Kizaki T, Oh-ishi S, Ookawara T, Yamamoto M, Izawa T & Ohno H (1996). Glucocorticoid-mediated generation of suppressor macrophages with high density FcγRII during acute cold stress. *Endocrinology* **137**, 4260–4267.

Liu D, Diorio J, Day JC, Francis D & Meaney MJ (2000). Maternal care, hippocampal synaptogenesis and cognitive development in rats. *Nat Neurosci* **3**, 799–806.

Liu D, Diorio J, Tannenbaum B, Caldji C, Francis D, Freedman A, Sharma S, Pearson D, Plotsky PM & Meaney MJ (1997). Maternal care, hippocampal glucocorticoid receptors, and hypothalamic-pituitary-adrenal responses to stress. *Science* **277**, 1659–1662.

McEwen BS (1998). Protective and damaging effects of stress mediators. *N Engl J Med* **338**, 171–179.

Meaney MJ, Aitken DH, Bhatnagar S, Van Berkel C & Sapolsky RM (1988). Postnatal handling attenuates neuroendocrine, anatomical, and cognitive impairments related to the aged hippocampus. *Science* **238**, 1766–1768.

Mitsuoka T, Sega T & Yamamoto S (1965). Eine verbesserte Methodik der qualitativen und quantitaven Analyse der Darmflora von Menschen und Tieren. *Zbl Bakt Hyg I Abt Orig* **195**, 445–469.

Neta R, Perlstein R, Vogel SN, Ledney D & Abrams J (1992). Role of interleukin 6 in protection from lethal irradiation and in endocrine responses to IL-1 and tumor necrosis factor. *J Exp Med* **175**, 689–694.

Nukina H, Sudo N, Aiba Y, Oyama N, Koga Y & Kubo C (2001). Restraint stress elevates the plasma interleukin-6 levels in germ-free mice. *J Neuroimmunol* **115**, 46–52.

Nukina H, Sudo N, Komaki G, Yu XN, Mine K & Kubo C (1998). The restraint stress-induced elevation in plasma interleukin-6 negatively regulates the plasma TNF-α level. *Neuroimmunomodulation* **5**, 323–327.

Paxinos G & Franklin KB (2001). *The Mouse Brain in Stereotaxic Coordinates*, 2nd edn. Academic Press, London.

Porter JR & Rettger LF (1940). Influence of diet on the distribution of bacteria in the stomach, small intestine and cecum of the white rat. *J Infect Dis* **66**, 104–110.

Rescigno M, Urbano M, Valzasina B, Francolini M, Rotta G, Bonasio R, Granucci F, Kraehenbuhl JP & Ricciardi-Castagnoli P (2001). Dendritic cells express tight junction proteins and penetrate gut epithelial monolayers to sample bacteria. *Nat Immunol* **2**, 361–367.

Roceri M, Hendriks W, Racagni G, Ellenbroek BA & Riva MA (2002). Early maternal deprivation reduces the expression of BDNF and NMDA receptor subunits in rat hippocampus. *Mol Psychiatry* **7**, 609–616.

Schmidt M, Oitzl MS, Levine S & de Kloet ER (2002). The HPA system during the postnatal development of CD1 mice and the effects of maternal deprivation. *Dev Brain Res* **139**, 39–49.

Shanks N, Larocque S & Meaney MJ (1995). Neonatal endotoxin exposure alters the development of the hypothalamic-pituitary-adrenal axis: early illness and later responsivity. *J Neurosci* **15**, 376–384.

Shanks N, Windle RJ, Perks PA, Harbuz MS, Jessop DS, Ingram CD & Lightman S (2000). Early-life exposure to endotoxin alters hypothalamic-pituitary-adrenal function and predisposition to inflammation. *Proc Natl Acad Sci U S A* **97**, 5645–5650.

Starnes HF, Pearce MK, Tewari A, Yim HH, Zou JC & Abrams JS (1990). Anti-IL-6 monoclonal antibodies protect against lethal *Escherichia coli* infection and lethal tumor necrosis factor-α challenge in mice. *J Immunol* **145**, 4185–4191.

Steffen EK, Berg RD & Deitch EA (1988). Comparison of translocation rates of various indigenous bacteria from the gastrointestinal tract to the mesenteric lymph node. *J Infect Dis* **157**, 1032–1038.

Sudo N, Aiba Y, Takaki A, Yu XN, Oyama N, Koga Y & Kubo C (2000). Dietary nucleic acids promote a shift in Th1/Th2 balance toward Th1-dominant immunity. *Clin Exp Allergy* **30**, 979–987.

Sudo N, Sawamura SA, Tanaka K, Aiba Y, Kubo C & Koga Y (1997). The requirement of intestinal bacterial flora for the development of an IgE production system fully susceptible to oral tolerance induction. *J Immunol* **159**, 1739–1745.

Suzuki K, Harasawa Y, Yoshitake Y & Mitsuoka T (1983). Effects of crowding and heat stress on intestinal flora, body weight gain, and feed efficiency of growing rats and chickens. *Jpn J Vet Sci* **45**, 331–338.

Tannock GW (1999). The normal microflora: an introduction. In *Medical Importance of the Normal Micro Flora*, ed. Tannock GW, pp. 1–23. Kluwer, Dordrecht, The Netherlands.

Tannock GW & Savage DC (1974). Influences of dietary and environmental stress on microbial populations in the murine gastrointestinal tract. *Infect Immun* **9**, 591–598.

Turnbull AV & Rivier CL (1999). Regulation of the hypothalamic-pituitary-adrenal axis by cytokines: actions and mechanisms of action. *Physiol Rev* **79**, 1–71.

Wang X, Wang BR, Zhang XJ, Xu Z, Ding YQ & Ju G (2002). Evidences for vagus nerve in maintenance of immune balance and transmission of immune information from gut to brain in STM-infected rats. *World J Gastroenterol* **8**, 540–545.

Zahradnik R, Brennam G, Hutchison JS & Odell WD (1989). Immunoradiometric assay of corticotropin with use of avidin-biotin separation. *Clin Chem* **35**, 804–807.

Acknowledgements

This work was supported in part by a Grant-in-Aid for General Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology, Japan (no. 13470014-0) and by a Grant-in-Aid from the Yakult Bioscience Research Foundation.

Biosci. Biotechnol. Biochem., 69 (3), 099-1–4, 2005

Note

Molecular Monitoring of the Developmental Bacterial Community in the Gastrointestinal Tract of Japanese Infants

Prapa SONGJINDA,[1] Jiro NAKAYAMA,[1,†] Yumiko KUROKI,[1] Shigemitsu TANAKA,[1] Sanae FUKUDA,[2] Chikako KIYOHARA,[3] Tetsuro YAMAMOTO,[2,4] Kunio IZUCHI,[5] Taro SHIRAKAWA,[2] and Kenji SONOMOTO[1]

[1]*Laboratory of Microbial Technology, Division of Microbial Science and Technology, Department of Bioscience and Biotechnology, Faculty of Agriculture, Graduate School, Kyushu University, 6-10-1 Hakozaki, Higashi-ku, Fukuoka 812-8581, Japan*

[2]*Department of Health Promotion and Human Behavior, Kyoto University Graduate School of Public Health, Yoshida-Konoe cho, Sakyo-ku, Kyoto 606-8501, Japan*

[3]*Department of Preventive Medicine, Division of Social Medicine, Graduate School of Medical Sciences, Kyushu University, Maidashi 3-1-1, Higashi-ku, Fukuoka 812-8582, Japan*

[4]*Total Technology Consultant, 1-20-2 Ebisunishi, Shibuya-ku, Tokyo 150-0021, Japan*

[5]*Izuchi Hospital, 4-15-6 Yakuin, Chuo-ku, Fukuoka 810-0022, Japan*

Received October 18, 2004; Accepted November 26, 2004

The dynamics of the developmental bacterial community in the Japanese neonatal gastrointestinal tract were examined by monitoring 16S ribosomal RNA gene (rDNA) diversity in fecal samples by PCR and denaturing gradient gel electrophoresis (DGGE). The results showed a certain pattern common in infants without antibiotic treatment, in which aerobes, *e.g.*, *Pseudomonas*, appeared first and were then immediately replaced by facultative anaerobe, *Enterococcus*, *Streptococcus*, and *Enterobacteriaceae* through the first month, and finally strictly anaerobic *Bifidobactrerium* appeared.

Key words: denaturing gradient gel electrophoresis (DGGE); gastrointestinal tract; infant; 16S rRNA; intestinal microbiota

Soon after birth, bacterial colonization starts in the previously germfree gastrointestinal tract and commensal host-microbial relationships begin.[1,2] The colonizing bacteria contribute to maintenance of the mucosal barrier, facilitate carbohydrate assimilation, and modulate the mucosal immune system. Thus, the initial development of intestinal microbiota is considered to have great influence on the health of the infant. In this study, the succession of the gastrointestinal bacterial community was examined for the first two months in nine Japanese infants by monitoring 16S ribosomal RNA gene (rDNA) diversity in fecal samples.

All infant subjects (infants nos. 1, 2, 5, 6, 10, 20, 24, 25, and 33) participated in this study were vaginally delivered. Infant no. 5 was fed formula milk and the other infants were fed both breast and formula milk during the sampling period. Infants no. 1 and no. 33 were subjected to antibiotic therapy, receiving cefalex (50 mg/kg, 4 times a day) the first four days, whereas infant no. 5 was treated on day 0 only. Fecal samples were collected on day 0/1, day 3, day 5, month 1, and month 2 (there was no month-2 sample from infant no. 5). All the parents of our subjects gave written informed consent and the Ethics Committee of the Faculty of Medicine of Kyoto University approved this study protocol.

DNA was isolated from each fecal sample using a bead beating method essentially as previously described,[3] except for 2–3 times washing of the fecal sample before the bead beating step. In order to construct 16S rDNA libraries, a V1–V3 region of 16S rDNA was amplified from each sample by PCR with 8UA (5′-AGAGTTTGATCCTGGCTCAG-3′)[4] and 519B (5′-ATTACCGCSGCTGCTG-3′)[5] primers, and cloned into a pGEM-T vector (Promega, Madison, WI), and transformed in *E. coli* JM109. About ten clones from each library were sequenced. In total, 357 clones were sequenced and the ribotypes found are summarized with the result of the database search in Table 1.

PCR-denaturing gradient gel electrophoresis (PCR-DGGE), which allowed rapid and efficient molecular fingerprinting of gut microbiota,[3,6] was performed in order to monitor the succession of the infant fecal bacterial community. The variable region V2–V3 of 16S rDNA was amplified by PCR using primers HDA1-GC (5′-CGC CCG GGG CGC GCC CCG GGC GGG GCG

† To whom correspondence should be addressed. Tel/Fax: +81-92-642-3020; E-mail: nakayama@agr.kyushu-u.ac.jp

Table 1. List of Ribotypes Obtained in This Study

Ribotype	Closest sequence relative[a] (species)	% identity	Numbers			Accession no.
			clones	subjects	DGGE[b]	
Gram-positive facultative anaerobes						
Ef	*Enterococcus faecalis*	99.4	25	4	7	AY635946
Ss	*Streptococcus salivarius*	99.8	14	6	3	AY635947
Sp	*Streptococcus parasanguis*	96.4	12	3	7	AY635948
Se	*Staphylococcus epidermidis*	100.0	11	4	8	AY635949
Pa	*Propionibacterium avidum*	99.0	4	3	0	AY635950
Em	*Enterococcus faecium*	99.8	4	2	6	AY635951
Sa	*Streptococcus anginosus*	99.8	4	1	1	AY635952
Lg	*Lactobacillus gasseri*	99.8	2	2	0	AY635953
St	*Streptococcus thermophilus*	99.8	2	1	5	AY635954
Sm'	*Streptococcus mitis*	96.6	2	1	0	AY635955
Sm	*Streptococcus mitis*	99.0	1	1	4	AY635956
Sc	*Streptococcus cremoris*	99.1	1	1	3	AY635957
Si	*Streptococcus infantarius*	99.6	1	1	1	AY635958
Gram-positive strict anaerobes						
Cb	*Clostridium butyricum*	100.0	25	4	4	AY635959
Bd	*Bifidobacterium dentium*	99.0	11	5	5	AY635960
Bp	*Bifidobacterium pseudocatanulatum*	100.0	11	2	5	AY635961
Fm	*Finegoldia magna*	98.8	2	2	0	AY635962
Rm	*Ruminococcus* sp.	99.0	2	1	1	AY635963
Ch	*Clostridium hathewayi*	98.6	3	1	0	AY635964
Gram-positive aerobes						
Mm	*Micrococcus mucilaqginosus*	98.8	5	2	1	AY635965
Ar	*Acinetobacter rhizospaerue*	98.7	5	1	0	AY635966
Gram-negative facultative anaerobes						
Es	*Enterobacter* sp. B901-2	99.8	32	5	6	AY635967
Kp	*Klebsiella pneumoniae*	99.2	31	6	2	AY635968
Km	*Klebsiella milletis*	99.0	19	8	6	AY635969
Ec	*Escherichia coli*	99.2	15	2	3	AY635970
Ko	*Klebsiella oxytoca*	99.6	12	2	2	AY635971
Cd	*Citrobacter diversus*	98.5	5	2	0	AY635972
Kr	*Klebsiella rennanqify*	99.0	9	3	4	AY635973
Cg	*Calymmatobacterium granulomatis*	99.0	7	4	1	AY635974
Ea	*Enterobacter aerogenes*	97.8	5	2	0	AY635975
Km'	*Klebsiella milletis*	99.0	3	3	1	AY635976
Sf	*Escherichia coli*	99.4	3	2	0	AY635977
Ko'	*Klebsiella oxytoca*	98.3	2	1	1	AY635978
Kr'	*Klebsiella rennanqify*	98.9	2	1	0	AY635979
Gram-negative strict anaerobes						
Bu	*Bacteroides uniformis*	99.4	8	3	1	AY635980
Vp	*Veillonella parvula*	98.9	1	1	1	AY635981
Vp'	*Veillonella parvula*	98.4	1	1	1	AY635982
Gram-negative aerobes						
Pm	*Pseudomonas marginalis/reactans/veronii*	99.4	28	5	5	AY635983
Fh	*Flavobacterium heparinum*	98.1	8	3	3	AY635984
Pt	*Pseudomonas tolaasii*	99.6	7	4	2	AY635985
Ad	*Acidovorax deflvii*	99.4	6	1	1	AY635986
Ph	*Phyllobacterium myrsinacearum*	99.6	4	1	0	AY635987
Pr	*Pseudomonas trivialis/poae*	99.6	2	2	2	AY635988

Ribotypes found with more than two clones in the 16S rDNA libraries or found only once but also detected in DGGE are listed.
[a]Cultured bacteria in Genbank showing highest identity as a result of Blast search.
[b]The number of bands corresponding to the indicated species in the DGGE (Fig. 1).

GGG GCA CGG GGG GAC TCC TAC GGG AGG CAG CAG T-3') and HDA2 (5'-GTA TTA CCG CGG CTG CTG GCA C-3').[7] The PCR condition was as follows: 94 °C for 5 min, 30 cycles consisting of 94 °C for 40 s, 58 °C for 20 s, and 72 °C for 1 min, and finally 72 °C for 5 min. DGGE analysis was performed as described by Muyzer *et al.*[8] and Heilig *et al.*[9] using a Dcode System apparatus (Bio-Rad, CA). Each band in the DGGE gel was assigned one of the ribotypes in Table 1 either by sequencing of DNA fragments excised from the DGGE gel or by comparing band positions with those of reference clones derived from the 16S rDNA clone library.

Figure 1 shows the DGGE profile of nine subjects. Although each subject showed individual banding patterns, a stepwise development from aerobic to

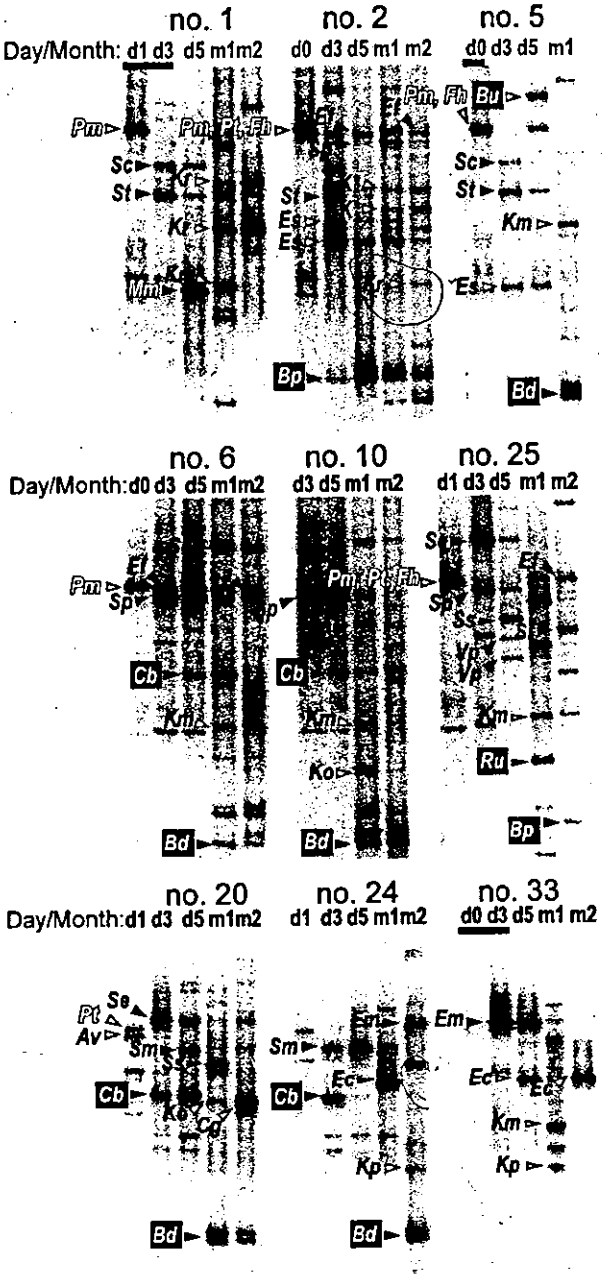

Fig. 1. PCR-DGGE Profiles Representing the Fecal Bacterial Community in the First Two Months of Nine Infants.

The bands identified with ribotypes in the 16S rDNA clone libraries are indicated by arrowheads with their names listed in Table 1, except K* which indicates unidentified *Klebsiella* spp. Open and black arrowheads represent Gram-negative and Gram-positive bacteria respectively. Open, black, and outlined letters represent aerobic, facultatively anaerobic, and strictly anaerobic bacteria respectively. Bold lines under the sampling days indicate the period of antibiotic treatment. Electrophoresis was done in 8% polyacylamide gel with a denaturing gradient of 30–65%, where 100% corresponds to 7 M urea and 40% formamide. The gel was run at 100 V for 6.5 h at 60°C and then stained in 1 × SYBR Gold (Molecular Probes, Eugene, OR).

anaerobic microbial ecosystem was observed in the succession of bacterial composition in the seven subjects (nos. 2, 5, 6, 10, 20, 24, and 25) without successive antibiotic treatment in the first four days. In the beginning, bands corresponding to aerobic Gram-negative bacteria such as *Pseudomonas* appeared and they were then replaced by facultatively anaerobic bacteria such as *Streptococcus*, *Enterococcus*, or *Staphylococcus epidermidis* and *Enterobacteriaceae*. Particularly, strong bands closely related to *Streptococcus parasanguis*, *Streptococcus cremoris* and *Streptococcus thermophilus* appeared on day 3 in many subjects. It is interesting to note that a large majority of the bacteria types such as *Streptococcus parasanguis*, *Streptococcus mitis*, *Streptococcus salivarius*, *Bifidobacterium dentium*, and *Veillonella parvula* detected in this period are regarded as oral-origin bacteria rather than intestinal species. This suggests that these oral-origin bacteria may transiently colonize the intestine during this period.

Bifidobacteria detected in the bottom part of the DGGE gel appeared within two months in most subjects. In infant no. 2, *Bifidobacterium pseudocatanulatum* colonized predominantly from day 3 and continued until 2 months of age. This subject was only the case which agreed with the finding of previous studies,[6,10,11] showing that bifidobacteria usually appear and become dominant within a week after birth. The appearance of bifidobacterial bands in infant no. 2 was concomitant with a decrease in *Enterobacteriaceae* bands, which appeared as dominant on day 3. A concomitant decrease in *Enterobacteriaceae* with an increase of bifidobacteria in breast-milk fed infants has been reported.[12] Bands related to *Clostridium butyricum* were also detected in infants nos. 6, 10, 20, and 24, in which they appeared earlier than bifidobacteria. The other strict anaerobes, *Veillonella parvula*-like bacteria, and *Ruminococcus* sp. were found only in infant no. 25. *Bacteroides uniformis* was detected only at day 5 in infant no. 5 (antibiotic treatment on day 0) who was the only subject brought up only on formula milk.

Infants nos. 1 and 33 treated with antibiotics in the first 3 days showed relatively simple microbiota, and the developmental patterns deviated remarkably from the trends observed in the other subjects without antibiotic treatment. In infant no. 1, a dominant band corresponding to *Micrococcus mucilaginosus*, which is not a common inhabitant of the intestine, appeared suddenly on day 5 and completely disappeared during month 1. In infant no. 33, a dominant band corresponding to *Enterococcus faecium* appeared on day 3 and disappeared during month 1. No bands corresponding to bifidobacteria and other strict anaerobes were found in the testing period in either baby and only bands corresponding to *Enterobacteriaceae* were found during month 1 and month 2, suggesting domination by *Enterobacteriaceae*. This was also indicated by the data of random sequencing of 16S rDNA clone libraries, which showed that all 30 clones sequenced from the month-1 and month-2 libraries of these two subjects belonged to *Enterobacteriaceae*. These data showed that antibiotic treatment at the beginning of life has strong

influence on the establishment of a normal microbial ecosystem in the intestine.

In conclusion, this molecular study indicates the stepwise development from aerobic to anaerobic microbial ecosystem with a variety of bacterial groups, although the process differed among individuals at the species level. The step of the bacterial colonization in the gastrointestinal tract is most likely a key to the developmental process. Strong antibiotic treatment interrupted the development of normal microbiota, including bifidobacteria. Further studies with modern molecular methods are needed for understanding of the environmental and host factors affecting the developmental process of neonatal microbiota.

Acknowledgment

We thank all the families who provided fecal samples for this study. This research was supported in part by a Research Grant for Immunology, Allergy, and Organ Transplants from the Ministry of Health and Welfare of Japan, by a grant from Danon Institute for the Promotion of Health and Nutrition, and by a grant from Takeda Science Foundation.

References

1) Mackie, R. I., Sghir, A., and Gaskins, H. R., Developmental microbial ecology of the neonatal gastrointestinal tract. *Am. J. Clin. Nutr.*, **69** (Suppl.), 1035S–1045S (1999).
2) Hooper, L. V., and Gordon, J. I., Commensal host-bacterial relationships in the gut. *Science*, **292**, 1115–1118 (2001).
3) Zoetendal, E. G., Akkermans, A. D. L., and de Vos, W. M., Temperature gradient gel electrophoresis analysis of 16S rRNA from human fecal samples reveals stable and host-specific communities of active bacteria. *Appl. Environ. Microbiol.*, **64**, 3854–3859 (1998).
4) Acinas, S. G., Anton, J., and Rodrigues-Valera, F., Diversity of free-living and attached bacteria in offshore western Mediterranean waters as depicted by analysis of genes encoding 16S rRNA. *Appl. Environ. Microbiol.*, **65**, 514–522 (1999).
5) Bertilsson, S., Cavanaugh, C. M., and Polz, M. F., Sequencing-independent method to generate oligonucleotide probes targeting a variable region in bacterial 16S rRNA by PCR with detachable primers. *Appl. Environ. Microbiol.*, **68**, 6077–6086 (2002).
6) Favier, C. F., Vaughan, E. E., de Vos, W. M., and Akkermans, A. D. L., Molecular monitoring of succession of bacterial communities in human neonates. *Appl. Environ. Microbiol.*, **68**, 219–226 (2002).
7) Tannock, G. W., Munro, K., Harmsen, H. J. M., Welling, G. W., Smart, J., and Gopal, P. K., Analysis of the fecal microflora of human subjects consuming a probiotic product containing *Lactobacillus rhamnosus* DR20. *Appl. Environ. Microbiol.*, **66**, 2578–2588 (2000).
8) Muyzer, G., de Vaal, E., and Uitterlinden, A., Profiling of complex microbial populations by denaturing gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Appl. Environ. Microbiol.*, **59**, 695–700 (1993).
9) Heilig, H. G. H. J., Zoetendal, E. G., Vaughan, E. E., Marteau, P., Akkermans, A. D. L,, and de Vos, W. M., Molecular diversity of *Lactobacillus* spp. and other lactic acid bacteria in the human intestine as determined by specific amplification of 16S ribosomal DNA. *Appl. Environ. Microbiol.*, **68**, 114–123 (2002).
10) Benno, Y., and Mitsuoka, T., Development of intestinal microflora in humans and animals. *Bifidobacteria Microflora*, **5**, 13–25 (1986).
11) Weiling, G. W., Wildeboer-Veloo, L., Raangs, G. C., Franks, A. H., Jansen, G. J., Tonk, R. H. J., Degener, J. E., and Harmsen, H. J. M., Variations of bacterial populations in human feces measured by FISH with group-sepcific 16S rRNA-targeted oligonucleotide probes. In "Molecular Ecological Detection and Identification of Intestinal Microflora", ed. Mitsuoka, T., Japan Scientific Societies Press, Tokyo, pp. 7–18 (2000).
12) Yoshioka, H., Iseki, K., and Fugita, K., Development and differences of intestinal flora in the neonatal period in breast-fed and bottle-fed infants. *Pediatrics*, **72**, 317–321 (1983).

High Levels of Urinary Pentosidine, an Advanced Glycation End Product, in Children With Acute Exacerbation of Atopic Dermatitis: Relationship With Oxidative Stress

Hirokazu Tsukahara, Rumiko Shibata, Naoko Ohta, Shuko Sato, Masahiro Hiraoka, Setsuko Ito, Eisei Noiri, and Mitsufumi Mayumi

Pentosidine is an advanced glycation end product formed by sequential glycation and oxidation. The formation of pentosidine is increased in diseases associated with oxidative stress, such as inflammatory conditions. The aim of the present study was to determine the urinary concentration of pentosidine in atopic dermatitis (AD) and its relationship to the inflammatory status of AD. Urine samples of 32 children with AD and 30 age-matched healthy control subjects were assayed for pentosidine, pyrraline (another advanced glycation end product formed by nonoxidative glycation), and 8-hydroxy-2'-deoxyguanosine (8-OHdG) (an established marker of oxidative stress). Of these 3 markers, urinary concentrations of pentosidine were significantly higher in patients with acute exacerbation of AD than in healthy controls and patients with stable AD. Urinary concentrations of 8-OHdG were significantly higher in AD patients with and without acute exacerbation than in healthy controls. Urinary pentosidine levels correlated significantly with those of 8-OHdG when all data of healthy controls and AD patients were plotted. In patients with acute exacerbation of AD, both urinary pentosidine and 8-OHdG significantly decreased after 7 to 9 days of treatment. Our findings in patients with acute exacerbation of AD suggest that pentosidine levels are partly determined by the prevailing oxidative stress in these patients.

PENTOSIDINE IS one of several chemically characterized advanced glycation end products (AGEs).[1,2] Pentosidine exists in human tissues, such as skin, kidney, cartilage, aorta, and lens protein and in blood and urine, and its concentrations in tissues increase with age and are increased in diabetes and, more overtly, in uremia. Blood pentosidine levels are also elevated in the latter 2 conditions.[3,4] Pentosidine is formed by sequential glycation and oxidation (thereby termed "glycoxidation") reactions between carbohydrate-derived carbonyl group and protein amino group, known as the Maillard reaction.[5] Its formation requires aerobic conditions, while an antioxidative condition inhibits such reaction. On the other hand, pyrraline, another AGE, is derived from nonoxidative chemistry.[6] Therefore, it is hypothesized that formation of pentosidine, but not of pyrraline, is accelerated in diseases accompanied by oxidative stress.

Atopic dermatitis (AD) is a chronic, relapsing inflammatory disease characterized by extreme pruritus, typically distributed eczematous skin lesions, and a personal or family history of atopic diseases.[7] Patients afflicted with AD suffer greatly, as it adversely affects their quality of life. Population studies suggest that the prevalence of AD has been increasing since World War II, and in most countries, it now affects more than 10% of children at some point during childhood. At present, there is no treatment directed at the basic cause of AD. Therefore, exploring the mechanisms involved in the complex inflammatory cascades could lead to the generation and acceptance of new improved therapies for this common and potentially debilitating disease.

AD may be associated with a state of increased oxidative stress due to systemic inflammation, as indicated by increased urinary concentrations of 8-hydroxy-2'-deoxyguanosine (8-OHdG),[8,9] as well as enhanced release of reactive oxygen intermediates (ROI) from leukocytes[10,11] in these patients. These findings suggest a possible increase in glycoxidation reaction and augmented pentosidine formation in AD patients, especially those with acute exacerbation.

Based on this background, the present study was designed to measure pentosidine concentrations in urine of AD patients with and without acute exacerbation of AD. Our results demonstrated for the first time the presence of significantly higher pentosidine concentrations in urine of patients with acute exacerbation of AD compared with those of patients with stable AD and normal healthy subjects. We also found that urinary pentosidine concentrations of AD patients correlated significantly with those of 8-OHdG. Therefore, a plausible explanation for the increased formation of pentosidine in our patients with acute exacerbation of AD is enhanced oxidative stress.

MATERIALS AND METHODS

Patients and Controls

Thirty-two patients with AD who fulfilled the criteria of the 1999 Guideline for Atopic Dermatitis (of the Ministry of Health, Labor and Welfare, Japan[9]) participated in this study. Twenty patients had stable AD without recent flare-up. These patients were using topical corticosteroids and/or nonsteroidal anti-inflammatory agents at the time of the study. The patients were 7 boys and 13 girls (age, 5.8 ± 2.8 years, mean ± SD; range, 2.8 to 12.1 years). The remaining 12 had acute exacerbation of AD with purulent skin infection caused by *Staphylococcal aureus* and were thus admitted to the hospital. The patients were 3 boys and 9 girls (age, 5.4 ± 2.4 years; range, 1.9 to 10.9 years). They were treated with systemic antibiotics, topical antiseptics, and corticosteroids. All showed satisfactory clinical response to treatment during hospitalization. All participating patients had normoglycemia and nor-

From the Department of Pediatrics, Fukui Medical University, Fukui; Department of Pediatrics, National Minami-Fukuoka Hospital, Fukuoka; Department of Pediatrics, Takeda Hospital, Kyoto; and the Department of Nephrology and Endocrinology, The University of Tokyo, Tokyo, Japan.

Submitted March 3, 2003; accepted May 21, 2003.

Address reprint requests to Hirokazu Tsukahara, MD, PhD, Department of Pediatrics, Fukui Medical University, Fukui 910-1193, Japan.

0026-0495/03/5212-0054$30.00/0
doi:10.1016/S0026-0495(03)00310-X

mal renal function. We also recruited 30 age-matched healthy control children (12 boys and 18 girls; age, 5.8 ± 2.3 years; range, 1.4 to 10.1 years) who were free of allergic or inflammatory diseases at the time of recruitment. All subjects in the present study were nonsmokers. Informed consent was obtained from the parents of the subjects, and the study was approved by the hospital ethics committee.

Urine Samples

Urine samples were obtained from each subject. For patients with acute exacerbation of AD, urine samples were obtained on admission and the seventh to ninth hospital days. The samples were centrifuged and the supernatants were stored at −20°C until analysis. All analyses were performed in duplicate, and the examiner was blinded to the clinical and laboratory results.

Determination of Urinary Pentosidine

Urinary concentrations of pentosidine were determined using the method of Yoshihara et al.[12] The washing solution was a mixture of *n*-butanol: acetic acid: hydrochloric acid (8:1:1, vol/vol). CF-1 slurry was prepared by making a 5% (wt/vol) suspension of CF-1 cellulose powder in the washing solvent. The pretreatment column was prepared by adding 8 mL CF-1 slurry to a Poly-Prep chromatography column (0.8 mm × 40 mm internal diameter [ID], Bio-Rad, Hercules, CA). A total of 250 μL urine was hydrolyzed with an equal volume of concentrated hydrochloric acid at 108°C for 18 hours. The cooled hydrolysate (250 μL) was mixed with 250 μL CF-1 slurry, 250 μL acetic acid, and 2 mL *n*-butanol and then loaded to the pretreatment column. After washing the column with 35 mL of the washing solvent, pentosidine was eluted from the column with 9 mL of 50 mmol/L hydrochloric acid and dried under N_2 gas flow. The dry residue was then dissolved in 250 μL 1% *n*-heptafluorobutyric acid (vol/vol), and an aliquot (10 μL) of each sample was applied to analytical high-performance liquid chromatography (HPLC). The HPLC eluent was 7% acetonitrile (vol/vol) containing 0.1% *n*-heptafluorobutyric acid. The HPLC system was equipped with an L-6200 intelligent pump (Hitachi, Ibaragi, Japan), an F-1050 fluorescence detector set at excitation and emission wavelengths of 335 nm and 385 nm, respectively, (Hitachi) and Symmetry RP18 column (3.5 μm, 4.6 mm × 150 mm, ID, Waters, Milford, MA). The flow rate was maintained at 0.8 mL/min, and the column was kept at 30°C. Standard pentosidine was synthesized and purified as described in detail previously.[12] The mean concentration of urinary pentosidine in healthy adults (n = 64) determined using this method is 28.2 ± 12.0 pmol/mg creatinine (Cr).

Determination of Urinary Pyrraline

Urinary concentrations of pyrraline were determined using the method of Yoshihara et al.[13] Urine samples were treated in solid-phase extraction cartridges (Oasis HLB, 3 mL, Waters). The cartridge was preconditioned with 1 mL methanol and equilibrated with 1 mL distilled water before loading the urine sample. A 500-μL urine sample was applied to the cartridge, followed by washing the cartridge with 1 mL 0.1% acetic acid (vol/vol). Pyrraline was eluted from the cartridge with 3 mL of 60% acetonitrile, and the eluent was dried under N_2 gas flow. The dried residue was then dissolved in 500 μL 0.1% trifluoroacetic acid (vol/vol) and the aliquot (20 μL) of each sample was applied to the analytical HPLC system. The HPLC eluent was 7% acetonitrile (vol/vol) containing 0.1% trifluoroacetic acid (vol/vol). The HPLC system was equipped with an L-7100 intelligent pump, an L-7400 UV detector set at 298 nm and Capcellpak UG120 column (3 μm, 4.6 mm × 150 mm, ID, Shiseido, Tokyo, Japan). The flow rate was maintained at 0.8 mL/min and the column was kept at 35°C. Standard pyrraline was synthesized and purified.[13] The mean concentration of urinary pyrraline in healthy adults (n = 27) determined using this method is 25.3 ± 30.0 nmol/mg Cr.

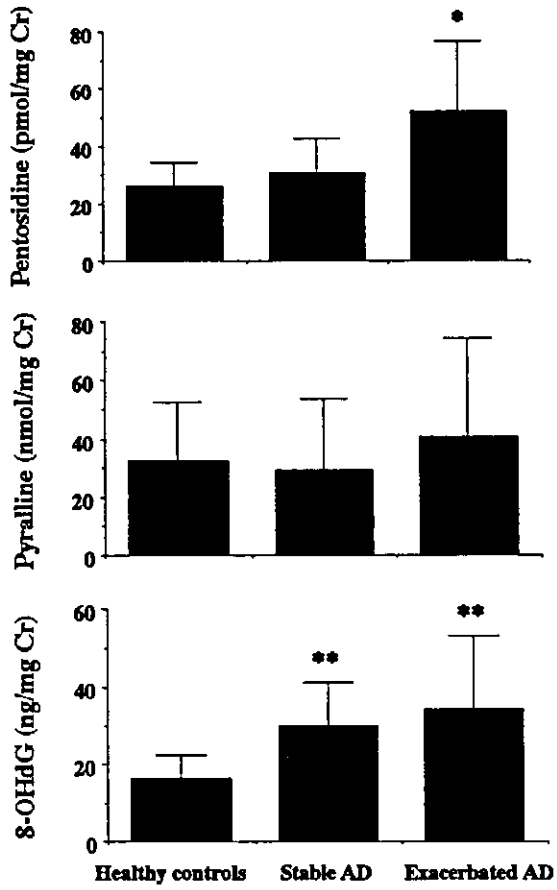

Fig 1. Comparisons of urinary concentrations of pentosidine, pyrraline, and 8-OHdG among healthy control subjects, patients with stable AD, and patients with acute exacerbation of AD. Data are shown as mean and SD. *P < .001 *v* healthy control subjects and patients with stable AD; **P < .001 *v* healthy control subjects.

Determination of 8-OHdG and Cr

The concentration of 8-OHdG was determined using a competitive enzyme-linked immunosorbent assay (8-OHdG Check, Institute for the Control of Aging, Shizuoka, Japan), as described previously.[9,14] All the above urinary markers were expressed relative to urinary Cr concentration, which was measured enzymatically using the Creatinine HR-II Test kit (Wako Pure Chemical, Osaka, Japan).

Statistical Analysis

Data are presented as mean ± SD and range. Differences between groups were examined for statistical significance using 1-way analysis of variance followed by the Scheffe test or the paired t test as appropriate. Correlations between variables were assessed by linear regression. A P value < .05 denoted the presence of a statistically significant difference.

RESULTS

As shown in Fig 1, the concentrations of pentosidine, pyrraline, and 8-OHdG in urinary samples of 30 healthy children

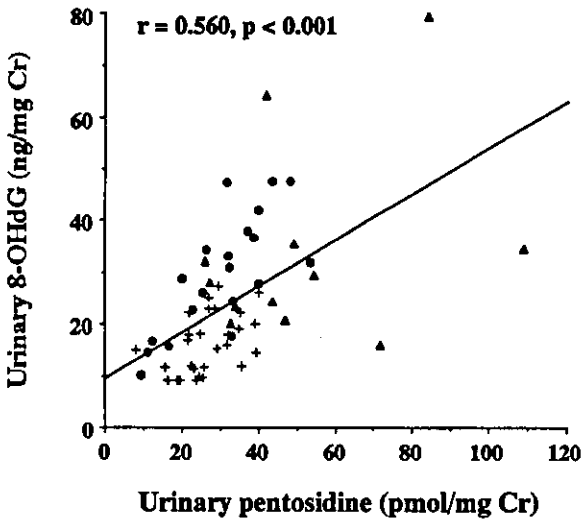

Fig 2. Relationship between urinary concentrations of pentosidine and 8-OHdG in all subjects studied. Healthy control subjects (+), patients with stable AD (●), and patients with acute exacerbation of AD (▲). The correlation between pentosidine and 8-OHdG was significant (n = 62, r = .560, P < .001).

were 25.9 ± 8.2 pmol/mg Cr (range, 8.0 to 40.0 pmol/mg Cr), 32.6 ± 19.7 nmol/mg Cr (range, 2.3 to 80.8 nmol/mg Cr), and 16.5 ± 5.9 ng/mg Cr (range, 9.1 to 27.2 ng/mg Cr), respectively. The concentrations of pentosidine, pyrraline, and 8-OHdG in urinary samples of 20 children with stable AD were 30.4 ± 12.3 pmol/mg Cr (range, 9.5 to 53.3 pmol/mg Cr), 29.1 ± 24.5 nmol/mg Cr (range, 5.4 to 93.3 nmol/mg Cr), and 29.7 ± 11.4 ng/mg Cr (range, 10.1 to 47.7 ng/mg Cr), respectively. Of these 3 markers, only urinary levels of 8-OHdG were significantly higher in these patients than in healthy controls (P < .001). The concentrations of pentosidine, pyrraline, and 8-OHdG in urinary samples of 12 children with acute exacerbation of AD on admission were 51.8 ± 25.0 pmol/mg Cr (range, 26.0 to 109.0 pmol/mg Cr), 40.5 ± 33.8 nmol/mg Cr (range, 3.6 to 97.0 nmol/mg Cr), and 34.0 ± 18.9 ng/mg Cr (range, 16.0 to 79.4 ng/mg Cr), respectively. Of these 3 markers, urinary levels of pentosidine and 8-OHdG, but not pyrraline, were significantly higher in these patients than in healthy controls (P < .001, each). Urinary pentosidine was also significantly higher in children with acute exacerbation of AD compared with those of patients with stable AD (P < .001). There was a significant positive correlation between pentosidine and 8-OHdG when all data of healthy controls and AD patients were entered into the analysis (n = 62, r = .560, P < .001) (Fig 2).

In children with acute exacerbation of AD, the concentrations of pentosidine and 8-OHdG significantly decreased from the day of admission to the seventh to ninth hospital day (P < .05, each) (Fig 3). The concentrations of pentosidine, pyrraline, and 8-OHdG on the seventh to ninth hospital day were 35.1 ± 15.0 pmol/mg Cr (range, 20.1 to 74.1 pmol/mg Cr), 28.2 ± 26.2 nmol/mg Cr (range, 3.7 to 96.0 nmol/mg Cr), and 20.9 ± 8.6 ng/mg Cr (range, 9.7 to 35.6 ng/mg Cr), respectively, in these patients.

DISCUSSION

Urinary 8-OHdG, derived from DNA or the nucleotide pool via an endonuclease or nucleotide excision repair, has been used as a marker of oxidative stress.[14] Our results showed that the urinary concentrations of 8-OHdG of AD patients with or without acute exacerbation were significantly higher than those of healthy children. This result supports previous findings of the involvement of oxidative stress in the pathophysiology of AD.[8,9] Of greater significance is the fact that the urinary concentrations of pentosidine of patients with acute exacerbation of AD were significantly higher than those of patients with stable AD and healthy control subjects.

Although we do not know the exact reason for the increased pentosidine in patients with acute exacerbation of AD, the involvement of redox imbalance and oxidative stress may be relevant. Yoshihara et al[12] (unpublished results), using the same methodologies applied in our study, examined the relationship between blood and urinary concentrations of pentosidine in 26 healthy subjects and in 44 patients with impaired renal function. Urinary pentosidine levels closely correlated with blood pentosidine concentrations, indicating that pentosi-

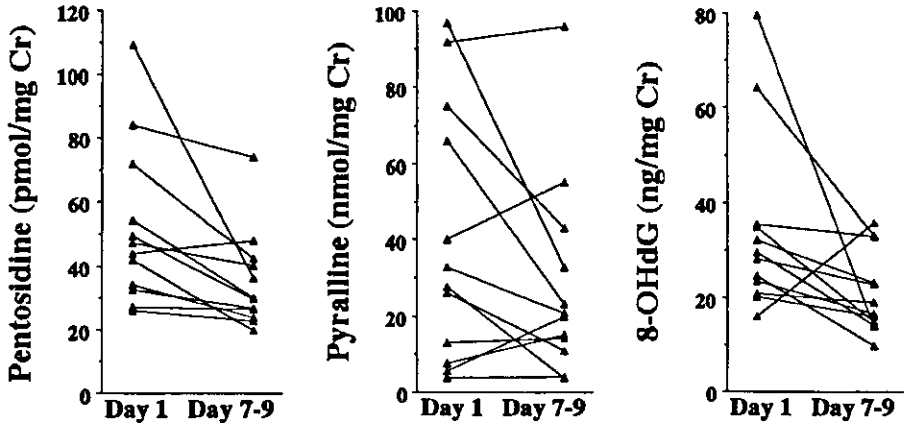

Fig 3. Effect of treatment on urinary concentrations of pentosidine, pyrraline, and 8-OHdG in patients with acute exacerbation of AD. The concentrations of pentosidine and 8-OHdG decreased significantly after 7 to 9 days of treatment (P < .05, each).

dine in the circulation is excreted into the urine according to its blood concentration. Therefore, we assume that the increased excretion of pentosidine observed in our patients reflects a greater synthesis and accumulation in the blood, tissues, and organs.

AGE accumulation, including pentosidine, is enhanced in diabetes as a result of sustained hyperglycemia.[1,2] However, hyperglycemia per se is not the direct reason of increased pentosidine levels in peripheral blood, because these levels are markedly increased in uremia, despite normal levels of blood glucose in patients with renal failure.[3,4] Furthermore, pentosidine levels in blood, synovial fluid, and urine are elevated in patients with rheumatoid arthritis in the absence of hyperglycemia or impaired renal function.[15-17] It is more plausible that the increased pentosidine levels in blood and synovial fluid are associated with the systemic inflammatory activity of rheumatoid arthritis. Pentosidine formation is closely linked not only to glycation, but also to oxidative processes, hence its qualification as a "glycoxidation" product. Wolff and Dean[18] demonstrated that reducing sugars could be autoxidized by metal-catalyzed oxidative processes and generate ROI and ketoaldehydes, which contribute to chromo- and fluorophoric alterations of proteins now taken as characteristic of AGEs. That pentosidine is the product of "glycoxidation" is further supported by in vitro evidence that the absence of oxygen in the incubation medium prevents pentosidine formation.[5] Because rheumatoid arthritis is associated with increased oxidative stress due to systemic inflammation, it appears likely that oxidative stress contributes to the generation of AGEs, including pentosidine.[15-17] The oxidative stress theory in increased pentosidine production is supported in uremia by the recent demonstration in uremic plasma of a close correlation between the levels of pentosidine and oxidative stress markers (ie, oxidized ascorbic acid, highly oxidized proteins).[19,20] In addition, the colocalization of AGEs with markers of lipid peroxidation in vascular and renal tissues indicates that both glycation and oxidation reactions contribute to pathologic lesions in diabetic atherosclerosis and nephropathy.[2,21] In the present study, we demonstrated that urinary pentosidine levels correlated significantly with those of 8-OHdG when all data of healthy controls and AD patients were plotted.

Acute skin inflammation of AD is characterized histopathologically by intense infiltration of T lymphocytes, monocyte-macrophages, and eosinophils.[7] These cells release bioactive substances, such as cytokines, chemokines, and tissue degradative enzymes and ROI, such as O_2^-, H_2O_2, $ONOO^-$, upon immunologic and nonimmunological stimulation.[7,11,22,23] Moreover, keratinocytes and endothelial cells can influence the inflammatory responses by elaborating cytokines, chemokines, adhesion molecules, and ROI. Purulent skin infection caused by *Staphylococcal aureus* infection may precipitate the exacerbation of AD.[24] Numerous infiltrating neutrophils are activated and show enhanced ROI release.[10] Activated neutrophils produce hypochlorite (HOCl) through the action of myeloperoxidase-H_2O_2-chloride system. HOCl can oxidize free amino acids to the corresponding aldehydes.[25] Anderson et al[25] showed that HOCl converts serine to glycolaldehyde, which in the presence of protein, generates carboxymethyllysine, another glycoxidation product. Similar reactions, initiated by HOCl or ROI, may lead to the generation of reactive carbohydrate intermediates, such as dicarbonyls or pentoses, which would readily react with proteins to generate pentosidine. In patients with acute exacerbation of AD, the high urinary concentrations of pentosidine, as well as 8-OHdG, decreased as the patients started to recover from the disease. The changing pattern of these markers allows us to suggest that some adaptation process occurred in these patients. Acute exacerbation of AD may be characterized by a state of enhanced oxidative stress, and this could lead to the upregulation of the antioxidant systems and the resistance to oxidative stress.[14] We observed no significant increase in the generation of pyrraline, which does not require oxidative conditions, in AD patients with or without acute exacerbation. Taken together, the enhanced prooxidant milieu may offer a reasonable explanation for the increased pentosidine formation in patients with acute exacerbation of AD.

Formation of AGEs, including pentosidine, is accompanied by a cross-linking of proteins causing alterations of structural and functional properties of macromolecules. Furthermore, AGEs are endowed with diverse biologic activities, such as increase in endothelial permeability, activation of macrophages and endothelial cells with secretion of cytokines and growth factors, which in turn, accelerate inflammation and enhancing oxidative stress.[1,2] Therefore, some of the possible mechanisms leading to acute exacerbation of AD may be related to an increase in the formation of AGEs, including pentosidine. Further studies are necessary to investigate whether the increased AGEs actively participate in the acute worsening of AD.

The observations described in the present study may not be specific to acute exacerbation of AD, but represent changes common to inflammatory or infectious conditions. However, in a series of preliminary studies, we found that the urinary concentrations of pentosidine, pyrraline, and 8-OHdG of children with acute bronchitis were not significantly different from those of aged-matched healthy controls (unpublished results). Further investigation is necessary to determine concentrations of AGEs in other conditions with activated immune and inflammatory responses.

In conclusion, our findings of increased urinary concentrations of pentosidine and 8-OHdG and their close relationship, together with normal pyrraline concentrations, in patients with acute exacerbation of AD are interpreted as evidence that pentosidine levels are partly determined by the prevailing oxidative stress in these patients. Pentosidine could thus be used as a marker of the disease status in patients with AD.

REFERENCES

1. Bucala R, Vlassara H: Advanced glycosylation end products in diabetic renal and vascular disease. Am J Kidney Dis 26:875-888, 1995

2. Suzuki D, Miyata T: Carbonyl stress in the pathogenesis of diabetic nephropathy. Intern Med 38:309-314, 1999

3. Makita Z, Bucala R, Rayfield EJ, et al: Reactive glycosylation

endproducts in diabetic uraemia and treatment of renal failure. Lancet 343:1519-1522, 1994

4. Miyata T, Kurokawa K, van Ypersele de Strihou C: Advanced glycation and lipoxidation end products: Role of reactive carbonyl compounds generated during carbohydrate and lipid metabolism. J Am Soc Nephrol 11:1744-1752, 2000

5. Dyer DG, Blackledge JA, Thorpe SR, et al: Formation of pentosidine during nonenzymatic browning of proteins by glucose: Identification of glucose and other carbohydrates as possible precursors of pentosidine in vivo. J Biol Chem 266:11654-11660, 1991

6. Hayase F, Nagaraj RH, Miyata S, et al: Aging of proteins: Immunological detection of a glucose-derived pyrrole formed during Maillard reaction in vivo. J Biol Chem 263:3758-3764, 1989

7. Leung DY: Atopic dermatitis: New insights and opportunities for therapeutic intervention. J Allergy Clin Immunol 105:860-876, 2000

8. Tsuboi H, Kouda K, Takeuchi H, et al: 8-hydroxydeoxyguanosine in urine as an index of oxidative damage to DNA in the evaluation of atopic dermatitis. Br J Dermatol 138:1033-1035, 1998

9. Omata N, Tsukahara H, Ito S, et al: Increased oxidative stress in childhood atopic dermatitis. Life Sci 69:223-228, 2001

10. Hoeger PH, Niggemann B, Schroeder C: Enhanced basal and stimulated PMN chemiluminescence activity in children with atopic dermatitis: Stimulatory role of colonizing staphylococci ? Acta Paediatr 81:542-546, 1992

11. Kapp A, Zeck-Kapp G, Czech W, et al: The chemokine RANTES is more than a chemoattractant: Characterization of its effect on human eosinophil oxidative metabolism and morphology in comparison with IL-5 and GM-CSF. J Invest Dermatol 102:906-914, 1994

12. Yoshihara K, Nakamura K, Kanai M, et al: Determination of urinary and serum pentosidine and its application to elder patients. Biol Pharm Bull 21:1005-1008, 1998

13. Yoshihara K, Kiyonami R, Shimizu Y, et al: Determination of urinary pyrraline by solid-phase extraction and high performance liquid chromatography. Biol Pharm Bull 24:863-866, 2001

14. Toyokuni S: Reactive oxygen species-induced molecular damage and its application in pathology. Pathol Int 49:91-102, 1999

15. Takahashi M, Suzuki M, Kushida K, et al: Relationship between pentosidine levels in serum and urine and activity in rheumatoid arthritis. Br J Rheumatol 36:637-642, 1997

16. Rodriguez-Garcia J, Requena JR, Rodriguez-Segade S: Increased concentrations of serum pentosidine in rheumatoid arthritis. Clin Chem 44:250-255, 1998

17. Miyata T, Ishiguro N, Yasuda Y, et al: Increased pentosidine, an advanced glycation end product, in plasma and synovial fluid from patients with rheumatoid arthritis and its relation with inflammatory markers. Biochem Biophys Res Commun 244:45-49, 1998

18. Wolff SP, Dean RT: Glucose autoxidation and protein modification. The potential role of 'autoxidative glycosylation' in diabetes. Biochem J 245:243-250, 1987

19. Witko-Sarsat V, Friedlander M, Capeillere-Blandin C, et al: Advanced oxidation protein products as a novel marker of oxidative stress in uremia. Kidney Int 49:1304-1313, 1996

20. Miyata T, Wada Y, Cai Z, et al: Implication of an increased oxidative stress in the formation of advanced glycation end products in patients with end-stage renal failure. Kidney Int 51:1170-1181, 1997

21. Miyata T, Inagi R, Asahi K, et al: Generation of protein carbonyls by glycoxidation and lipoxidation reactions with autoxidation products of ascorbic acid and polyunsaturated fatty acids. FEBS Lett 437:24-28, 1998

22. Taha RA, Minshall EM, Leung DY, et al: Evidence for increased expression of eotaxin and monocyte chemotactic protein-4 in atopic dermatitis. J Allergy Clin Immunol 105:1002-1007, 2000

23. Wakita H, Sakamoto T, Tokura Y, et al: E-selectin and vascular cell adhesion molecule-1 as critical adhesion molecules for infiltration of T lymphocytes and eosinophils in atopic dermatitis. J Cutan Pathol 21:33-39, 1994

24. Nomura I, Tanaka K, Tomita H, et al: Evaluation of the staphylococcal exotoxins and their specific IgE in childhood atopic dermatitis. J Allergy Clin Immunol 104:441-446, 1999

25. Anderson MM, Hazen SL, Hsu FF, et al: Human neutrophils employ the myeloperoxidase-hydrogen peroxide-chloride system to convert hydroxy-amino acids into glycolaldehyde, 2-hydroxypropanal, and acrolein. A mechanism for the generation of highly reactive α-hydroxy and α, β-unsaturated aldehydes by phagocytes at sites of inflammation. J Clin Invest 99:424-432, 1997

Full Title:

Prevalence of Atopic Dermatitis and Serum IgE Values in Nursery School Children in Ishigaki Island, Okinawa, Japan

Running Head:

Atopic dermatitis and IgE in children in Ishigaki

Authors:

Maki Hamada *, Norihiro Furusyo *†, Kazunori Urabe §, Keisuke Morita §, Takeshi Nakahara§, Naoko Kinukawa ¶, Yoshiaki Nose ¶, Jun Hayashi *†, and Masutaka Furue §

Affiliations of Authors:

* Department of General Medicine, Kyushu University Hospital, Fukuoka, Japan.

† Department of Environmental Medicine and Infectious Disease, Faculty of Medical Sciences, Kyushu University, Fukuoka, Japan.

¶ Department of Medical Informatics, Kyushu University Hospital, Fukuoka, Japan.

§ Department of Dermatology, Kyushu University Hospital, Fukuoka, Japan.

Address for Correspondence:

Masutaka Furue, MD., PhD.

Department of Dermatology, Kyushu University Hospital, Fukuoka, Japan.

Key Words: Atopic dermatitis, Epidemiology, Questionnaires, Immunoglobulin E

Abbreviations:

AD; atopic dermatitis

Acknowledgement

This work was supported by grants from the Ministry of Health, Labor and Welfare.

Abstract.

There have been many studies of the prevalence of atopic dermatitis (AD), but few population-based epidemiologic studies measure the prevalence in Japan among children aged 5 years and younger. We examined the prevalence of AD, serum total IgE levels and specific IgE antibodies to 10 common allergens among children in Ishigaki Island, Okinawa, Japan in 2001. We also obtained information on the predictability of the U.K. Working Party diagnostic questionnaire criteria for AD in this population. Five hundred and sixty five children aged 5 years and younger were enrolled in this study with informed consent from their parents. The questionnaire of the U.K. Working Party diagnostic criteria for AD was translated into Japanese, and the parents completed the questionnaire sheet. Physical examination and blood sampling were done for all children. Thirty-nine out of the 565(6.9 %) children were diagnosed with AD by physical examination. The total and specific IgE levels were significantly higher in the children with AD than in those without AD. High levels of total IgE were found in 33.3% of the children with AD. A specific IgE to one or more allergens were detected in 64.1% of children with AD. However, a substantial population of children without AD also had high levels of total IgE (12.7%) and a specific IgE to one or more allergens (30.2%), and the increment of total and specific IgE levels was significantly associated with age. The percentage of positive answers to the questionnaire of the U.K. Working Party diagnostic criteria for AD was significantly higher in children with AD (59.0 %) than in children without AD (5.3 %) ($P<0.0001$). Its specificity was 94.7%. The false negative rate was 41%. In conclusion, the prevalence of AD was relatively low in children in Ishigaki Island. High levels of total IgE were found in only one third of children with AD under 5 years of age. The Japanese translated form of the questionnaire of the U.K. Working Party diagnostic criteria for AD should be refined to improve its sensitivity.

Introduction

Atopic dermatitis (AD) is a common chronic inflammatory skin disease that is characterized by relapsing itch and eczema. It is a major skin disease of children that is increasing in both developed (1-3) and developing counties (4). A similar trend has been documented in Japan (5); however, one study has reported that AD is no longer increasing (6). There have been many studies of the prevalence of AD (6-13), but few population-based epidemiologic studies that measure the prevalence in Japan among children aged 5 years and younger.

The first set of standardized diagnostic criteria for AD arose from the work of Hanifin and Lobits, and it was revised by Hanifin and Rajka (14, 15). The Japanese Dermatological Association criteria for the diagnosis of AD were established in 1995 (16). In order to set more useful criteria for mass-screening, the United Kingdom (U.K.) Working Party furthered the development of a standardized questionnaire defining the diagnostic criteria for AD (17). This questionnaire was composed of only 5 questions that were easy to answer by parents.

The aim of the present study was to determine the prevalence of AD, serum total IgE, and specific IgE antibodies among children aged 5 years and younger living in a relatively isolated area, Ishigaki Island. An additional aim of the study was to obtain information on the predictability of the questionnaire of the U.K. Working Party diagnostic criteria for AD when used in combination with physical examination in a Japanese population.

Methods

Study population

A large-population, long-term study in residents of the Yaeyama District of Okinawa, Japan for hepatitis B virus markers has been ongoing since 1968 (18-20). The present study was done as a part of the above-mentioned epidemiologic study in 2001. We visited 15 nursery schools in Ishigaki Island, which has a population of 45,000, in the Yaeyama District of Okinawa Prefecture, Japan. Approval for the study was obtained from the Ethics Committee of Kyushu University Hospital as well as from the directors and class teachers of the schools. Informed consent to allow participation of the children was obtained from the parents and guardians. The yearly average temperature and humidity were 25.4 °C and 76 % on Ishigaki Island.

Six hundred and five children were originally enrolled in the study. There were 40 exclusions because of insufficient physical and laboratory examination or incomplete answers to questionnaires. The remaining 565 children were 302 boys and 263 girls, with a mean age of 3.1 years, and represented 13.7 % of the 4112 kindergarten pupils in Ishigaki City. Physical examinations with questionnaires concerning histories of symptoms and family history were completed, and venous blood samples were obtained between July 30 and August 3, 2001.

Physical and laboratory examination

The medical examinations for all children were done by two dermatologists from the Department of Dermatology at Kyushu University Hospital. AD was diagnosed according to the Japanese Dermatological Association criteria for the diagnosis of AD (16). All children were tested for total and specific IgE antibodies. Total IgE level was determined by a radioimmunoassay with a detection limit of 20 IU/ml (Shionoria IgE, Shionogi & Co., Ltd. Japan). A total IgE level over 230 IU/ml was considered abnormal for statistical analysis. Specific IgE antibodies against aeroallergens such as house dust, Japanese cedar pollen, *Dermatophagoides pteronyssinus*, *Dermatophagoides farinae*, *Candida*, *Malassezia,* and food allergens, such as chicken egg white, cow's milk, rice, and soy were tested with the Pharmacia Enzyme CAP procedure (Pharmacia CAP System Specific IgE FEIA, Pharmacia Diagnostics AB, Sweden). A level of specific IgE antibodies over 0.7 UA/ml was considered abnormal for statistical analysis.

Questionnaire

The questionnaire of The U.K. Working Party diagnostic criteria for AD was translated into Japanese by a staff member of Kyushu University Hospital. The questionnaire has 5 questions regarding the present and past history of skin conditions (17). Each one-page questionnaire was completed by parents on behalf of their children. Children with suitable positive answers were diagnosed as AD using the same evaluation method proposed by the U.K. Working Party (17).

Statistical analysis

Continuous data were expressed as mean values ± standard deviation (SD) or standard error (SE) of the mean. Unpaired t-test and Mann-Whitney U-test were used to compare the means of samples between two groups. The chi-square test or Fisher's exact test was used for categorical variables for comparisons between two groups. The Cochran-Armitage test was used to determine the relationship between the increase or decrease in the prevalence rate of AD or the IgE abnormality rate. $P < 0.05$ was considered statistically significant.

Results

Prevalence of AD

Table 1 shows the overall prevalence of AD in the study population. Out of 565 children, 39 (6.9 %) were diagnosed with AD by physical examination. The prevalence peaked at age 3 (10%), and was lowest at age 5 (5%); however, the age-related difference was not statistically significant (P=0.7146 by the Cochran-Armitage test). No significant differences were found when boys (19 of 302, 6.3 %) and girls (28 of 263, 7.6 %) were compared for disease prevalence.

Total IgE levels

The mean (± SE) total IgE levels were significantly higher in children with AD (451.1 ± 120.4 IU/ml) than in those without AD (139.2 ± 14.7 IU/ml) (P<0.001 by Mann-Whitney U-test) (**Fig.1**). The total IgE levels were quite variable in each age group, and significant differences in mean IgE levels were found at ages 1, 3, and 4 between children with and without AD (1 year old, P=0.0026; 3 years old, P=0.0272; and 4 years old, P=0.0037, by Mann-Whitney U-test)(**Fig.1**). As shown in **Table 2**, the occurrence of abnormal total IgE levels of over 230 IU/ml was significantly higher in children with AD (13 of 39, 33.3 %) than in those without AD (67 of 526, 12.7 %)(P=0.0029 by the chi-square test). Interestingly, the rate of abnormal total IgE levels in children with AD did not significantly increase with age, however; the rate of abnormal total IgE levels in children without AD significantly increased with age (P=0.0007 by the Cochran-Armitage test)(**Table 2**).

Positivity of specific IgE antibodies against aeroallergens and food allergens

Antigen-specific IgE antibodies against aeroallergens and food allergens, as indicated by values over 0.7 UA/ml, were found in 184 (32.6 %) of the total of 565 children. **Table 3** shows the differences in specific IgE antibody between children with and without AD. A positive response for one or more specific IgE antibodies was significantly higher in children with AD (64.1 %) than in those without AD (30.2 %) (P<0.0001). Specific IgE antibody positivities, with the exceptions of Japanese cedar pollen, *Malassezia*, cow's milk and rice, were significantly higher in children with AD than those without AD (**Table 3**). The percentage positivity of specific IgE antibodies in children with AD did not significantly differ according to age (**Table 4**) (38.5% to 100%, P=0.3618 by the Cochran-Armitage test). However, the percentage positivity of specific IgE antibodies significantly increased with age in children without AD (**Table 4**)(25.8% to 42.7%,

P=0.0394 by the Cochran-Armitage test).

Questionnaire

We determined the sensitivity and specificity of the translated questionnaire of the U.K. Working Party diagnostic criteria for AD (**Table 5**). Fifty-one out of 565 children (9%) fulfilled the criteria for AD by the questionnaire. When compared to the actual diagnosis by physical examination, the sensitivity was 59% (23 out of 39), and the specificity was 94.7%(498 out of 526). The false positive and negative rates were 5.3% and 41%, respectively (**Table 5**).

Discussion

Symptoms of AD began during the first year of life in 65 % of the children and in 85 % during the first 5 years (21); it is thus worthwhile to determine the prevalence in children under the age of 5 years. In 2000 to 2002, the research team of Japanese Ministry of Welfare (chief researcher; Dr. S. Yamamoto) performed physical examinations of 39,755 children living in Asahikawa, Iwate, Tokyo, Gifu, Osaka, Hiroshima, Kochi, and Fukuoka (22). They reported that national average prevalence rate of AD was 12.8% in 4-month-old children, 9.8 % in 18-month-old, 13.2 % in 3-year-old, 11.8 % in 6- to 7-year-old, and 10.6 % in 11- to 12-year-old children. In our study, the prevalence of AD (6.9 %) in children aged 5 years and younger in Ishigaki Island, which is located in the subtropical zone of Japan, was lower than the average rate on the mainland of Japan. It is also interesting that the present result, like Yamamoto's study, showed that the prevalence peaked at age 3. A worldwide survey has reported that AD is increasing in the developed countries in cooler climates (8). Japanese investigators also reported that the prevalence (17.3 %) of AD was significantly higher in the cooler climate of Gifu than in the warmer climate of Itoman, Okinawa (3.4 %), even after controlling for genetic and environmental factors (9, 10). The reason for the lower prevalence in Okinawa (Itoman and Ishigaki) remains to be elucidated.

IgE levels have been reported to be elevated in 80 to 85 % of children who developed AD (23, 24). In the present study, the total IgE levels were significantly higher in children with AD than in those without disease. The children with AD also had higher positive rates of most specific IgE antibodies against aeroallergens and food allergens than the children without AD. However, the positive percentage was lower than expected (high levels of total IgE; 33.3%, one or more specific IgE; 64.1%). None of the children had specific IgE antibody to Japanese cedar pollen, probably because there are no cedars in Ishigaki. Approximately 20 % of children with AD have been reported to show allergic reactions to food constituents (25). In infancy allergic sensitization is predominantly to food. In later childhood, allergic sensitization to aeroallergens, such as house dust mites and pollen, is common (26). We also confirmed that the major allergens (specific IgE positive rates) were house dust mites, egg white, and milk in children with AD in Ishigaki. It should be emphasized that high serum levels of IgE was detected in 12.7% (67/526) of children without AD, and that 30.2% of these non-atopic children had one or more positive specific IgE antibodies to common allergens in our study. House dust mites, milk, and egg white