

Antibiotic use during infancy promotes a shift in the T_H1/T_H2 balance toward T_H2 -dominant immunity in mice

Naomi Oyama, MD, Nobuyuki Sudo, MD, PhD, Hiroshi Sogawa, MD, PhD, and Chiharu Kubo, MD, PhD *Fukuoka, Japan*

Background: Recent epidemiologic studies indicate that antibiotic use in infancy may be associated with an increased risk of development of atopy; however, its precise mechanism remains to be elucidated.

Objective: The purpose of this study is to clarify whether one such antibiotic, kanamycin, affects the T_H1/T_H2 balance.

Methods: BALB/c mice at 3 and 52 weeks of age were orally administered 600 mg/d kanamycin sulfate for 7 consecutive days. Blood samples were collected on weeks 0, 10, 18, and 26 after the cessation of kanamycin treatment, and the effect of the kanamycin treatment on the T_H1/T_H2 balance was evaluated on the basis of both the *in vivo* antibody levels and the *in vitro* splenocyte cytokine secretion pattern.

Results: The administration of kanamycin increased the serum levels of total IgG1 and IgE while decreasing the serum IgG2a levels. Moreover, when spleen cells were stimulated with immobilized anti-CD3 antibody *in vitro*, such kanamycin treatment enhanced the *in vitro* IL-4 secretion while reducing the *in vitro* IFN- γ secretion. The basal IL-12 p70 secretion levels of splenic dendritic cells in the kanamycin-treated mice were lower than those in the control mice, although no significant difference was seen in IL-12 p40 levels between either group of mice.

Conclusion: These results suggested that antibiotic use during infancy may indeed quantitatively disturb, qualitatively disturb, or both the intestinal microflora and thereby prevent postnatal T_H1 cell maturation, thus resulting in a T_H2 -polarized immune deviation. (*J Allergy Clin Immunol* 2001;107:153-9.)

Key words: Antibiotics, IFN- γ , IgE, IL-4, IL-12, intestinal microflora, kanamycin

Atopic disease is showing a steadily increasing prevalence in modern highly industrialized countries.¹ Changes over such a short genetic interval suggest the influence of important environmental determinants. Recently, a declining exposure to infections early in life has been proposed to be one candidate mechanism determining such an increase of allergy.² In fact, a wide body of epidemiology literature has shown an inverse associa-

tion between the exposure to intracellular infections, including tuberculosis,³ measles,⁴ and hepatitis A,⁵ and allergic disease. In addition to such microbial pathogens, the principal drive for postnatal maturation of the mammalian immune system has also been recognized to be provided by confrontation with the microbial environment, particularly through stimulation by means of indigenous microflora, which colonize the infant gastrointestinal tract.^{6,7} In fact, our previous studies of animals⁸ showed that if such stimuli are not available to the developing immune system during infancy, the maturation of T_H1 -dependent immune deviation mechanisms is inhibited, thus resulting in a possible lifelong dysregulation of the T_H2 responses. Moreover, some recent studies have also indicated that antibiotic use in infancy may be associated with an increased risk of development of atopic disorders.^{9,10} Because antibiotic use usually depletes the normal commensal microflora, in addition to targeting microbial pathogens, it is therefore interesting to speculate that such a depletion of microflora by antibiotics may be responsible for the increased risk of development of atopy.

The purpose of this study is to determine whether one such antibiotic, kanamycin, may influence the direction of the T_H1/T_H2 balance as evaluated on the basis of the serum antibody levels and splenic cytokine synthesis patterns in mice.

METHODS

Animals

Male BALB/c mice (3 and 52 weeks of age) were obtained from Charles River Japan, Inc (Shizuoka, Japan). All animals were maintained on a 12-h light/12-h dark cycle with food and water freely available. The temperature of the colony room was maintained at 22°C to 23°C. This experiment was reviewed by the committee of Ethics on Animal Experiments in the Faculty of Medicine, Kyushu University, and was carried out under the control of the Guidelines for Animal Experiments of the Faculty of Medicine, Kyushu University, Fukuoka, Japan, and The Law (No. 105) and Notification (No. 6) of the government.

Study protocol

To eliminate bacteria from the intestine, the mice were orally administered kanamycin (600 μ g/d per mouse; Meiji Seika Kaisha Ltd, Tokyo, Japan) for 7 consecutive days. We selected kanamycin because this antibiotic is not absorbed from the intestine into the body.¹¹ Control mice were similarly treated but did not receive

From the Department of Psychosomatic Medicine, Graduate School of Medical Sciences, Kyushu University, Fukuoka.

Received for publication May 22, 2000; revised August 21, 2000; accepted for publication August 24, 2000.

Reprint requests: Nobuyuki Sudo, MD, Department of Psychosomatic Medicine, Graduate School of Medical Sciences, Kyushu University, 3-1-1 Maidashi, Higashi-Ku, Fukuoka 812-8582, Japan.

Copyright © 2001 by Mosby, Inc.

0091-6749/2001 \$35.00 + 0 1/111142

doi:10.1067/mai.2001.111142

kanamycin. Fecal samples were processed for bacteriologic examination at weeks 0 (immediately) and 10 after the termination of kanamycin treatment. Blood samples were obtained from the orbital plexus at weeks 0, 10, 18, and 26 after the cessation of kanamycin treatment, and thereafter the sera were kept for -80°C until antibody levels were measured. The thymus, spleen, and Peyer patches were also removed for further analysis, as described below.

Determination of fecal bacterial flora

Approximately 1 μg (wet weight) of feces was suspended in an anaerobic diluent, and then serial 10-fold dilutions from 10^{-1} to 10^{-8} were prepared. From the appropriate dilutions, a 0.05-mL aliquot was then spread on 3 agar plates (all from Nissui Pharmaceutical Co, Tokyo, Japan). After incubation for either 1 day (aerobes) or 2 days (anaerobes), the bacterial groups were thus recognized on the basis of both the colonial and cellular morphology, Gram staining, spore formation, and aerobic and anaerobic growth.¹²

Flow cytometric analysis

Single cell suspensions from the thymus, spleen, or Peyer patches were counted in a Bürker hemocytometer and thereafter were preincubated for 30 minutes at 4°C with rat serum to prevent any nonspecific binding. The cells were then washed twice with PBS containing 2% FCS plus 0.1% NaN_3 and incubated at 1×10^6 cells per sample for 30 minutes at 4°C with FITC-conjugated anti-B220 (clone RA3-6B2), phycoerythrin-conjugated anti-CD3 (145-2C11), FITC-anti-CD8 (Ly-2), or FITC anti-CD4 (GK1.5) mAb (all from Pharmingen, San Diego, Calif). A flow cytometric analysis of 10^4 cells was performed by using a FACS caliber (Becton Dickinson, San Jose, Calif). Any residual erythrocytes, dead cells, and debris were excluded from the analysis.

Measurement of total antibody levels of IgG1, IgG2, and IgE in the serum

The amounts of total IgG1, IgG2a, and IgE in the serum were determined by using a sandwich ELISA, as previously described.^{13,14} Briefly, 96-well plates for ELISA were coated with each isotype-specific anti-mouse Ig antibodies by incubation overnight at 4°C and then were treated further with 2% (wt/vol) BSA dissolved in PBS containing 0.05% Tween-20 for 2 hours at room temperature to block any nonspecific binding. Next, serial dilutions of the serum samples were incubated in the wells in duplicate for 1 hour at room temperature. After being washed 3 times with PBS containing 0.05% Tween-20, each of the isotype-specific peroxidase-conjugated anti-mouse Ig antibodies (all from Caltag Laboratories, Burlingame, Calif) were added to the wells. In the case of the IgE assay, biotin-conjugated rat anti-mouse IgE antibody (LO-ME-2; Serotec Ltd, Kidlington, Oxford, United Kingdom) was added to the wells, followed by the addition of streptavidin-peroxidase (Seikagaku Corp, Tokyo, Japan). After washing 3 times, the plates were developed by using a substrate solution containing 0.04% *o*-phenylenediamine dissolved in phosphate citrate buffer (pH 5.0). The reactions were terminated by the addition of sulfuric acid. The plates were read in a microplate reader at 490 nm, and the amount of the Ig isotype was calculated by comparing it with the individual Ig standard (monoclonal mouse Ig panel; Zymed Laboratories Inc, San Francisco, Calif). For the assay of each Ig isotype, the following coating antibodies were used: IgG1, LO-MG-1-2; IgG2a, MG2a-3RT; and IgE, LO-ME-3 (all from Serotec Ltd).

Cytokine measurement

For a cytokine production assay of the spleen, a single cell suspension from the spleen was suspended at a final concentration of

$2.5 \times 10^6/\text{mL}$ and cultured in 1-mL aliquots in 24-well plates, either in a complete RPMI medium alone or with stimulation by immobilized anti-CD3 antibody (145-2C11). The complete RPMI medium consisted of RPMI-1640 containing 10% FCS and antibiotics but contained no 2-mercaptoethanol. The supernatants were collected after 48 hours for measurements of IL-2 and IFN- γ and 72 hours for measurements of IL-4 and IL-10 because the cytokine production exhibited its peak level at these times. These cytokine concentrations in the supernatants were quantified by using the sandwich ELISA technique, as described in our previous report.¹⁵ For an assay of each cytokine, the following coated antibodies, biotinylated antibodies, and standards were used: IL-2, JES6-1A12 (Pharmingen), JES6-5H4 (Pharmingen), and rIL-2 (Genzyme, Cambridge, Mass); IL-4, BVD4-1D11 (Pharmingen), BVD6-24G2 (Pharmingen), and rIL-4 (Life Technologies, Gaithersburg, Md); IL-10, JES5-2A5 (Pharmingen), SXC-1 (Pharmingen), and rIL-10 (Pharmingen); and IFN- γ , R4-6A2 (Pharmingen), XMG1.2 (Pharmingen), and rIFN- γ (Life Technologies).

Isolation of splenic dendritic cells

Splenic dendritic cells were magnetically isolated by using magnetic microbead-conjugated anti-mouse CD11c mAbs (N418; Miltenyi Biotec, Auburn, Calif), as previously reported.¹⁶ In brief, CD11c⁺ dendritic cells were separated by passing the cell suspension over a magnetic-activated cell sorter MS+ column held in mini-MACS magnetic separator. The CD11c⁺ dendritic cells adhering to the columns were then used for both the *in vitro* IL-12 production and a flow cytometric analysis.

IL-12 is a heterodimeric cytokine composed of 2 disulfide-linked p40 and p35 subunits, and both subunits have to be expressed within the same cell to produce a biologically active p70 heterodimer. Because excessive p40 production leads to the formation of p40-p40 homodimers and thereafter inhibits the biologic activity of IL-12,¹⁷ both the IL-12 p40 and p70 concentrations in the culture supernatants were quantified by using a commercially available ELISA kit (Opt EIA, Pharmingen). In some experiments the surface expression levels of CD11c, MHC class II, F4/80, and CD80 were also analyzed by using flow cytometry.

Statistical analysis

All data are expressed as the mean $\pm$ SE. Statistical differences between the groups were determined by using a factorial ANOVA followed by an unpaired *t* test.

RESULTS

Kanamycin treatment during infancy reduces the total cell number of lymphocytes in the spleen and Peyer patches but not in the thymus

We first examined the effect of kanamycin treatment on the composition of the intestinal flora. As shown in Fig 1, almost all gram-negative bacteria in the stool were eliminated in the kanamycin-treated mice immediately after the termination of kanamycin administration. However, no such elimination of gram-negative bacteria was found at 10 weeks after the termination of kanamycin treatment, which thus suggests that such kanamycin treatment only temporarily changes the composition of the microflora. On the other hand, there were no significant differences in the number of gram-positive bacteria between the kanamycin-treated and control animals.

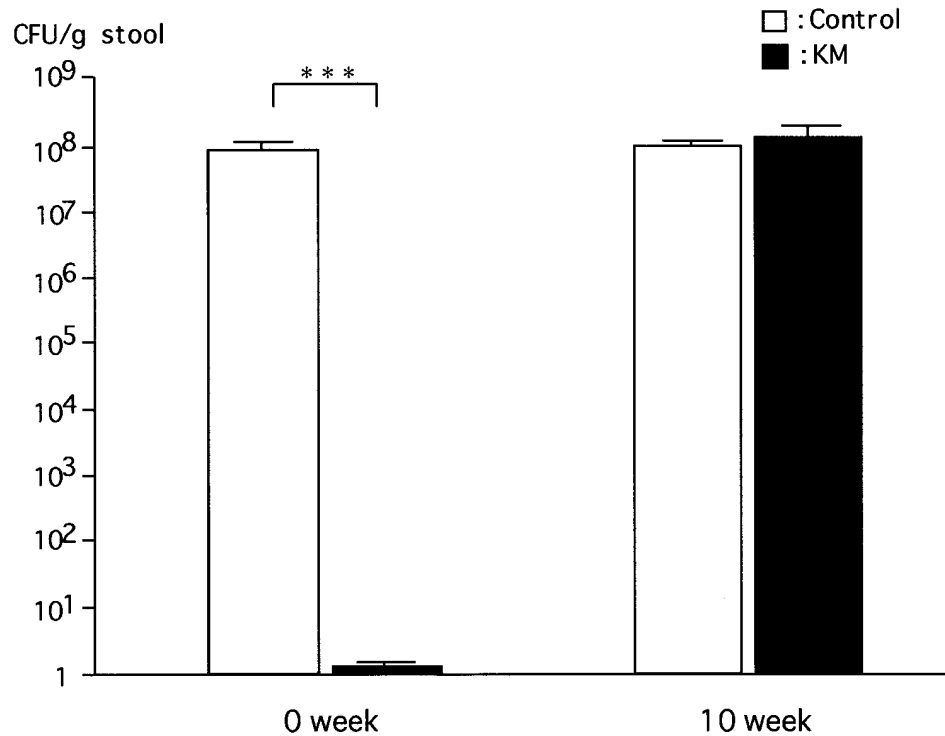


FIG 1. Elimination of gram-negative bacteria by kanamycin. Mice at 3 weeks of age were orally administered kanamycin for 7 consecutive days. Stool specimens were collected either immediately or 10 weeks after the cessation of kanamycin treatment. All data are expressed as the mean $\pm$ SE (n = 4). *** P < .001 was considered to be significantly different from the corresponding control mice.

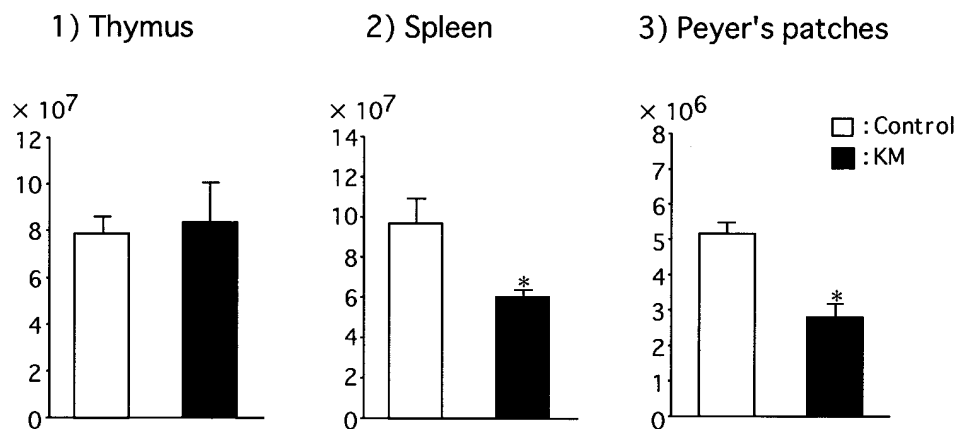


FIG 2. Effect of the kanamycin treatment on the total cell number of lymphocytes in the thymus, spleen, and Peyer patches. All mice were processed to count the total number of lymphocytes in the thymus, spleen, and Peyer patches at 10 weeks after the termination of the kanamycin treatment, as described in the "Methods" section. All data are expressed as the mean $\pm$ SE (n = 6). * P < .05 was considered to be significantly different from the corresponding control mice.

We next investigated the effects of kanamycin treatment on the number of lymphocytes in the spleen, Peyer patches, and thymus. As shown in Fig 2, the total cell number of lymphocytes in the spleen and Peyer patches in the kanamycin-treated animals were significantly lower than that in the control animals, even at 10 weeks after the

cessation of kanamycin administration, although there was no significant difference in the proportion of CD3⁺CD4⁺, CD3⁺CD8⁺, and B220⁺ cells between the groups of animals. In contrast, there was no significant difference in the number of thymocytes between both groups of animals.

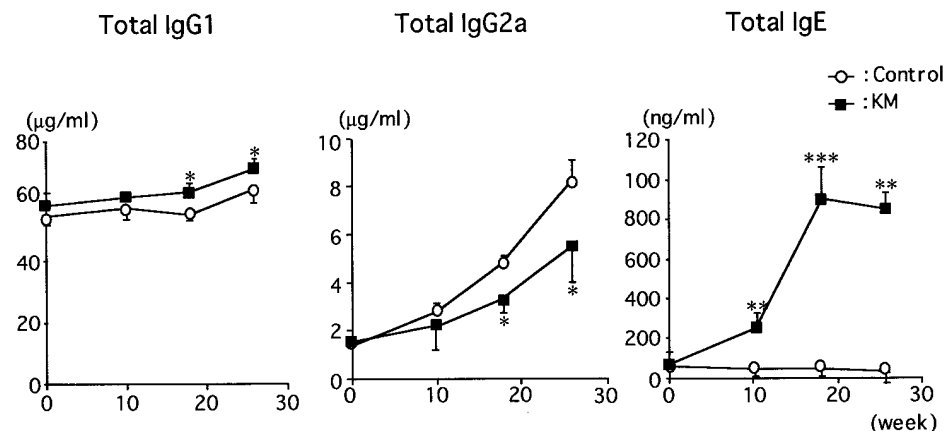


FIG 3. Effects of the kanamycin treatment on the serum IgE, IgG1, and IgG2a levels. Blood samples for measuring the antibody titer in the serum were collected on weeks 0 (immediately), 10, 18, and 26 after the cessation of kanamycin treatment. All data are expressed as the mean $\pm$ SE ($n = 6$). * $P < .05$, ** $P < .01$, and *** $P < .001$ were considered to be significantly different from the corresponding time point value in the control mice.

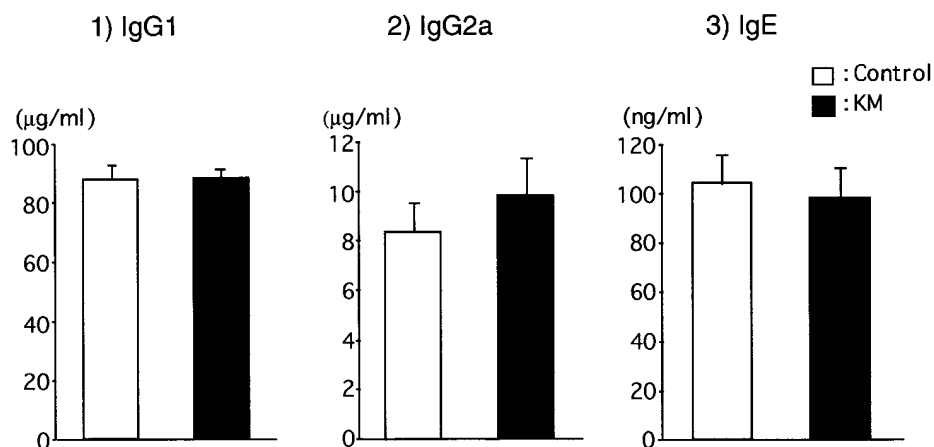


FIG 4. The kanamycin treatment during adolescence does not cause a shift in the T_H1/T_H2 balance toward T_H2 -dominant immunity. Mice at 52 weeks of age were orally administered kanamycin for 7 consecutive days. Blood samples for measuring the antibody titer in the serum were collected at 10 weeks after the cessation of kanamycin treatment. All data are expressed as the mean $\pm$ SE ($n = 6$).

Kanamycin treatment during infancy elevates the serum IgG1 and IgE antibody levels while decreasing the IgG2a antibody levels

To clarify the effect of antibiotic treatment on T_H1/T_H2 balance, we thus compared the difference in the serum IgE, IgG1, and IgG2a levels between the groups of either the kanamycin-treated or control mice. As shown in Fig 3, the serum levels of both the total IgG1 and IgE in the kanamycin-treated mice were significantly higher than those in the control mice at weeks 10, 18, and 26 after the cessation of kanamycin administration. In contrast, the serum level of total IgG2a in the kanamycin-treated mice was significantly lower than that in the control mice at weeks 18 and 26 after the cessation of kanamycin administration.

Interestingly, such an influence on serum antibody levels was only effective when kanamycin treatment

was performed in infants but not in mice at an older age (Fig 4).

Effect of kanamycin treatment on in vitro cytokine production

To further verify such kanamycin-induced T_H2 -skewed immune response, spleen cells from either the kanamycin-treated or control mice were stimulated with immobilized anti-CD3 antibody, and then the cytokine contents in the culture supernatant were examined.

As summarized in Table I, the level of IL-4 in the culture supernatant in the kanamycin-treated mice was higher than that in the control mice after the stimulation through anti-CD3 antibody, although the difference was not immediately statistically significant after terminating the kanamycin treatment. Similarly, the level of IL-10 in the kanamycin-treated mice was also higher than that in the control mice at weeks 0 and 10 after stopping the

TABLE I. Anti-CD3–stimulated cytokine production in spleen cells

Cytokine	Time measured	Control		Kanamycin	
		Medium	Anti-CD3	Medium	Anti-CD3
IL-2 (U/mL)	0 week*	1.21 ± 0.31	12.4 ± 2.05	1.15 ± 0.51	13.1 ± 2.31
	10 weeks†	1.03 ± 0.06	11.6 ± 1.38	0.91 ± 0.11	12.7 ± 3.64
IL-4 (pg/mL)	0 week	1.56 ± 0.48	19.8 ± 7.88	2.42 ± 0.96	34.2 ± 9.65
	10 weeks	2.11 ± 0.75	26.7 ± 9.71	2.16 ± 0.56	54.7 ± 22.3‡
IL-10 (ng/mL)	0 week	2.14 ± 0.62	6.21 ± 2.13	3.02 ± 0.98	8.25 ± 2.45
	10 weeks	3.16 ± 0.29	5.78 ± 1.23	3.25 ± 0.27	7.37 ± 1.29
IFN-γ (U/mL)	0 week	69.5 ± 9.26	1956 ± 432	58.8 ± 5.64	1250 ± 216‡
	10 weeks	81.3 ± 0.99	2963 ± 397	84.0 ± 1.53	1516 ± 342§

Spleen cells in the mice treated either with or without kanamycin were stimulated with immobilized anti-CD3 antibody at weeks 0 and 10 after the termination of kanamycin treatment. Culture supernatants were thereafter processed for the cytokine measurements, as described in the “Methods” section. For one group, 4 individual spleens were cultured separately to obtain the samples of the supernatant. All data are expressed as the mean ± SE (n = 4).

*Immediately after the cessation of kanamycin treatment.

†Ten weeks after the cessation of kanamycin treatment.

‡ $P < .05$ and § $P < .01$ were considered to be significantly different from the corresponding control values.

kanamycin treatment, although the difference was not of statistical significance. In contrast, the level of IFN-γ in the kanamycin-treated mice was significantly lower than that in the control mice at weeks 0 and 10 after stopping the kanamycin treatment. However, there was no significant difference in the IL-2 levels between the kanamycin-treated and control mice regardless of whether the cells were stimulated with immobilized anti-CD3 antibody or not.

Kanamycin treatment suppressed the IL-12 p70 production in splenic dendritic cells

To further clarify the mechanisms whereby decontamination with kanamycin treatment elicits such a T_H2 -polarized immune deviation, we next examined the ability of splenic dendritic cell to produce IL-12, which is a critical regulator in the development of maturation in T_H1 cells.¹⁸

The IL-12 p70 production in the kanamycin-treated animals was significantly lower than that in the control animals immediately after the termination of kanamycin treatment, although there was no significant difference in IL-12 p40 production between both groups of animals (p70: control 88.6 ± 19.5 ; kanamycin, 47.0 ± 6.8 [$P < .01$]; p40: control 243 ± 96.4 ; kanamycin 256 ± 45.8). On the other hand, no significant difference in the surface expression levels of MHC class II, CD80, and F4/80 was found between the groups of animals (data not shown).

DISCUSSION

Recently, accumulating evidence suggests that antibiotic use during infancy may be associated with the subsequent development of atopic disorders.^{9,10} Although the underlying mechanism to explain such an association still remains to be clarified, it is possible that an alteration in the size, composition, or both of the commensal microflora induced by such antibiotics may prevent the postnatal maturation of the immune system, thus inducing a T_H2 -polarized immune deviation. In the present

study kanamycin treatment was also suggested to possibly reduce the number of lymphocytes in the spleen and Peyer patches by altering the size, composition, or both of the microflora. The precise mechanism whereby a decreased number of lymphocytes may affect the T_H1/T_H2 balance was not definitely determined in this study; however, these findings may suggest that the adequate maturation of T_H1 cells requires the normal development of lymphoid tissues. Further studies are still needed to clarify the effect that such a reduction in the number of lymphocytes may have on the T_H1/T_H2 balance.

The administration of kanamycin increased the serum levels of total IgG1 and IgE while decreasing the serum IgG2a levels. Moreover, when spleen cells were stimulated with immobilized anti-CD3 antibody in vitro, such kanamycin treatment enhanced the in vitro IL-4 secretion while reducing the in vitro IFN-γ secretion. Because the production of IgE and IgG1 has been demonstrated to be dependent on T_H2 cells, whereas the production of IgG2a is required for the participation of T_H1 cells, these results suggest that such elimination of microflora by kanamycin treatment may prevent the T_H1 -mediated immune response and drive a shift in the T_H1/T_H2 balance toward T_H2 -dominant immunity.

Although IL-12 was initially recognized as a cytokine capable of synergizing with IL-2 to augment the cytotoxic lymphocyte responses,¹⁹ many reports have detailed the critical role of IL-12 in the regulation of T_H1/T_H2 balance, such as the upregulation of T_H1 cytokines, especially IFN-γ, and the downregulation of T_H2 cytokines.^{18,20} Because IL-12 is mainly produced by monocytes and dendritic cells in response to microbial stimulation,²¹ the capacity of monocytes and dendritic cells to produce IL-12 is thus considered to be a crucial factor in the regulation of T_H1 development. In fact, Tineke et al²² showed the production of IL-12 p70 to be reduced in patients with allergic asthma after stimulation of whole blood cultures with a *Staphylococcus aureus* Cowan I strain. Together with the fact that the IL-12 production in germ-free mice is dramatically lower than that

in specific-pathogen-free mice (our unpublished observation), the present results suggest that the depletion of the commensal microflora by kanamycin treatment reduces the IL-12 production of dendritic cells in response to microbial stimulation, thus inducing a subsequent T_H2 -skewed immune deviation. Nonetheless, the possibility that the positive selection of spleen dendritic cells may affect the functionality of the cells, such as the cytokine production, cannot be ruled out. Further investigation with negative selection is thus called for to elucidate the ability of dendritic cells to produce IL-12.

It is also possible that kanamycin may exert some direct action on the regulation of the T_H1/T_H2 balance independently of indigenous microflora; however, this possibility seems unlikely because neither an increase in IL-4 nor a decrease in IFN- γ production in the spleen cells was induced even by a sufficiently high concentration of kanamycin when the cells were cultured in the presence of various concentrations of kanamycin. Moreover, the non-specific effect by kanamycin was also unlikely because such a T_H2 -skewed effect was not seen when the mice were administered kanamycin at a later stage. Further studies are now in progress to determine whether the oral introduction of probiotics, defined as a live microbial feed supplement, can reverse such kanamycin-induced immune deviation by improving the intestinal microbial balance.

One of the central enigmas of allergy literature is the concept of a *sensitization window* during infancy.²³ This concept is based on a wide body of epidemiologic literature, indicating that allergen challenge of the immune system during infancy predisposes the child toward development of a long-term, T_H2 -skewed, allergen-specific, immunologic memory. This concept is also supported in the present study, in which kanamycin treatment in adolescents had no effect on the serum IgE, IgG1, and IgG2a levels. More importantly, it has recently been demonstrated that the key causative factor in atopic disease may not be the initial acquisition of allergen-specific T_H2 -skewed immunity per se but instead may be the efficiency of immune deviation mechanisms, which in normal (nonatopic) individuals redirect these fetal immune responses toward the T_H1 cytokine phenotype.²⁴⁻²⁶ The gastrointestinal tract is colonized with more than 10^{14} microorganisms that weigh more than 1 kg,⁷ which thus suggests that the intestinal microflora is quantitatively the most important source of microbial stimulation and may provide a primary signal for inducing the postnatal maturation of T_H1 -like immunity.

If a causal link does exist between increased antibiotic use and the increasing frequency of atopy, then this phenomenon could lead to further interesting possibilities. Because it is widely acknowledged that many antibiotic courses used in childhood are unnecessary or ineffective,²⁷ more appropriate guidelines for antibiotic use might be useful in preventing the continuing rise of atopy. In addition, the identification of the commensal organisms and their products, which are most important in the postnatal maturation of T_H1 cells, might also help us to eventually develop useful preventive agents in atopy.²⁸

In conclusion, we have provided evidence that the elimination of indigenous microflora by antibiotic use during infancy may induce a shift in the T_H1/T_H2 balance toward T_H2 -dominant immunity. Clearly, further studies are still called for, but this work may provide a useful animal model for elucidating the precise mechanism of the complex interaction between antibiotic use and atopic disorders.

REFERENCES

1. The International Study of Asthma and Allergies in Childhood (ISSAC) Steering Committee. World variation in prevalence of symptoms of asthma, allergic rhinoconjunctivitis, and atopic eczema: ISAAC. *Lancet* 1998;351:1225-32.
2. Mutius EV. The environmental predictors of allergic disease. *J Allergy Clin Immunol* 2000;105:9-19.
3. Shirakawa T, Enomoto T, Shimazu SI, Hopkins JM. The inverse association between tuberculin responses and atopic disorder. *Science* 1997;275:77-9.
4. Shaheen SO, Aaby P, Hall AJ, Barker DJP, Heyes CB, Shiell AW, et al. Measles and atopy in Guinea-Bissau. *Lancet* 1996;347:1792-6.
5. Matricardi PM, Rosmini F, Ferrigno L, Nisini R, Rapicetta M, Chionne P. Cross sectional retrospective study of prevalence of atopy among Italian military students with antibodies against hepatitis A virus. *BMJ* 1997;314:999-1003.
6. Holt PG, Macaubas C, Prescott SL, Sly PD. Microbial stimulation as an aetiological factor in atopic disease. *Allergy* 1999;54(Suppl 49):12-6.
7. Björkstén B. The intrauterine and postnatal environments. *J Allergy Clin Immunol* 1999;104:1119-27.
8. Sudo N, Sawamura S, Tanaka K, Aiba Y, Kubo C, Koga Y. The requirement of intestinal bacterial flora for the development of an IgE production system fully susceptible to oral tolerance induction. *J Immunol* 1997;159:1739-45.
9. Wickens K, Pearce N, Crane J, Beasley R. Antibiotic use in early childhood and the development of asthma. *Clin Exp Allergy* 1999;29:766-71.
10. Farooqi IS, Hopkins JM. Early childhood infection and atopic disorder. *Thorax* 1998;53:927-32.
11. Tada T, Yamamura S, Kuwano Y, Abo T. Level of myelopoiesis in the bone marrow is influenced by intestinal flora. *Cell Immunol* 1996;173:155-61.
12. Benno Y, Mitsuoka T. Impact of *Bifidobacterium longum* on human fecal microflora. *Microbiol Immunol* 1992;36:683-94.
13. Fukui Y, Sudo N, Yu XN, Nukina H, Sogawa H, Kubo C. The restraint stress-induced reduction in lymphocyte cell number in lymphoid organs correlates with the suppression of in vivo antibody production. *J Neuroimmunol* 1997;79:211-7.
14. Garside P, Steel M, Worthey EA, Satoskar A, Alexander J, Bluethmann H, et al. T helper 2 cells are subject to high dose oral tolerance and are not essential for its induction. *J Immunol* 1995;154:5649-55.
15. Kruszezwska B, Felten SY, Moynihan JA. Alterations in cytokines and antibody production following chemical sympathectomy in two strains of mice. *J Immunol* 1995;155:4613-20.
16. Marriott I, Hammond TG, Thomas EK, Bost KL. Salmonella efficiently enter and survive within cultured CD11c+ dendritic cells initiating cytokine expression. *Eur J Immunol* 1999;29:1107-15.
17. Gillissen S, Carvajal D, Ling P, Podlaski FJ, Stremlo DL, Familletti PC, et al. Mouse interleukin 12 (IL-12) p40 homodimer—a potent IL-12 antagonist. *Eur J Immunol* 1995;25:200-6.
18. Gavett SH, O'Hearn DJ, Li X, Huang SK, Finkelman FD, Wills-Karp M. Interleukin 12 inhibits antigen-induced airway hyperresponsiveness, inflammation, and Th2 cytokine expression in mice. *J Exp Med* 1995;182:1527-36.
19. Stern AS, Podlaski FJ, Hulmes JD, Pan Y-CE, Quinn PM, Wolitzky AG, et al. Purification to homogeneity and partial characterization of cytotoxic lymphocyte maturation factor from human B-lymphoblastoid cells. *Proc Natl Acad Sci U S A* 1990;87:6808-12.
20. Brunda MJ. Interleukin-12. *J Leukoc Biol* 1994;55:280-8.
21. Verhasselt V, Buelens C, Willems F, Groote DD, Haeffer-Cavaillon N, Goldman M. Bacterial lipopolysaccharide stimulates the production of

- cytokines and the expression of costimulatory molecules by human peripheral blood dendritic cells. *J Immunol* 1997;158:2919-25.
22. Tineke CTM, Boeijs LCM, de Groot ER, Stapel SO, Sniijders A, Kapsenberg ML, et al. Reduced production of IL-12 and IL-12-dependent IFN- γ release in patients with allergic asthma. *J Immunol* 1997;158:5560-5.
 23. Holt PG. Immunoprophylaxis of atopy: light at the end of the tunnel. *Immunol Today* 1994;15:484-9.
 24. Lin H, Mosmann TR, Guilbert L, Tuntipopipat S, Wegmann TG. Synthesis of T helper 2-type cytokines at the maternal-fetal interface. *J Immunol* 1993;151:4562-73.
 25. Chen N, Gao Q, Field EH. Expansion of memory Th2 cells over Th1 cells in neonatal primed mice. *Transplantation* 1995;60:1187-93.
 26. Prescott SL, Macaubas C, Holt BJ, Smallacombe TB, Loh R, Sly PD, et al. Transplacental priming of the human immune system to environmental allergens: universal skewing of initial T cell responses toward the Th2 cytokine profile. *J Immunol* 1998;160:4730-7.
 27. Nyquist AC, Gonzales R, Steiner JF, Sande MA. Antibiotic prescribing for children with colds, upper respiratory tract infections, and bronchitis. *JAMA* 1998;279:875-7.
 28. Majamaa H, Isolauri E. Probiotics: a novel approach in the management of food allergy. *J Allergy Clin Immunol* 1997;99:179-85.