^{**}P < 0.01$, $^{***}P < 0.001$ by Dunnett's *post hoc* test.

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mice was partly reversed by the addition of a complete SPF faeces at an early stage of development, indicating that either a complete indigenous microbiota containing aerobic and anaerobic bacteria or some strains of anaerobic bacteria including *Bifidobacterium* may play a protective role against the deleterious effects of elevated plasma glucocorticoid levels.

In summary, the present study shows that commensal microbiota are an environmental determinant that regulates the development of the HPA stress response. These findings indicate that the series of events in the gastrointestinal tract following postnatal microbial colonization can have a long-lasting impact on the neural processing of sensory information regarding the endocrine stress axis. Our concept, based on *in vivo* findings, provides evidence of a novel link between indigenous microorganisms and the nervous system and shows a new aspect of the brain–gut axis.

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