cells: functional evidence that both cytokines act through the same receptor. Int Immunol 1996;8:1915–25.
[7] Hilton DJ, Zhang J-G, Metcalf D, Alexander WS, Nicola NA, Willson TA. Cloning and characterization of a binding subunit of the interleukin 13 receptor that is also a component of the interleukin 4 receptor. Proc Nat Acad Sci 1996;93:497–501.
[8] Obiri NI, Debinski W, Leonarud WJ, Puri RK. Receptor for interleukin 13. J Biol Chem 1995;270:8797–804.
[9] Orchansky PL, Kwan R, Lee F, Schradu JW. Characterization of the cytoplasmic domain of interleukin-13 receptor alpha. J Biol Chem 1999;275:20818–25.
[10] Miloux B, Laurent P, Bonnin O, Lupker J, Caput D, Vita N, et al. Cloning of the human IL-13R alpha1 chain and reconstitution with the IL-4R alpha of a functional IL-4/IL-13 receptor complex. FEBS Lett 1997;401:163–6.
[11] Zurawski SM, Chomarat P, Djossou O, Bidaud C, Mckenzie AN, Miossec P, et al. The primary binding subunit of the human interleukin-4 receptor is also a component of the interleukin 13 receptor. J Biol Chem 1995;270:13869–78.
[12] Donaldson DD, Whitters MJ, Fitz LJ, Neben TV, Finnerty H, Henderson SL, et al. The murine IL-13 receptor alpha2: molecular cloning, characterization, and comparison with murine IL-13 receptor alpha1. J Immunol 1998;161:2317–24.
[13] Akaiwa M, Yu B, Umeshita-Suyama R, Tera N, Suto H, Koga T, et al. Localization of human interleukin-13 receptor in non-haematopoietic cells. Cytokine 2001;13:75–84.
[14] Graber P, Gretener D, Herren S, Aubry JP, Elson G, Poudrier J, et al. The distribution of IL-13 receptor alpha1 expression on B cells, T cells and monocytes and its regulation by IL-13 and IL-4. Eur J Immunol 1998;28:4286–98.
[15] Hart PH, Bonder CS, Balogh J, Dickensheets HL, Vazquez N, Davies KV, et al. Diminished responses to IL-13 by human monocytes differentiated in vivo: role of the IL-13Rα1 chain and STAT6. Eur J Immunol 1999;29:2087–97.
[16] So EY, Park HH, Lee CE. IFN-γ and IFN-α posttranscriptionally down-regulate the IL-4-induced IL-4 receptor gene expression. J Immunol 2000;165:5472–9.
[17] Vita N, Lefort S, Laurent P, Caput D, Ferrara P. Characterization and comparison of the interleukin 13 receptor with the interleukin 4 receptor on several cell types. J Biol Chem 1995;270:3512–7.
[18] Daines MO, Hershey GK. A novel mechanism by which interferon-γ can regulate interleukin (IL)-13 responses. J Biol Chem 2002;277:10387–93.
[19] Palmer-Crocher RL, Hughes CC, Pober JS. IL-4 and IL-13 activate the JAK2 tyrosine kinase and STAT6 in cultured human vascular endothelial cells through a common pathway that does not involve the gamma c chain. J Clin Invest 1996;98:604–9.
[20] Izuhara K, Umeshita-Suyama R, Akaiwa M, Shirakawa T, Deichmann KA, Arima K, et al. Recent advances in understanding how interleukin-13 signals are involved in the pathogenesis of bronchial asthma. Arch Immunol Ther Exp 2000;48:502–12.
[21] Umeshita-Suyama R, Sugimoto R, Akaiwa M, Arima K, Yu B, Wada M, et al. Characterization of IL-4 and IL-13 signals dependent on the human IL-13 receptor alpha chain 1: redundancy of requirement of tyrosine residue for STA3 activation. Int Immunol 2000;12:1499–509.
[22] Grunig G, Warnock M, Wakil AE, Venkayya R, Brombacher F, Rennick DM, et al. Requirement for IL-13 independently of IL-4 in experimental asthma. Science 1998;282:2261–3.
[23] Laporte JC, Moore PE, Baraldo S, Jouvin MH, Church TL, Schwartzman IN, et al. Direct effects of interleukin-13 on signaling pathways for physiological responses in cultured human airway smooth muscle cells. Am J Respir Crit Care Med 2001;164:141–8.
[24] Hamid Q, Naseer T, Minshall EM, Song YL, Boguniewicz M, Leung DY. In vivo expression of IL-12 and IL-13 in atopic dermatitis. J All Clin Immunol 1996;98:225–31.
[25] Taha RA, Leung DYM, Ghaffar O, Boguniewicz M, Hamid Q. In vivo expression of cytokine receptor mRNA in atopic dermatitis. J All Clin Immunol 1998;102:245–50.
[26] Skov L, Chan L, Fox DA, Lansen JK, Voorhees JJ, Cooper KD, et al. Lesional psoriatic T cell contain the capacity to induce a T cell activation molecule CDw60 on normal keratinocytes. Am J Pathol 1997;150:675–83.
[27] Barlow Y, Pye RJ. Keratinocyte culture. Method Mol Biol 1997;75:117–29.
[28] Moser R, Scheleiffenbaum B, Groscurth P, Fehr J. Interleukin 1 and tumor necrosis factor stimulate human vascular endothelial cells to promote transendothelial neutrophil passage. J Clin Invest 1989;83:444–55.
[29] Beltrani VS. The clinical spectrum of atopic dermatitis. J Allergy Clin Immunol 1999;104:S87–98.
[30] Wery-Zennaro S, Letourneur M, David M, Bertoglio J, Pierre J. Binding of IL-4 to the IL-13Rα1/IL-4Rα receptor complex leads to STAT3 phosphorylation but not to its nuclear translocation. FEBS Lett 1999;464:91–6.
[31] Ghoreschi K, Thomas P, Breit S, Dugas M, Mailhammer R, Van Eden W, et al. Interluekin-4 therapy of psoriasis induced Th2 responses and improves human autoimmune disease. Nature Med 2003;9:40–6.
[32] Blumberg H, Conklin D, Xu WF, Grossmann A, Brender T, Carollo S, et al. Interelukin-20: discovery, receptor identification and the role in epidermal function. Cell 2001;104:9–19.
[33] Pastore S, Mascia F, Giustizieri ML, Giannetti A, Girolomoni G. Pathogenetic mechanism of atopic dermatitis. Arch Immunol Ther Exp 2000;48:497–504.
[34] Nickoloff BJ, Bonish B, Huang BB, Porcelli SA. Characterization of a T cell line bearing natural killer receptors and capable of creating psoriasis in a SCID mouse model system. J Dermatol Sci 2000;24:212–25.
[35] Cancino-Diaz JC, Reyes-Maldonado E, Carmen A, Banuelos-Panuco CA, Jimenez-Zamudio L, Garcia-Latorre E. Interleukin-13 receptor in psoriatic keratinocytes: overexpression of the mRNA and underexpression of the protein. J Invest Dermatol 2002;119:1114–20.
[36] Joshi BH, Kawakami K, Leland P, Puri RK. Heterogeneity in interleukin-13 receptor expression and subunit structure in squamous cell carcinoma of head and neck: differential sensitivity to chimeric fusion proteins comprised of interleukin-13 and a mutated form of pseudomonas exotoxin. Clin Cancer Res 2002;8:1948–56.
[37] Doucet C, Giron-Michel J, Canonica GW, Azzarone B. Human lung myofibroblasts as effectors of the inflammatory process: the common receptor γ chain is induced by Th2 cytokines, and CD40 ligand is induced by lipopolysaccharide, thrombin and TNF-α. Eur J Immunol 2002;32:2437–49.
[38] Prens E, Hgmans J, Lien RC, Debets R, Van Joost T, Benner R. Increased expression of interleukin-4 receptors on psoriatic epidermal cells. Am J Pathol 1996;148:1493–502.
[39] Schnyder B, Lugli S, Feng N, Etter H, Lutz RA, Ryffel B, et al. Interleukin-4 (IL-4) and IL-13 bind to a shared heterodimeric complex on endothelial cells mediating vascular cell adhesion molecule-1 induction in the absence of the common gamma chain. Blood 1996;15:4286–95.
[40] Wakugawa M, Nakamura K, Akatsuka M, Nakagawa H, Tamaki K. Interferon-gamma-induced RANTES production by human keratinocytes is enhanced by IL-1β, TNF-α, IL-4

and IL-13 and is inhibited by dexamethasone and tacrolumus. Dermatology 2001;202:239–45.
[41] David M, Ford D, Bertoglio J, Maizel AL, Pierre J. Induction of the IL-13 receptor α2-chain by IL-4 and IL-13 in human keratinocyte: involvement of STAT6, ERK and p38 MAPK pathways. Oncogene 2001;20:6660–8.
[42] Matsuura H, Sakaue M, Subbaramaiah K, Kamitani H, Eling TE, Dannenberg AJ, et al. Regulation of cyclooxygenase-2 by interferon γ and transforming growth factor α in normal human epidermal kerationcyte and squamous carcinoma cell. J Biol Chem 1999;274:29138–48.
[43] Huang BB, Bonish BK, Chaturvedi V, Qin J-Z, Nickoloff BJ. Keratinocyte CDw60 expression is modulated by both a Th-1 type cytokine IFN-gamma and Th-2 cytokines IL-4 and IL-13: relevance to psoriasis. J Invest Dermatol 2001;116:305–12.

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Description of a New Yeast Species, *Malassezia japonica*, and Its Detection in Patients with Atopic Dermatitis and Healthy Subjects

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Lipophilic yeasts of the genus *Malassezia* are part of the normal cutaneous microflora and are considered one of the factors that trigger atopic dermatitis (AD). We isolated two strains of *Malassezia* from a healthy Japanese female. Analysis of the D1/D2 26S ribosomal DNA and internal transcribed spacer region sequences of the isolates suggested that they are new members of the genus *Malassezia*. We propose the name *Malassezia japonica* sp. nov. for the isolates. *M. japonica* is easily distinguished from the seven known lipophilic species by its ability to assimilate Tween 40 and Tween 60 and its inability to assimilate Tween 20 and Tween 80 and to grow at 40°C. Furthermore, by applying transparent dressings to the skin lesions of 36 patients with AD and the skin of 22 healthy subjects, *M. japonica* DNA was detected by a non-culture-based method consisting of nested PCR with *M. japonica* species-specific primers. *M. japonica* DNA was detected from 12 of the 36 patients (33.3%) and 3 of the 22 healthy subjects (13.6%). Although it is not known whether *M. japonica* plays a role in AD, this species was part of the microflora in both patients with AD and healthy subjects.

Lipophilic yeasts of the genus *Malassezia* are members of the normal human cutaneous microflora and are also associated with several skin diseases. It is strongly suspected that *Malassezia* species are responsible for pityriasis versicolor, seborrheic dermatitis, *Malassezia* folliculitis, and atopic dermatitis (AD) (2, 4, 5, 19). The genus *Malassezia* includes eight species: *Malassezia globosa*, *M. restricta*, *M. obtusa*, *M. slooffiae*, *M. furfur*, *M. sympodialis*, *M. dermatis*, and *M. pachydermatis* (7, 22). *M. pachydermatis* is not a lipophilic species and is associated several animal skin diseases (1, 8). Recently, our research group found *M. dermatis* on Japanese patients with AD (22). Much research has examined the relationships between these eight species and their roles as causative agents of disease or factors that trigger disease. Most studies indicate that pityriasis versicolor and seborrheic dermatitis are likely affected by *M. globosa* and *M. sympodialis* (3, 9, 10, 15). The distribution of *Malassezia* species in the skin lesions of AD patients and healthy subjects was previously compared by a non-culture-based method (nested PCR) that is not affected by the isolation medium (21). Of the members of the genus *Malassezia*, *M. globosa* and *M. restricta* were associated with disease in more than 90% of AD patients, while the other species were detected in less than 50% of the patients. In our survey of the cutaneous *Malassezia* microflora, we isolated a new *Malassezia* species from a healthy subject. In the present study, we propose a new species, *M. japonica*, for the isolates from this subject and investigated the skin surfaces of patients with AD and healthy subjects for the presence of this species.

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MATERIALS AND METHODS

Yeast isolate. *Malassezia* strains were isolated from the left forearm of a healthy 22-year-old Japanese female. OpSite transparent dressings (3 by 7 cm; Smith and Nephew Medical Ltd., Hull, United Kingdom) were applied to the scalp, back, arm, and nape of the neck of the subject. The samples were then transferred onto modified Leeming and Notman agar (LNA; 20 g of glucose, 50 g of malt extract, 1 g of polypeptone, 20 g of bile salts [Oxoid, Basingstoke, United Kingdom], 1% Tween 40, 0.2% glycerol, and 50 μg of chloramphenicol [Sankyo, Tokyo, Japan]) and incubated at 32°C.

Direct DNA sequencing of rRNA genes. Nuclear DNA was extracted by the method of Makimura et al. (14). The D1/D2 26S rRNA and internal transcribed spacer (ITS) regions of the rRNA gene were sequenced directly from the PCR products by using primer pair NL-1 (5′-GCATATCAATAAGCGGAGGAA AAG) and NL-4 (5′-GGTCCGTGTTTCAAGACGG) (13) and primer pair pITS-F (5′-GTCGTAACAAGGTTAACCTGCGG) and pITS-R (5′-TCCTCC GCTTATTGATATGC) (20), respectively. The PCR products were sequenced with an ABI 310 DNA sequencer and a BigDye Terminator Cycle Sequencing Ready Reaction kit (Perkin-Elmer Applied Biosystems, Foster, Calif.), according to the instructions of the manufacturer.

Molecular phylogenetic analysis. The sequences were aligned by using Clustal W software (23). For the neighbor-joining analysis (18), the distances between sequences were calculated by using the two-parameter model of Kimura (12). A bootstrap analysis was conducted with 100 replications (6).

Morphological, physiological, and chemotaxonomic characteristics. Morphology was examined on LNA after incubation at 32°C for 7 days. Tween 20, 40, 60, and 80 utilization, catalase reactions, and diazonium blue B reactions were performed as described by Guého et al. (7). Ubiquinone molecules were identified by the method of Nakase and Suzuki (16).

Direct detection of DNA in samples from patients with AD and healthy subjects. (i) Subjects. The microfloras of 36 AD outpatients (24 men and 12 women; age range, 20 to 64 years; mean age, 33.3 ± 10.5 years) at Tokyo Medical University Hospital and 22 healthy students (7 men and 15 women; age range, 19 to 25 years; mean age, 20.7 ± 1.6 years old) at Meiji Pharmaceutical University were analyzed. AD was diagnosed according to the criteria of Hanifin and Rajka

TABLE 1. Specificities of the primers for *M. japonica* and related species

Species	Strain[a]	Specificity of primer pair: 26SBF and Mala-R	Specificity of primer pair: JP-IGSIF and JP-IGSIR
M. japonica sp. nov.	M 9966	+	+
M. japonica sp. nov.	M 9967	+	+
M. dermatis	M 9927	−	−
M. dermatis	M 9929	−	−
M. furfur	CBS 4162	+	−
M. furfur	CBS 6000	+	−
M. furfur	CBS 7982	+	−
M. globosa	CBS 7966	+	−
M. globosa	M 9972	+	−
M. obtusa	CBS 7876	+	−
M. obtusa	Clinical isolate 2-17	+	−
M. pachydermatis	CBS 1879	+	−
M. restricta	CBS 7991	+	−
M. restricta	M 9976	+	−
M. slooffiae	CBS 7956	+	−
M. slooffiae	M 9980	+	−
M. sympodialis	CBS 7222	−	−
M. sympodialis	M 9978	−	−
Candida albicans	CBS 562	−	−
Candida guilliermondii	CBS566	−	−
Candida parapsilosis	CBS 604	−	−
Rhodotorula mucilaginosa	CBS 17	−	−
Staphylococcus aureus	Clinical isolate 4-2	−	−

[a] CBS, Centraal Bureau voor Schimmelcultures, Baarn, The Netherlands; M, Meiji Pharmaceutical University, Tokyo, Japan.

(11). Routine skin care, including intermittent applications of mild steroid ointment or petrolatum, was administered before sampling. Written informed consent was obtained from each subject.

(ii) Design of *M. japonica* species-specific primers for PCR. The sequences of the intergenic spacer (IGS) 1 region, which is located between the 18S and 5.8S rRNA genes of *M. japonica* and the phylogenetically closely related species *M. furfur* and *M. obtusa*, were determined. The IGS 1 region was amplified with primer 26SBF (5′-AGCT GCTGCCAATGCTAGCTC), which hybridizes to a sequence located at the end of the 26S rRNA gene, and primer Mala-R (5′-T ACTGCTGTGAATGCTCCAGC), which hybridizes to a sequence located in the 5.8S rRNA gene, and by use of the following program: 94°C for 3 min, followed by 30 cycles of 94°C for 30 s, 58°C for 30 s, and 72°C for 30 s, with a final extension at 72°C for 10 min. The amplified PCR products were directly sequenced with the primer pair 26SBF and Mala-R. A species-specific primer pair was designed on the basis of IGS 1 sequence analysis: primer JP-IGS1F (5′-G ACTGCTGATAATGCTCCAGT) and primer JP-IGS1R (5′-GTCTGCTG AT AAGTCTCACTG). To investigate the specificities of these primers, we used the other *Malassezia* and relevant species listed in Table 1.

(iii) Analysis of *M. japonica* microflora. Samples of *Malassezia* were collected by applying OpSite transparent dressings (3 by 7 cm) to erythematous lesions on the faces and necks of patients with AD and the faces and necks of healthy subjects. *Malassezia* DNA was extracted from the OpSite dressing by a previously described method (21). The DNA extracted (3 μl) from each sample was added to 47 μl of the PCR master mixture, which consisted of 5 μl of 10× PCR buffer (100 mM Tris-HCl [pH 8.3], 500 mM KCl, 15 mM $MgCl_2$; Takara Inc., Shiga, Japan), 4 μl of 200 μM deoxynucleoside triphosphates (an equimolar mixture of dATP, dCTP, dGTP, and dTTP; Takara), 10 pmol of each primer, and 2.5 U of Ex *Taq*DNA polymerase (Takara). PCR was performed with primers 26SBF and Mala-R, with an initial denaturation at 94°C for 3 min, followed by 30 cycles of 30 s at 94°C, 1 min at 57°C, and 30 s at 72°C, with a final extension at 72°C for 10 min. In the nested PCR step, 1 μl of the first amplification product was added to a new reaction mixture with the same composition as the first one. The PCR with primers JP-IGS1F and JP-IGS1R consisted of an initial denaturation at 94°C for 3 min, followed by 30 cycles of 30 s at 94°C, 1 min at 44°C, and 30 s at 72°C, with a final extension at 72°C for 10 min.

Nucleotide sequence accession numbers. The nucleotide sequences of the D1/D2 26S rRNA, ITS, and IGS regions determined in this study have been deposited with DDBJ (DNA Data Bank of Japan) and are listed in Table 2.

RESULTS AND DISCUSSION

Molecular phylogenetic analysis and taxonomic characteristics. The isolates formed a cluster with *M. furfur* and *M. obtusa*, with 100 and 99% bootstrap support on trees constructed by using the D1/D2 26S rRNA gene and ITS 1 sequences, respectively (Fig. 1A and B). The dissimilarities between the D1/D2 regions of the 26S rRNA genes of the isolates and those of the *M. furfur* and *M. obtusa* strains were 4.6% (27 of 582 bp) and 6.9% (40 of 580 bp), respectively. The ITS 1 regions of the isolates had 12.5 to 24.2% and 15.3 to 20.3% dissimilarities to those of the *M. furfur* and *M. obtusa* strains, respectively; and the ITS 2 regions of the isolates had 15.6 to 17.9% and 20.2 to 21.3% dissimilarities to those of the the *M. furfur* and *M. obtusa* strains, respectively. Since the divergence

TABLE 2. Accession numbers of the IGS, ITS, and D1/D2 26S rRNA sequences

Species	Strain	Accession no.: D1/D2 26S rRNA gene	Accession no.: ITS region	Accession no.: IGS 1 region
M. japonica sp. nov.	M 9966[Ta]	AB100599	AB100599	AB105063
	M 9967	AB105199	AB105199	AB105064
M. furfura	CBS 4162			AB111460
	CBS 6000			AB111459
	CBS 7982[T]	AB105198	AB105150	
	Clinical isolate 2–9		AB105151	
	Clinical isolate 2–4		AB105152	
	Clinical isolate 2–521		AB105153	
	Clinical isolate 2–52		AB105154	
M. obtusa	CBS 7876[T]	AB105197	AB105158	AB111461
	Clinical isolate 2–17		AB105155	
	Clinical isolate 2–20		AB105156	
	Clinical isolate 2–35		AB105157	

[a] T, type strain.

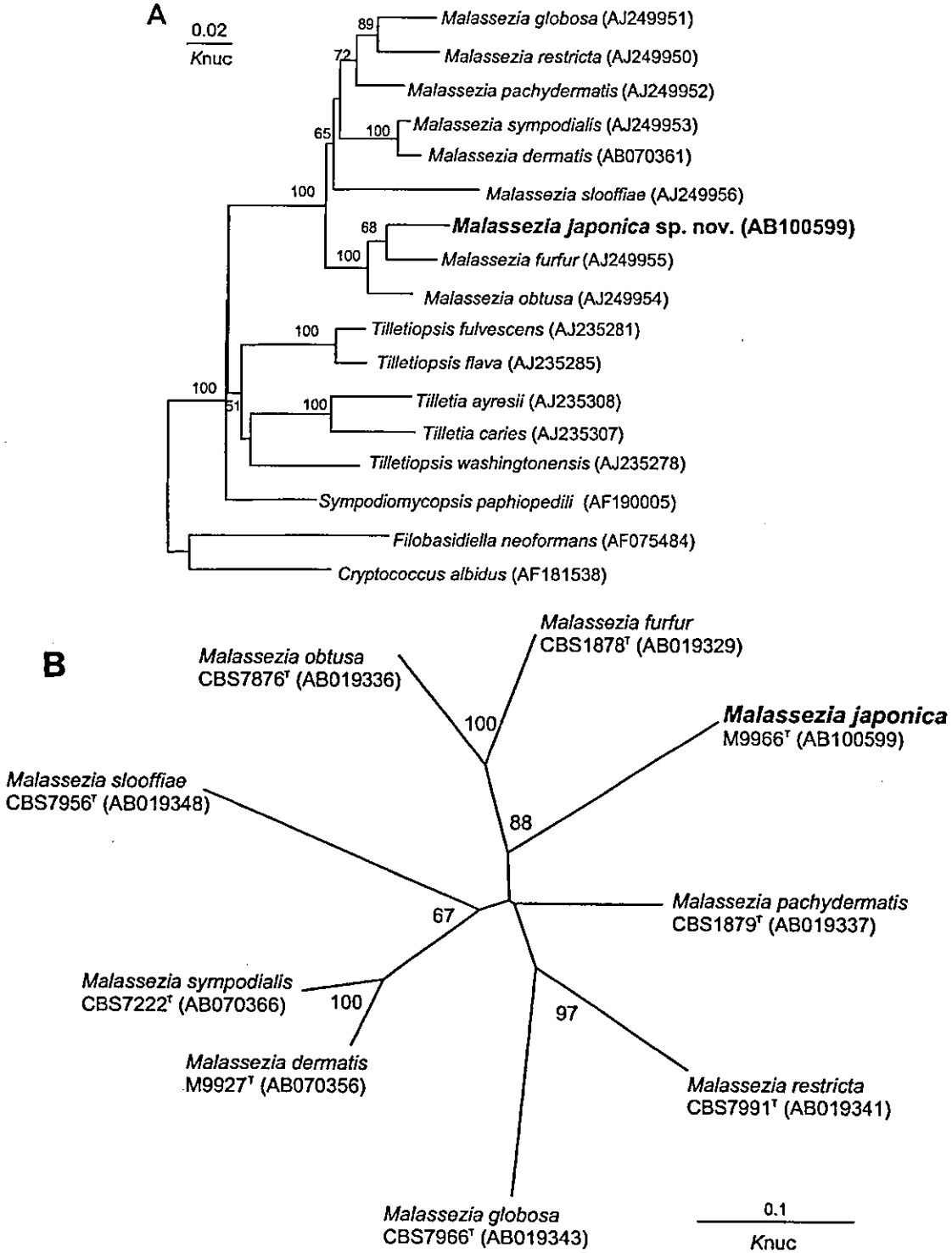

FIG. 1. Phylogenetic trees constructed by using the D1/D2 26S rRNA sequences of *M. japonica* sp. nov. and related species of the class *Ustilaginomycetes* (A) and the ITS1 region of *M. japonica* sp. nov. and other members of the genus *Malassezia* (B). The DDBJ and GenBank accession numbers are indicated in parentheses. The numerals represent the confidence levels from 100 replicate bootstrap samplings (frequencies less than 50% are not indicated). *K*nuc, Kimura's parameter (12).

between the *M. furfur* and *M. obtusa* strains and our isolates is sufficient to resolve them as individual species, we propose the name *M. japonica* for the isolates (17, 20). The species epithet used here refers to the country where the species was discovered. The characteristics differentiating the new species, *M. japonica*, and the known *Malassezia* species are summarized in Table 3. *M. japonica* is easily distinguished from the other species by its ability to assimilate Tween 40 and Tween 60 and

TABLE 3. Physiological characteristics of *M. japonica sp. nov.* and other *Malassezia* species[a]

Species	Growth on SA[b] at 32°C	Growth on mDixon[c] at			Catalase reaction	Utilization of:			
		32°C	37°C	40°C		10% Tween 20	0.5% Tween 40	0.5% Tween 60	0.1% Tween 80
M. japonica sp. nov.	−	+	+	−	+	−	±	+	−
M. slooffiae[d]	−	+	+	+	+	± or +	+	+	−
M. sympodialis[d]	−	+	+	+	+	−	+	+	+
M. furfur[d]	−	+	+	+	+	+	+	+	+
M. dermatis	−	+	+	+	+	+	+	+	+
M. globosa[d]	−	+	± or −	−	+	−	−	−	−
M. obtusa[d]	−	+	± or +	−	+	−	−	−	−
M. restricta[d]	−	+	+	−	−	−	−	−	−
M. pachydermatis[d]	+	+	+	+	± or +	−	+	+	+

[a] +, positive; −, negative; ±, weakly positive.
[b] SA, Sabouraud dextrose agar.
[c] mDixon, modified Dixon agar.
[d] Data are from Guého et al. (7).

its inability to assimilate Tween 20 and Tween 80 and to grow at 40°C.

Direct detection of *M. japonica* DNA by a non-culture-based method. The sensitivity of the nested PCR assay was examined by using *M. japonica* DNA purified from the culture. The limit of detection for purified DNA was approximately 10 fg by the nested PCR assay. The specificities of the *M. japonica* species-specific primers are shown in Table 1. Our PCR primers amplified only the targeted *M. japonica* DNA and did not amplify the DNA of any other *Malassezia* species. We confirmed the absence of false-positive reactions by determining the DNA sequences of the PCR products after they were cloned in the pCR2.1 vector (Invitrogen), since various species, including bacteria and filamentous fungi, colonize the skin surface. We collected and analyzed 142 samples from 36 patients with AD and 66 samples from 22 healthy subjects. *M. japonica* DNA was detected in 12 patients (33.3%) and 3 healthy subjects (13.6%). A non-culture-based method (PCR) was previously developed to analyze the *Malassezia* microflora on the skin surface, since the isolation media and technique influence the growth of *Malassezia* species and the growth rates of each species differ. As a result, the detection rate is higher by the PCR-based method than by traditional culture methods (21). The previous study suggests that *M. globosa* and *M. restricta* make up the major part of the microflora in patients with AD. The frequency of detection of the new species, *M. japonica*, is the same as that of *M. sympodialis* and *M. furfur*.

In conclusion, we have described a novel species, *M. japonica*, isolated from the skin surface of a healthy Japanese subject. It is not known whether this microorganism plays a significant role in AD or other skin diseases. *M. japonica* was part of the microfloras in both patients with AD and healthy subjects.

Latin description of *Malassezia japonica* Sugita, Takashima, Kodama, Tsuboi, et Nishikawa. In LNA, post dies 6 ad 32°C, cellulae vegetativae sphaericae, ovoideae vel ellipsoideae 2–5 × 2–7 μm; sympodiales gemmantes proferentes. Cultura xanthoalba, semi-nitida aut hebetata, rugosa, et butyracea et margo glabra aut lobulata. In agaro glucoso-peptonico Tween 40 et Tween 60 (0.5%) addito crescit. H_2O_2 hydrolysatur. Commutatio colori per diazonium caeruleum B positiva. GTC acid: deoxyribonucleati 60.4 mol%. Ubiquinonum majus Q-9 est. Teleomorphis ignota. Typus: JCM 11963^T, ex cute, feminae sani, Tokyo, Japonia, 2002, T. Kodama (originaliter ut M 9966), conservatur in collectionibus culturarum quas "Japan Collection of Microorganisms," Saitama, Japan, sustentat.

Description of *Malassezia japonica* Sugita, Takashima, Kodama, Tsuboi, et Nishikawa sp. nov. On LNA, after 6 days at 32°C, the vegetative cells are spherical, oval, or ellipsoidal and 2 to 5 by 2 to 7 μm, and sympodial budding is observed (Fig. 2). The colony is pale yellowish, semishining to dull, wrinkled, and butyrous and has an entire to lobed margin. The organism grows on glucose-peptone agar with 0.5% Tween 40 and Tween 60 as the sole source of lipid. The catalase reaction is positive. The diazonium blue B reaction is positive. The G+C content of nuclear DNA is 60.4 mol%, and the major ubiquinone is Q-9. The teleomorph is unknown.

JCM 11963^T (CBS 9431^T; originally strain M 9966) was isolated from the skin of a healthy Japanese subject in Tokyo, Japan, by M. Kodama in November 2002 and is maintained in the Japan Collection of Microorganisms (JCM), Saitama, Japan. The other strain, M 9967, has also been deposited in the JCM and CBS collections as strain 11964 and strain 9432, respectively.

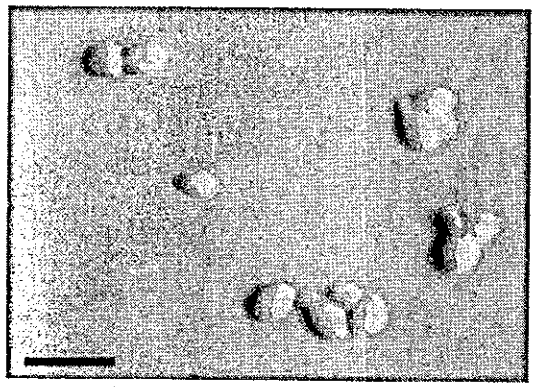

FIG. 2. Vegetative cells of *M. japonica* M 9966 grown in LNA for 7 days at 32°C. Bar, 10 μm.

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REFERENCES

1. **Akerstedt, J., and I. Vollset.** 1996. *Malassezia pachydermatis* with special reference to canine skin disease. Br. Vet. J. **152**:269–281.
2. **Ashbee, H. R., and E. G. Evans.** 2002. Immunology of diseases associated with *Malassezia* species. Clin. Microbiol. Rev. **15**:21–57.
3. **Aspiroz, C., M. Ara, M. Varea, A. Rezusta, and C. Rubio.** 2002. Isolation of *Malassezia globosa* and *M. sympodialis* from patients with pityriasis versicolor in Spain. Mycopathologia **154**:111–117.
4. **Faergemann, J.** 1998. The role of *Malassezia* species in the ecology of human skin and as pathogens. Med. Mycol. **36**(Suppl. 1):220–229.
5. **Faergemann, J.** 2002. Atopic dermatitis and fungi. Clin. Microbiol. Rev. **15**:545–563.
6. **Felsenstein, J.** 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution **39**:783–791.
7. **Guého, E., G. Midgley, and J. Guillot.** 1996. The genus *Malassezia* with description of four new species. Antonie Leeuwenhoek **69**:337–355.
8. **Guillot, J., and R. Bond.** 1999. *Malassezia pachydermatis*: a review. Med. Mycol. **37**:295–306.
9. **Gupta, A. K., Y. Kohli, J. Faergemann, and R. C. Summerbell.** 2001. Epidemiology of *Malassezia* yeasts associated with pityriasis versicolor in Ontario, Canada. Med. Mycol. **39**:199–206.
10. **Gupta, A. K., Y. Kohli, R. C. Summerbell, and J. Faergemann.** 2001. Quantitative culture of *Malassezia* species from different body sites of individuals with or without dermatoses. Med. Mycol. **39**:243–251.
11. **Hanifin, J. M., and G. Rajka.** 1980. Diagnostic features of atopic dermatitis. Acta Dermatol. Venereol. **92**:4–47.
12. **Kimura, M.** 1980. A simple method for estimation evolutionary rate of base substitutions through comparative studies of nucleotide sequences. J. Mol. Evol. **16**:111–120.
13. **Kurtzman, C. P., and C. J. Robnett.** 1997. Identification of clinically important ascomycetous yeasts based on nucleotide divergence in the 5′ end of the large-subunit (26S) ribosomal DNA gene. J. Clin. Microbiol. **35**:1216–1223.
14. **Makimura, K., Y. S. Murayama, and H. Yamaguchi.** 1994. Detection of a wide range of medically important fungal species by polymerase chain reaction (PCR). J. Med. Microbiol. **40**:358–364.
15. **Nakabayashi, A., Y. Sei, and J. Guillot.** 2000. Identification of *Malassezia* species isolated from patients with seborrhoeic dermatitis, atopic dermatitis, pityriasis versicolor and normal subjects. Med. Mycol. **38**:337–341.
16. **Nakase, T., and M. Suzuki.** 1986. *Bullera megalospora*, a new species of yeast forming large ballistospores isolated from dead leaves of *Oryza sativa*, *Miscanthus sinensis*, and *Sasa* sp. in Japan. J. Gen. Appl. Microbiol. **32**:225–240.
17. **Peterson, S. W., and C. P. Kurtzman.** 1991. Ribosomal RNA sequence divergence among sibling species of yeasts. Syst. Appl. Microbiol. **14**:124–129.
18. **Saitou, N., and M. Nei.** 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. **4**:406–425.
19. **Scheynius, A., C. Johansson, E. Buentke, A. Zargari, and M. T. Linder.** 2002. Atopic eczema/dermatitis syndrome and *Malassezia*. Int. Arch. Allergy Immunol. **127**:161–169.
20. **Sugita, T., A. Nishikawa, R. Ikeda, and T. Shinoda.** 1999. Identification of medically relevant *Trichosporon* species based on sequences of internal transcribed spacer regions and construction of a database for *Trichosporon* identification. J. Clin. Microbiol. **37**:1985–1993.
21. **Sugita, T., H. Suto, T. Unno, R. Tsuboi, H. Ogawa, T. Shinoda, and A. Nishikawa.** 2001. Molecular analysis of *Malassezia* microflora on the skin of atopic dermatitis patients and healthy subjects. J. Clin. Microbiol. **39**:3486–3490.
22. **Sugita, T., M. Takashima, T. Shinoda, H. Suto, T. Unn, R. Tsuboi, H. Ogawa, and A. Nishikawa.** 2002. New yeast species, *Malassezia dermatis*, isolated from patients with atopic dermatitis. J. Clin. Microbiol. **40**:1363–1367.
23. **Thompson, J. D., D. G. Higgins, and T. J. Gibson.** 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. **22**:4673–4680.

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The Basidiomycetous Yeasts *Cryptococcus diffluens* and *C. liquefaciens* Colonize the Skin of Patients with Atopic Dermatitis

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Abstract: **Our previous research showed that lipophilic yeasts, *Malassezia* species, colonize the skin of patients with atopic dermatitis (AD) at a high frequency. In this study, we found that two basidiomycetous yeasts, *Cryptococcus diffluens* and *C. liquefaciens*, colonize the skin significantly more frequently in AD patients than in healthy subjects. Transparent dressings were applied to the skin of 36 AD patients and 30 healthy subjects and then transferred onto Sabouraud dextrose agar. Colonies recovered from the medium were identified by DNA sequence analysis of internal transcribed spacer regions and the D1/D2 26S rRNA gene. *C. diffluens* and *C. liquefaciens* were isolated from 42% (15/36) and 33% (12/36) of AD patients and from 20% (6/30) and 20% (6/30) of healthy subjects, respectively. In addition, fungal DNA was extracted directly from the dressings and amplified in a specific nested PCR assay. *C. diffluens* and *C. liquefaciens* DNA were detected in dressings from 97% (35/36) and 86% (31/36) of the AD patients and 47% (14/30) and 37% (11/30) of the healthy subjects, respectively. These findings show that *Malassezia* spp. are not the only yeasts that colonize the skin of AD patients; *Cryptococcus* spp. also are present in a high proportion of patients. The role of these microorganisms in AD is as yet unknown, but the current findings, in combination with previous results, indicate that *C. diffluens*, *C. liquefaciens*, *M. globosa*, and *M. restricta* together colonize the skin surface of AD patients at a high frequency.**

Key words: ***Cryptococcus diffluens*, *Cryptococcus liquefaciens*, Atopic dermatitis, Microflora**

Most research on the relationship between atopic dermatitis (AD) and fungal flora have focused on *Malassezia* species and *Candida albicans*. *M. globosa* and *M. restricta* colonize the skin surface of AD patients at a frequency of approximately 90% (14). Although *Malassezia* species are common cutaneous microflora in both AD patients and healthy subjects, specific anti-*Malassezia* IgE is produced only in AD patients, and the titer of IgE correlates well with the severity of AD. In addition, antifungal agents sometimes are effective in treating AD. Based on this evidence, *Malassezia* spp. are considered an exacerbating factor in AD (1, 2). *Candida albicans* is a component of the fungal flora of the human gastrointestinal tract. Specific anti-*C. albicans* IgE is produced in 25–87% of AD patients (2).

During a survey of the cutaneous fungal flora of the skin of AD patients, we found the basidiomycetous yeasts, *Cryptococcus diffluens* and *C. liquefaciens*, at high frequencies. These two microorganisms previously were classified as *C. albidus*, a species that has been found to be taxonomically heterogeneous (12, 15, 18). In 2000, Fonseca et al. (3) reclassified *C. albidus* into 12 distinct species (*C. aerius*, *C. arrabidensis*, *C. chernovii*, *C. cylindricus*, *C. diffluens*, *C. liquefaciens*, *C. oeirensis*, *C. phenolicus*, *C. saitoi*, *C. terricola*, *C. uzbekistanensis*, and *C. wieringae*) based on rDNA sequence analysis. Though *C. albidus* sensu lato is distributed widely in the environment, infection due to this microorganism only has been reported rarely in immunocompromised patients (6, 8, 9). In addition, *C. albidus* is considered one of the causative antigens of Japanese summer-type hypersensitivity pneumonitis (10).

This paper describes the cutaneous distribution of *C. diffluens* and *C. liquefaciens* in AD patients and healthy

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Abbreviations: AD, atopic dermatitis; IGS, intergenic spacer.

subjects.

Materials and Methods

Subjects. Thirty-six AD outpatients (24 males, 12 females; ages 20 to 64 years, mean 33.3±10.5 years) at Tokyo Medical University Hospital and 30 healthy students (10 males, 20 females; ages 19 to 25 years, mean 20.9±1.4 years) at Meiji Pharmaceutical University participated in this study. AD was diagnosed according to the criteria of Hanifin and Rajka (4). Samples were collected from erythematous lesions on the faces and necks of the patients and from normal skin on the faces and necks of the healthy subjects. Routine skin care, including intermittent applications of mild steroid ointment or petrolatum, was given prior to sampling. Written informed consent was obtained from each subject.

Sample collection. OpSite transparent dressings (3 by 7 cm; Smith and Nephew Medical, Ltd., Hull, United Kingdom) were strapped to the skin and then transferred onto modified Sabouraud dextrose agar (20 g glucose, 10 g polypeptone, 5 g yeast extract, 15 g agar) containing 50 μg/ml chloramphenicol (Sankyo, Tokyo). The plates were incubated at 32 C for 5 days.

Identification of isolates. The strains recovered from the agar plates tentatively were identified using CHROMagar Candida (Kanto Chemical Corp., Tokyo) according to the manufacturer's instructions. The ITS region and D1/D2 26S rDNA of strains that could not be identified using CHROMagar Candida (i.e., dark purple colonies) were sequenced for accurate identification (7, 13). ITS and D1/D2 26S rRNA genes of all the dark purple yeast colonies were amplified by PCR using the oligonucleotide primers ITS1F (5'-GTCGTAACAAGGTTAACCTGCGG) and NL4R (5'-GGTCCGTGTTTCAAGACGG). PCR was performed in a Thermocycler (model 9700, Applied Biosystems, Calif., U.S.A.) with an initial 3 min denaturation at 94 C, followed by 30 cycles that consisted of 30 s at 94 C, 30 s at 54 C, and 1 min at 72 C, and a final 10 min extension at 72 C. The PCR products were sequenced with the ITS1F, NL1F (5'-GCATATCAATAAGCGGAGGAAAAG), ITS4R (5'-TCCTCCGCTTATTGATATGC), and NL4R primers using the ABI 310 DNA sequencer with an ABI PRISM BigDye Terminator Cycle Sequencing kit, version 3.1 (Applied Biosystems) according to the manufacturer's instructions.

Direct DNA detection in samples from patients with atopic dermatitis and healthy subjects. i) Design of C. diffluens *and* C. liquefaciens *species-specific primers for PCR.* Since the ITS and D1/D2 26S rDNA sequences of *C. diffluens* and *C. liquefaciens* are very similar, the sequences of the intergenic spacer (IGS) 1 region, located between the 26S and 5.8S rRNA genes of *C. diffluens* (CBS 160, CBS 926, and CBS 2824), *C. liquefaciens* (CBS 968), and the phylogenetically closely related species *C. albidosimilis* (CBS 7711) also were determined to design highly specific primer pairs for each species. The IGS 1 region was amplified using the primers 26SBF (5'-AGCTGCTGCCAATGCTAGCTC), which is located at the end of the 26S rRNA gene (16), and Crpt-5SR (5'-ACCCAGGATTCCCACGTGGTCC), which is located in the 5 S rRNA gene, with the following program: 94 C for 3 min, followed by 30 cycles of 94 C for 30 s, 58 C for 30 s, and 72 C for 30 s, with a final extension at 72 C for 10 min. The amplified PCR prod-

Table 1. Oligonucleotides used in the nested PCR

Species and primers used	Sequence (5'→3')	Reaction program	DNA fragment
1st PCR for *C. diffluens*		30 cycles of	207 bp
C. dif-IGS1-1F	TCGACTTGTCGACAATGGCAGTG	94 C for 20 s	
C. dif-IGS1-1R	AGAGTCCGAATGCGAGTCCAG	51 C for 1 min	
		72 C for 30 s	
2nd PCR for *C. diffluens*		30 cycles of	123 bp
C. dif-IGS1-2F	CTTAGTTAGTCAGTGCTGTCG	94 C for 20 s	
C. dif-IGS1-2R	TCCAGGACTTGAGCCTAAGCT	59 C for 30 s	
		72 C for 20 s	
1st PCR for *C. liquefaciens*		30 cycles of	256 bp
C. liq-IGS1-1F	CTCTGTGGGTGATGACTGAAG	94 C for 20 s	
C. liq-IGS1-1R	CTGCTTCAGATTCCTGTGTC	54 C for 1 min	
		72 C for 30 s	
2nd PCR for *C. liquefaciens*		30 cycles of	143 bp
C. liq-IGS1-2F	CTGGTTATCCTCTCGAACCAC	94 C for 20 s	
C. liq-IGS1-2R	CAGCTGACTTGCGTACTGTCT	57 C for 30 s	
		72 C for 20 s	

ucts directly were sequenced using the primer pair 26SBF and Crypt-5SR. From the IGS1 sequence analysis, a species-specific primer pair was designed as shown in Table 1. The specificity of the oligonucleotide primers was demonstrated using skin-related microorganisms as shown in Table 2. The fungal rRNA gene schemati-

Table 2. Specificity of the primers for *C. diffluens* and *C. liquefaciens*

Species	Strain[a]	Species-specific oligonucleotide primers for[b]			
		C. dif-IGS1-1F and C. dif-IGS1-1R	C. dif-IGS1-2F and C. dif-IGS1-2R	C. liq-IGS1-1F and C. liq-IGS1-1R	C. liq-IGS1-2F and C. liq-IGS1-2R
Cryptococcus diffluens	CBS 160	+	+	−	−
	CBS 926	+	+	−	−
	CBS 2824	+	+	−	−
	Isolate 5-11-1	+	+	−	−
	Isolate 11-1	+	+	−	−
Cryptococcus liquefaciens	CBS 968	−	−	+	+
	Isolate 3-2-2	−	−	+	+
	Isolate 3-29	−	−	+	+
	Isolate 5-4	−	−	+	+
	Isolate 23-4	−	−	+	+
Cryptococcus albidus	CBS 142	−	−	−	−
Cryptococcus albidosimilis	CBS 7711	−	−	−	−
Cryptococcus curvatus	CBS 570	−	−	−	−
Cryptococcus magnus	CBS 140	−	−	−	−
Candida albicans	CBS 562	−	−	−	−
Candida parapsilosis	CBS 604	−	−	−	−
Malassezia furfur	CBS 1878	−	−	−	−
Malassezia globosa	CBS 7966	−	−	−	−
Malassezia obtusa	CBS 7876	−	−	−	−
Malassezia restricta	CBS 7991	−	−	−	−
Malassezia slooffiae	CBS 7956	−	−	−	−
Malassezia sympodialis	CBS 7222	−	−	−	−
Rhodotorula mucilaginosa	CBS 17	−	−	−	−
Staphylococcus aureus	Isolate SA1	−	−	−	−

[a] Abbreviations: CBS, Centraalbureau voor Schimmelcultures, Baarn, The Netherlands.
[b] +, product obtained; −, no product obtained.

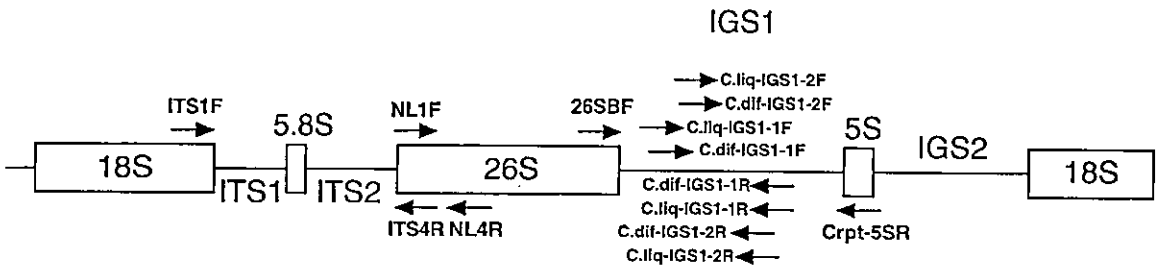

Fig. 1. Schematic representation of the fungal rRNA gene. ITS, internal transcribed spacer region; IGS, intergenic spacer region.

cally is shown in Fig. 1.

ii) Analysis of the C. diffluens *and* C. liquefaciens *microflora. C. diffluens* and *C. liquefaciens* DNA were detected using a nested PCR assay with each species-specific oligonucleotide primer. Fungal DNA was extracted from the OpSite dressings using a previously described method (14). PCR was performed with an initial denaturation at 94 C for 3 min, followed by conditions as listed in Table 1, and a final extension at 72 C for 10 min.

Results

Identification of the Yeast Isolates

Sixty and 21 dark-purple yeast colonies were recovered from the medium used for AD patients and healthy subjects, respectively. The ITS and D1/D2 26S rDNA sequences of the isolates were identical to those of *Cryptococcus diffluens* (AF145330 and AF075502) or *C. liquefaciens* (AF444345 and AF181515). *C. diffluens* and *C. liquefaciens* were isolated from 42% (15/36) and 33% (12/36) of the AD patients, respectively, and from 20% (6/30) and 20% (6/30) of the healthy subjects, respectively, as shown in Table 3. The numbers of colonies of *C. diffluens* and *C. liquefaciens* per individual were 2.5±1.3 and 1.9±1.0 for the AD patients, respectively, and 2.2±1.3 and 1.2±0.5 of the healthy subjects, respectively. The difference in the numbers of colonies between the AD patients and the healthy subjects was not significant. Colonies of these two species are shown in Fig. 2. Colony morphology also is described in the footnote to Fig. 2.

Direct DNA Detection in Samples

Before detecting *C. diffluens* and *C. liquefaciens* DNA in the samples, we demonstrated the specificity of our designed primers using DNA from representative skin-related microorganisms (*Malassezia, Rhodotorula*, and *Candida* species) and phylogenetically closely related *Cryptococcus* species, as shown in Table 2. Each oligonucleotide primer amplified the DNA only of the targeted species. The sensitivity of the nested PCR assay was examined using purified *C. diffluens* and *C. liquefaciens* DNA from the cultures. The limit of detection for purified DNA was approximately 10 fg in the nested PCR assay. *C. diffluens* and *C. liquefaciens* DNA were detected in samples from approximately 90% of the AD patients and approximately 40% of the healthy subjects (Table 3).

Nucleotide Sequence Accession Numbers

The nucleotide sequences of the IGS1 region determined in this study have been deposited with the DNA Data Bank of Japan (DDBJ) as AB115700 (*C. diffluens* CBS 160), AB115701 (*C. liquefaciens* CBS 968), and AB115702 (*C. albidosimilis* CBS 7711).

Table 3. Colonization frequency (%) of *Cryptococcus diffluens* and *C. liquefaciens* on the skin of patients with atopic dermatitis and healthy subjects

Species	Culture method			Non-culture method		
	AD patient	Healthy subject	$P^{a)}$	AD patient	Healthy subject	$P^{a)}$
C. diffluens	42% (15/36)	20% (6/30)	>0.05	97% (35/36)	47% (14/30)	<0.05
C. liquefaciens	33% (12/36)	20% (6/30)	>0.05	86% (31/36)	37% (11/30)	<0.05

a) Fisher exact test.

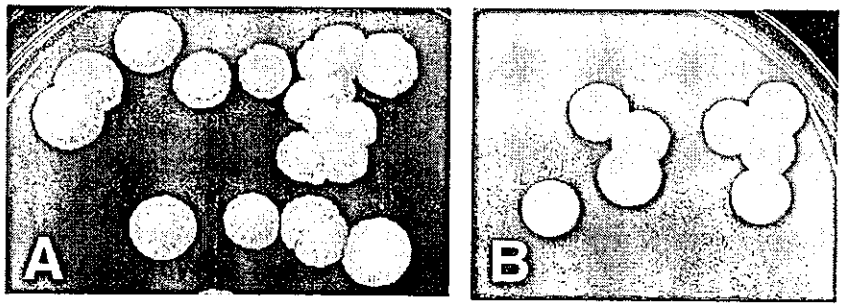

Fig. 2. Colonies of *C. diffluens* and *C. liquefaciens* isolated from patients with atopic dermatitis. The isolates were grown in modified Sabouraud dextrose agar for 5 days at 27 C. (A) *C. diffluens*; cream to pale yellow, semi-shining and shining near the margin, radially wrinkled to cerebriform and elevated in the center, butyrous with a lobate margin. (B) *C. liquefaciens*; yellowish white, glossy, smooth, soft, with an entire margin.

Discussion

It is well known that *Staphylococcus aureus* colonizes the skin lesions of patients with AD. In recent years, a ceramidase-producing bacterium, *Pseudomonas aeruginosa*, has been found in skin lesions. This microorganism is significantly more likely to colonize the skin lesions of AD patients than the skin of healthy subjects. The ceramidase from *P. aeruginosa* requires detergents for hydrolysis of human ceramide; however, there is no detergent on human skin. *P. aeruginosa* secretes a staphylolytic protease to lyse *S. aureus* cells, which results in the release of cardiolipin and phosphatidylglycerol. It is believed that *S. aureus*-derived glycerolphospholipids stimulate the hydrolysis of ceramide in atopic skin by bacterial ceramidase (5). These findings suggest that several types of microorganisms on the skin surface might participate in the development of AD, with each playing a different role. Among the fungal microflora, only *Malassezia* on the skin and *Candida albicans* in the gastrointestinal tract have been studied in AD patients (1, 2). In the present study, our comprehensive analysis showed that not only *Malassezia* but also *Cryptococcus* species colonize the skin of AD patients. Our findings, in combination with previous results, indicate that at least four fungal species, *M. globosa*, *M. restricta*, *C. diffluens*, and *C. liquefaciens*, colonize the skin of most AD patients. At present, the roles of the two *Cryptococcus* species in AD are unclear. However, Savolainen et al. (11) have detected specific IgE antibodies against several yeasts as aeroallergens, including *C. albidus*. Of a total of 12 patients with AD and 2 with rhinitis, 4 had specific IgE antibodies to *C. albidus*, while 10 patients had specific IgE antibodies to *C. albicans*. These results suggest that *C. albidus* might play a role in the etiology of AD in some patients. However, it is not known how the organisms identified as *C. albidus* by Savolainen et al. would be classified based on current taxonomic criteria. Therefore, it remains necessary to demonstrate specific IgE antibodies against *C. diffluens* and *C. liquefaciens* in order to elucidate the role of these species in AD. The cutaneous microflora include multiple species. By taking the interactions among various species into consideration, new roles of microorganisms in AD may be elucidated.

In the present study, we analyzed an IGS region because *C. diffluens*, *C. liquefaciens*, and *C. albidosimilis* are phylogenetically very closely related. The nucleotide differences of their ITS regions (total 379 bp) are 0 to 1 bp (0–0.3%), and those of their D1/D2 26S rDNA (total 658 bp) are 3 to 9 bp (0.46–1.37%). However, as the IGS1 sequences among the three species are only approximately 40–60% similar, the species easily can be distinguished by IGS1 sequence analysis. In addition, the IGS analysis has further analytical significance because IGS polymorphisms may exist within a species. We have reported that the genotype of *M. globosa*, a major component of the microflora of healthy skin, differs between AD patients and healthy subjects (17). This finding suggests that the specific genotypic strain may play a significant role in AD. The IGS sequences of the *C. diffluens* and *C. liquefaciens* isolates obtained from AD patients and healthy subjects in this study also were determined as identical, therefore, there was no IGS sequence diversity in either species.

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References

1) Ashbee, H.R., and Evans, E.G. 2002. Immunology of diseases associated with *Malassezia* species. Clin. Microbiol. Rev. **15**: 21–57.
2) Faergemann, J. 2002. Atopic dermatitis and fungi. Clin. Microbiol. Rev. **15**: 545–563.
3) Fonseca, A., Scorzetti, G., and Fell, J.W. 2000. Diversity in the yeast *Cryptococcus albidus* and related species as revealed by ribosomal DNA sequence analysis. Can. J. Microbiol. **46**: 7–27.
4) Hanifin, J.M., and Rajka, G. 1980. Diagnostic features of atopic dermatitis. Acta Dermato-Venereol. **92**: 4–47.
5) Kita, K., Sueyoshi, N., Okino, N., Inagaki, M., Ishida, H., Kiso, M., Imayama, S., Nakamura, T., and Ito, M. 2002. Activation of bacterial ceramidase by anionic glycerophospholipids: possible involvement in ceramide hydrolysis on atopic skin by *Pseudomonas* ceramidase. Biochem. J. **362**: 619–626.
6) Kordossis, T., Avlami, A., Velegraki, A., Stefanou, I., Georgakopoulos, G., Papalambrou, C., and Legakis, N.J. 1998. First report of *Cryptococcus laurentii* meningitis and a fatal case of *Cryptococcus albidus* cryptococcaemia in AIDS patients. Med. Mycol. **36**: 335–339.
7) Kurtzman, C.P., and Robnett, C.J. 1997. Identification of clinically important ascomycetous yeasts based on nucleotide divergence in the 5' end of the large-subunit (26S) ribosomal DNA gene. J. Clin. Microbiol. **35**: 1216–1223.
8) Loison, J., Bouchara, J.P., Gueho, E., de Gentile, L., Cimon, B., Chennebault, J.M., and Chabasse, D. 1996. First report of *Cryptococcus albidus* septicaemia in an HIV patient. J. Infect. **33**: 139–140.
9) Melo, J.C., Srinivasan, S., Scott, M.L., and Raff, M.J. 1980. *Cryptococcus albidus* meningitis. J. Infect. **2**: 79–82.
10) Miyagawa, T., Hamagami, S., and Tanigawa, N. 2000. *Cryp-*

tococcus albidus-induced summer-type hypersensitivity pneumonitis. Am. J. Respir. Crit. Care Med. **161**: 961–966.
11) Savolainen, J., Kortekangas-Savolainen, O., Nermes, M., Viander, M., Koivikko, A., Kalimo, K., and Terho, E.O. 1998. IgE, IgA, and IgG responses to common yeasts in atopic patients. Allergy **53**: 506–512.
12) Sugita, T., Nishikawa, A., and Shinoda, T. 1992. DNA relatedness among three varieties of *Cryptococcus albidus*. J. Gen. Appl. Microbiol. **40**: 397–408.
13) Sugita, T., Nishikawa, A., Ikeda, R., and Shinoda, T. 1999. Identification of medically relevant *Trichosporon* species based on sequences of internal transcribed spacer regions and construction of a database for *Trichosporon* identification. J. Clin. Microbiol. **37**: 1985–1993.
14) Sugita, T., Suto, H., Unno, T., Tsuboi, R., Ogawa, H., Shinoda, T., and Nishikawa, A. 2001. Molecular analysis of *Malassezia* microflora on the skin of atopic dermatitis patients and healthy subjects. J. Clin. Microbiol. **39**: 3486–3490.
15) Sugita, T., Takashima, M., Ikeda, R., Nakase, T., and Shinoda, T. 2001. Intraspecies diversity of *Cryptococcus albidus* isolated from humans as revealed by sequences of the internal transcribed spacer regions. Microbiol. Immunol. **45**: 291–297.
16) Sugita, T., Nakajima, M., Ikeda, R., Matsushima, T., and Shinoda, T. 2002. Sequence analysis of the ribosomal DNA intergenic spacer 1 regions of *Trichosporon* species. J. Clin. Microbiol. **40**: 1826–1830.
17) Sugita, T., Kodama, M., Saito, M., Ito, T., Kato, Y., Tsuboi, R., and Nishikawa, A. 2003. Sequence diversity of the intergenic spacer region of the rRNA gene of *Malassezia globosa* colonizing the skin of patients with atopic dermatitis and healthy individuals. J. Clin. Microbiol. **41**: 3022–3027.
18) Vancanneyt, M., Coopman, R., Tytgat, R., Hennebert, G.L., and Kersters, K. 1994. Whole-cell protein patterns, DNA base compositions and coenzyme Q types in the yeast genus *Cryptococcus* Kutzing and related taxa. Syst. Appl. Microbiol. **17**: 65–75.

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Transforming growth factor-β_1 suppresses atopic dermatitis-like skin lesions in NC/Nga mice

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Summary

Background Atopic dermatitis is a chronic, relapsing inflammatory disorder characterized by pruritic and eczematous skin lesions. Transforming growth factor (TGF)-β_1 has been implicated in the suppression of inflammatory responses.

Objective The purpose of this study is to determine whether TGF-β_1 suppresses skin lesions in a mouse model of atopic dermatitis.

Methods We used the NC/Nga strain of mice as an *in vivo* model of atopic dermatitis. The effects of exogenous TGF-β_1 on atopic dermatitis-like skin lesions in NC/Nga mice were evaluated clinically, histologically and immunologically.

Results Subcutaneous injection of recombinant TGF-β_1 macroscopically suppressed eczematous skin lesions in NC/Nga mice associated with reduced serum immunoglobulin E (IgE) levels. Histological analysis showed that TGF-β_1 significantly inhibited the infiltration of inflammatory cells such as mast cells and eosinophils into the skin of NC/Nga mice. Spontaneous interferon (IFN)-γ production from splenocytes of NC/Nga mice was down-regulated by the treatment with TGF-β_1 and neutralizing antibody against IFN-γ inhibited skin lesions in NC/Nga mice. The inhibitory effect of TGF-β_1 on the skin lesions lasted at least 1 week after cessation of the treatment.

Conclusion These findings indicate that TGF-β_1 suppressed atopic dermatitis-like skin lesions in NC/Nga mice at least in part through down-regulation of IFN-γ. These results suggest that TGF-β_1 may have a therapeutic potential for atopic dermatitis.

Keywords atopic dermatitis, TGF-β_1, NC/Nga mice, inflammation, IFN-γ
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Introduction

Atopic dermatitis is a chronically relapsing inflammatory disorder that is characterized by pruritic and eczematous skin lesions often associated with elevated serum immunoglobulin (Ig)E levels [1–3]. It has been suggested that T lymphocytes, mast cells, monocytes/macrophages, and eosinophils infiltrated into skin lesions of atopic dermatitis are involved in the development of atopic dermatitis through the release of various cytokines/chemokines including interleukin (IL)-4, IL-5, and, interferon (IFN)-γ [4].

Transforming growth factor (TGF)-β_1 is a member of the TGF-β superfamily that exerts a wide range of biological functions [5]. Importantly, TGF-β_1 null mice develop extensive inflammation in various organs and die shortly after birth, suggesting that TGF-β_1 plays a critical role in the suppression of the immune system *in vivo* [6]. In fact, TGF-β_1 has been implicated in the suppression of various inflammatory disorders [7]. However, it remains unclear whether TGF-β_1 suppresses skin lesions of atopic dermatitis.

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In this study, we determined the efficacy of exogenous TGF-β_1 to suppress atopic dermatitis-like skin lesions in NC/Nga mice which are a model of atopic dermatitis [8]. We showed that the administration of TGF-β_1 suppressed atopic dermatitis-like skin lesions in NC/Nga mice at least in part through the down-regulation of IFN-γ. The results suggest that TGF-β_1 may have a therapeutic potential for atopic dermatitis.

Materials and methods

Mice

The NC/Nga strain of mice originated from Japanese fancy mice and was established as an inbred strain by K. Kondo [9]. Male 18-weeks-old NC/Nga mice with fully developed skin lesions were purchased from SLC (Tokyo, Japan). These mice were kept in conventional surroundings. As a control for *in vivo* and *in vitro* experiments examining the effects of TGF-β_1 on atopic dermatitis-like skin lesions and on IFN-γ production from splenocytes, male 18-weeks-old NC/Nga mice that were kept in specific pathogen free (SPF) condition and showed no skin lesions were also employed. Animal experiments were approved by the Institutional Review Board of Juntendo University.

Study design

Mice at the age of 18 weeks with fully developed skin lesions and kept in conventional surroundings were treated with subcutaneous injection of recombinant human TGF-β_1 (1 μg/mouse) (R & D, Minneapolis, MN, USA) (Group 1) or intraperitoneal injection of water soluble dexamethasone (1 mg/mouse) (Sigma, St Louis, MO, USA) for comparison (Group 2). As a control, mice of the same age with similar lesions were treated with saline alone (Group 3). In each group of mice, injection of TGF-β_1 or dexamethasone was repeated three times at a 1-week interval. 1 week after the last injection, the mice (at the age of 21 weeks) from each group were killed for evaluation of the treatments. To determine the role of endogenous IFN-γ on the development of skin lesions in NC/Nga mice, neutralizing monoclonal antibody against IFN-γ (500 μg/mouse) (XMG-1.2) [10] was administered once intraperitoneally into the mice at the age of 18 weeks with fully developed skin lesions and that were kept in conventional surroundings. As a control, isotype-matched rat IgG antibody (PharMingen, San Diego, CA, USA) was administered.

Clinical scoring of skin lesions

A total clinical severity score for skin lesions of NC/Nga mice was defined as the sum of the individual score grades as 0 (none), 1 (mild), 2 (moderate), and 3 (severe) for each of five signs and symptoms (itch, erythema/haemorrhage, oedema, excoriation/erosion, and scaling/dryness) as previously described [8]. Itching was evaluated by observing scratching behaviour for > 2 min The clinical scoring was performed in a blinded fashion by one investigator in order to prevent inter-investigator variation.

Skin biopsy

The mice were killed by cervical dislocation, and the hairs of the face were removed. An area of approximately $1.5 \times 1.5\,cm^2$ of the skin was excised.

Histological analysis

Skin sections were resected, fixed in 10% paraform-aldehyde, embedded into paraffin, sectioned, stained with haematoxylin-eosin solution, and examined by light microscopy for histological evaluation. Mast cells, and eosinophils were stained with toluidine blue or eosin Y methylene blue, respectively. The number of infiltrating mast cells and eosinophils were counted in 5 randomly chosen consecutive fields at × 400 magnification in sections from two different mice. Skin fibrotic changes were evaluated with Elastica van Gieson staining, a specific histochemical staining for collagen and elastin.

Plasma IgE levels

Blood was collected from the retro-orbital plexus of NC/Nga mice before (at the age of 18 weeks) and after (at the age of 21 weeks) the treatment with TGF-β_1, as described above, while under ether anaesthesia and heparinized immediately thereafter. Plasma samples were obtained by centrifugation and stored at −20 °C until use. Total IgE levels were measured by a sandwich murine IgE ELISA kit (Yamasa, Japan).

Cytokine ELISA

Spleen cells (5×10^6 cells) isolated from NC/Nga mice which were treated with TGF-β_1 or dexamethasone as described above were re-stimulated with plate-bound anti-CD3 antibody (2C11) (10 μg/mL) (PharMingen) 7 days after the last treatment. 72 h after the culture, supernatants were collected for measurement of cytokines. The amount of IL-5, IL-13 and IFN-γ in the culture supernatant of spleen cells was determined using murine IL-5, IL-13, and IFN-γ ELISA kit (ENDOGEN, Woburn, MA, USA).

Data analysis

Data are summarized as mean ± SD. The statistical analysis of the results was performed by the amount of variance using Fisher's least significant difference test for multiple comparisons. $P < 0.05$ was considered to be significant.

Results

Administration of TGF-β_1 suppresses atopic dermatitis-like skin lesions and serum IgE levels in NC/Nga mice.

The NC/Nga strain of mice has been reported to develop an eczematous condition that is clinically and histologically very similar to atopic dermatitis when kept in conventional surroundings [8]. The eczema develops at the age of 8 weeks, with maximum activity at around 17 weeks with lesions characterized by oedema, haemorrhage, erosion, and dryness typically localized on the ears, back, neck and facial region. Marked hyperkeratosis and infiltration of CD4 T cells, macrophages, eosionophils, and mast cells are observed in the skin lesions.

To determine the efficacy of TGF-β_1 to suppress skin lesions of NC/Nga mice, we treated the NC/Nga mice with fully developed skin lesions at the age of 18 weeks with recombinant TGF-β_1 or dexamethasone for comparison. As a control, the mice with fully developed skin lesions were treated with saline. 3 weeks after the initiation of treatment with TGF-β_1, the skin lesions on the back, ears and facial regions of the NC/Nga mice showed extensive regression when compared with saline-treated mice (Figs 1a and b). The skin lesions of dexamethasone-treated mice also showed extensive regression (Figs 1c and d). Scoring of the clinical skin severity revealed marked reduction of the score in the TGF-β_1-treated mice (Fig. 1e). The reduction in score in TGF-β_1-treated mice was comparable to that in dexamethasone-treated mice (Fig. 1e).

Histologically, NC/Nga mice with fully developed skin lesions showed hyperkeratosis, partial defect of epidermis and dense infiltration of leucocytes in the dermis (Fig. 2a). After the treatment with TGF-β_1, the mice showed acanthosis and reduced infiltration of leucocytes in the dermis (Fig. 2e). Dexamethasone-treated mice also showed significant reduction of infiltrating leucocytes in the dermis (Fig. 2i). Toluidine blue staining showed decreased mast cell number in the skin lesions in TGF-β_1-treated mice (mast cell number: saline 46.7 ± 5.9 vs. TGF-β_1 32.1 ± 9.5, $n = 5$, mean ± SD, $P < 0.05$, Figs 2b and f). Eosinophil number was also significantly decreased in the skin lesions of TGF-β_1-treated mice (eosinophil number: saline 48.3 ± 8.2 vs. TGF-β_1 35.7 ± 5.4, $n = 5$, mean ± SD, $P < 0.05$, Figs 2c and g). The reduction of mast cell and eosinophil number in TGF-β_1-treated mice was also comparable to that in dexamethasone-treated mice (Figs 2j and k). Elastica van Gieson staining of skin tissue sections showed that treatment of skin lesions in NC/Nga mice with recombinant human TGF-β_1 did not induce any significant increase of collagen and elastic fibres in the skin tissue compared with saline-treated or

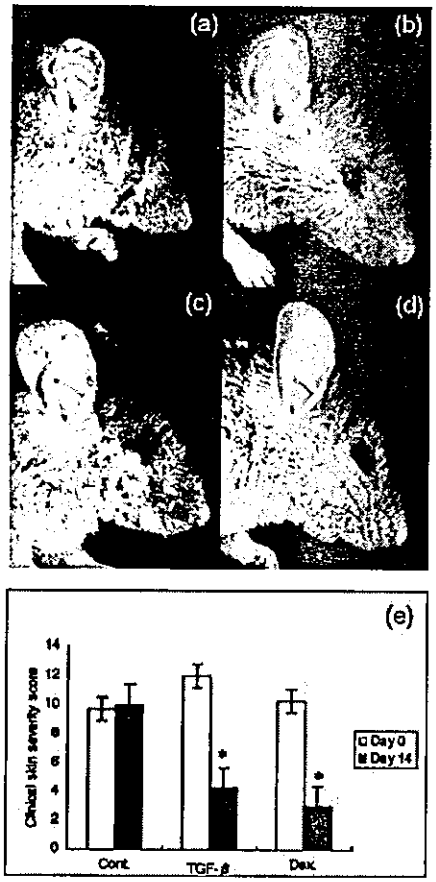

Fig. 1. Inhibition of skin lesions in NC/Nga mice by TGF-β_1. (a–d) Appearance of skin lesions. NC/Nga mice at the age of 18-weeks-old with fully developed skin lesions were treated with subcutaneous injection of TGF-β_1 (upper panels) or intraperitoneal injection of dexamethasone (lower panels) three times at a 1-week interval. The photograph of mice was taken at the age of 18 weeks (left) or 21 weeks (right). (e) Clinical skin severity scores of NC/Nga mice. Clinical scores were measured 3 weeks after the treatment with TGF-β_1 and dexamethasone (the mice were at the age of 21-weeks-old of age.) Values represent the mean ± SD ($n = 4$). *$P < 0.05$, significantly different from the mean value of the corresponding control response.

dexamethasone-treated mice although some minor increase of disorganized elastic fibres were observed in TGF-β_1-treated mice (Figs 2d, h and l).

It was previously shown that the serum IgE levels of NC/Nga mice were markedly elevated from the age of 8 weeks correlating with clinical severity of the dermatitis [8]. We then examined the effect of TGF-β_1 on serum IgE levels of NC/Nga mice. Blood samples were collected from NC/Nga mice with fully developed skin lesions before (at the age of 18 weeks) and after (at the age of 21 weeks) the treatment with TGF-β_1 and serum IgE levels were determined by ELISA. We found that the administration of recombinant TGF-β_1 significantly reduced serum IgE levels of NC/Nga mice after the treatment (serum IgE levels before treatment: 14193 ± 1072 ng/mL vs. after treatment: 8432 ± 590 ng/mL; $n = 4$, mean ± SD, $P < 0.05$). These results indicated that the administration of recombinant TGF-β_1 macroscopically and histologically suppressed skin lesions of NC/Nga mice associated with reduced serum IgE levels.

Administration of TGF-β_1 suppresses spontaneous IFN-γ production from splenocytes of NC/Nga mice

To explore possible mechanisms for TGF-β_1-mediated suppression of skin lesions in NC/Nga mice, we examined capacity of splenocytes to produce Th1 or Th2 type cytokines. As shown in Fig. 3(a), spontaneous IFN-γ production was observed in splenocytes from saline-treated mice with fully developed skin lesions whereas the spontaneous IFN-γ production was significantly suppressed in TGF-β_1 or dexamethasone-treated mice. There was no significant difference in IL-5 and IL-13 production from the splenocytes between saline-treated mice and TGF-β_1-treated mice regardless of the presence or absence of stimulation with anti-CD3 antibody (Fig. 3a). In addition, spontaneous IFN-γ production was not observed in splenocytes isolated from NC/Nga mice that were kept in SPF condition and showed no skin lesions (Fig. 3b). These findings suggested that spontaneous IFN-γ production from the splenocytes of NC/Nga mice was associated with the development of skin lesions in NC/Nga mice.

To further determine whether endogenous IFN-γ contributed to the development of skin lesions in NC/Nga mice, we examined the effect of neutralizing antibody against IFN-γ on the development of skin lesions. As shown in Fig. 4, we found that clinical severity of skin lesions was partially, but significantly reduced 1 week after the treatment with anti-IFN-γ antibody. These findings indicated that TGF-β_1 suppressed IFN-γ production in NC/Nga mice, thereby, at least in part, inhibited the atopic dermatitis-like skin lesions.

Duration of the inhibitory effect of TGF-β_1 on skin lesions of NC/Nga mice

Skin lesions in NC/Nga mice were suppressed at least for 1 week after the last injection of TGF-β_1 (Fig. 5). 2 weeks after the last injection of TGF-β_1, scoring of clinical severity of skin lesions became worse to the level before the treatment (Fig. 5).

Discussion

In this study, we demonstrated that the systemic administration of recombinant TGF-β_1 suppressed atopic dermatitis-like skin lesions in NC/Nga mice associated with reduced serum IgE levels and down-regulation of IFN-γ production from the splenocytes. Since treatment with anti-IFN-γ antibody partially ameliorated skin lesions in NC/Nga mice, these findings strongly suggested that TGF-β_1 suppressed skin lesions in NC/Nga mice at least in part through down-regulation of IFN-γ.

The pathophysiology of atopic dermatitis-like skin lesions in NC/Nga mice has not been fully understood. Previous studies showed that FK506 and persimmon leaf extract that suppressed mast cell functions improved skin lesions in NC/Nga mice [11,12], suggesting that T cells and mast cells contributed to the development of skin lesions. Our findings suggested that IFN-γ also contributed to the development of skin lesions in NC/Nga mice. Matsuda et al. previously showed that IFN-γ-producing cells were infiltrated in the skin lesions of NC/Nga mice [8]. In addition, Vestergaard et al. recently suggested that IFN-γ induced TARC (thymus- and activation-regulated chemokine) expression in the skin lesions of NC/Nga mice,

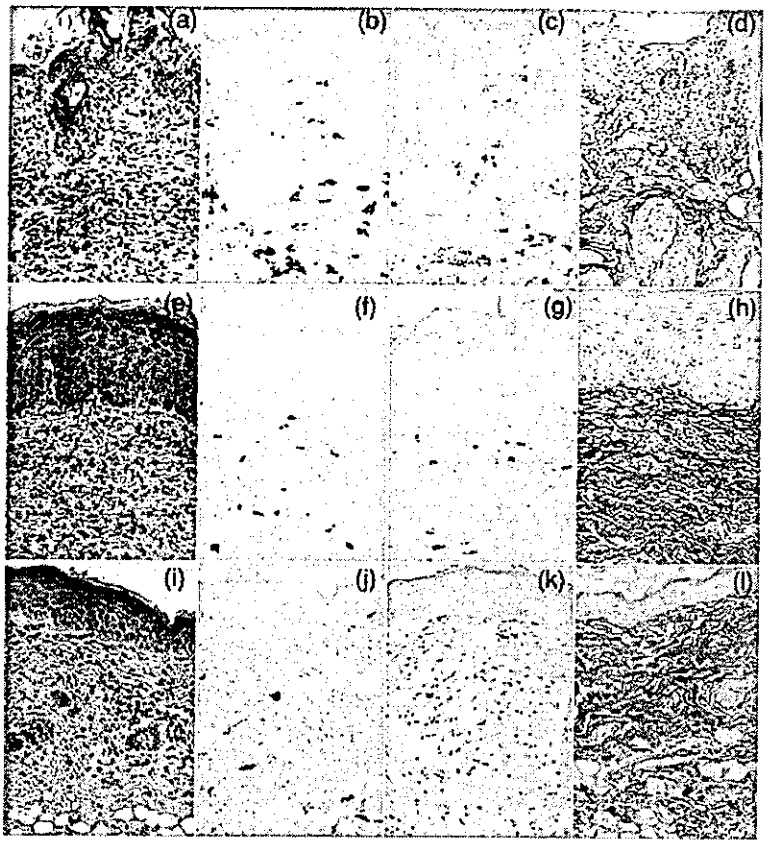

Fig. 2. Reduced skin inflammation in NC/Nga mice by TGF-β_1. Facial skin sections from saline-treated mice (a–d), TGF-β_1-treated mice (e–h), and dexamethasone-treated mice (i–l) were stained with haematoxylin and eosin (a, e and i), toluidine blue (b, f and j), eosin Y methylene blue (c, g and k), and Elastica van Gieson (collagen stained red and elastin black) (d, h and l).

thereby contributing to the development of the skin lesions [13]. IFN-γ was also implicated in the pathophysiology of atopic dermatitis [14]. The lymphocytes infiltrating into the skin lesions of atopic dermatitis produced not only Th2 cytokines but also Th1 type cytokines such as IFN-γ in later stages of the disease [4]. Taken together, IFN-γ appeared to play an important role in the pathophysiology of fully developed skin lesions in NC/Nga mice as well as skin lesions in atopic dermatitis.

We found that IFN-γ was spontaneously produced from splenocytes isolated from NC/Nga mice with fully developed skin lesions, but not from splenocytes from NC/Nga mice without skin lesions. The findings suggested that spontaneous IFN-γ production was associated with the development of skin lesions in NC/Nga mice. Our preliminary data showed that depletion of T cells with anti-thy1 antibody did not affect spontaneous IFN-γ production from splenocytes from NC/Nga mice with fully developed skin lesions, suggesting that the major cellular source of IFN-γ was non-T cells (possibly macrophages or dendritic cells). The factors that induce spontaneous IFN-γ production from splenocytes in NC/Nga mice remain to be determined.

Down-regulation of IFN-γ by exogenous TGF-β_1 was previously reported in animal models such as autoimmune insulitis and experimental colitis [15,16], which was consistent with our results. Treatment with TGF-β_1 inhibited IFN-γ production from lamina propria T cells obtained from a mouse model of colitis [16]. Intracellular mechanisms underlying TGF-β_1-mediated suppression of IFN-γ production should be investigated in future.

Since TGF-β_1 acts on multiple targets affecting the immune system [17–19], TGF-β_1-mediated suppression of skin lesions in NC/Nga mice could not be only attributed to down-regulation of IFN-γ. Because TGF-β_1 suppresses the survival of eosinophils and mast cells [18,19], the decreased number of these cells in the skin lesions might be a result of the suppression of their survival. Alternatively, TGF-β_1 inhibits expression of adhesion molecules involved in leucocyte–endothelial cell interaction [20]. Thus, inhibition of leucocyte infiltration into the skin at the level of leucocyte–endothelial cell interaction by TGF-β_1 could explain the decreased inflammation in the skin lesions of NC/Nga mice. Reduction of serum IgE levels by TGF-β_1 in NC/Nga mice might also contribute to the immunomodulating actions. However, it is most likely that these events occur in concert.

The ability of TGF-β_1 to induce fibrosis is a potential concern. Persistent activity of TGF-β_1 is thought to be important for inducing tissue fibrosis [21]. We did not observe any significant fibrosis in muscles, kidneys, lungs, liver, heart, pancreas, as well as skin in mice treated with TGF-β_1. We speculate that the activity of TGF-β_1 is not enough to induce tissue fibrosis at the dosage or duration of TGF-β_1 used in the current study. In addition, we do not know about the clinical significance of acanthosis observed in the TGF-β_1-treated mice and if it is beneficial or not. Acanthosis disappeared at least 4 weeks after

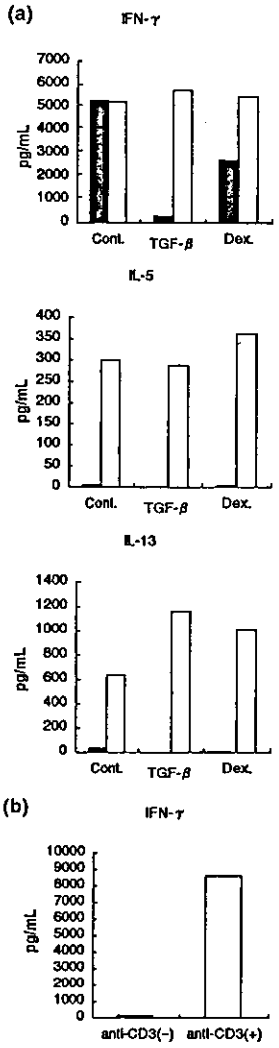

Fig. 3. Suppression of IFN-γ production from splenocytes obtained from NC/Nga mice by TGF-β_1. (a) Spleen cells from mice with fully developed skin lesions that were non-treated or treated with TGF-β_1 or dexamethasone were cultured in the presence (open column) or absence (closed column) of plate-bound anti-CD3 antibody for 3 days. IFN-γ, IL-5 and IL-13 productions in the culture supernatant were measured by ELISA. Data from one representative experiment are shown. Similar results were obtained from at least other two independent experiments. (b) Spleen cells from mice without skin lesions were cultured in the presence or absence of plate-bound anti-CD3 antibody for 3 days. IFN-γ levels in the culture supernatant were measured by ELISA. Data from one representative experiment are shown. Similar results were obtained from at least two other independent experiments.

the cessation of TGF-β_1 treatment (data not shown). We thus think so far that acantosis would not cause unbeneficial outcome in the TGF-β_1-treated mice.

In summary, we demonstrated that TGF-β_1 suppressed atopic dermatitis-like skin lesions in NC/Nga mice. These findings suggest that TGF-β_1 may have a therapeutic potential for atopic dermatitis.

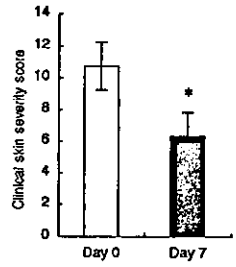

Fig. 4. Suppression of skin lesions in NC/Nga mice by anti-IFN-γ antibody.NC/Nga mice with fully developed skin lesions at the age of 18 weeks were treated with anti-IFN-γ antibody or rat IgG and clinical skin scores were measured 1 week after the treatments. Values represent the mean ± SD ($n = 4$). *$P < 0.05$, significantly different from the mean value of the corresponding control response.

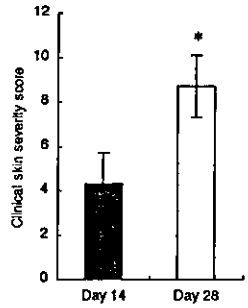

Fig. 5. Duration of TGF-β_1-mediated suppression of skin lesions in NC/Nga mice.NC/Nga mice with fully developed skin lesions were treated with TGF-β_1 three times at a 1-week interval. Clinical severity of skin lesions was evaluated 1 or 2 weeks after the last injection of TGF-β_1 to determine the duration of the treatment. Values are mean ± SD for 5 mice in each group. *$P < 0.05$, significantly different from the mean value of the corresponding control response.

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References

1 Hanifin J, Rajka G. Dianostic features of atopic dermatitis. Acta Derm Venereol Suppl 1980; 92:44–7.
2 Rudikoff D, Lebwohl M. Atopic dermatitis. Lancet 1998; 351:1715–21.
3 Cooper KD. Atopic dermatitis. recent trends in pathogenesis and therapy. J Invest Dermatol 1994; 102:128–37.
4 Leung DYM. Atopic dermatitis. new insights and opportunities for therapeutic intervention. J Allergy Clin Immunol 2000; 105:860–76.
5 Roberts AB, Sporn MB. The transforming growth factors-β. In: Sporn MB, Roberts AB. eds. Handbook of Experimental Pharmacology. Springer-Verlag, Heidelberg, 1990; 95:418–72.

6 Shull MM, Ormsby I, Kier AB et al. Targeted disruption of the mouse transforming growth factor-beta 1 gene results in multifocal inflammatory disease. Nature 1992; 359:693–9.
7 Wahl S. Transforming growth factor-β. the good, the bad, and the ugly. J Exp Med 1994; 180:1587–90.
8 Matsuda H, Watanabe N, Geba GP et al. Development of atopic dermatitis-like skin lesion with IgE hyperproduction in NC/Nga mice. Int Immunol 1997; 9:461–6.
9 Kondo K, Nagami T, Tadokoro S. Differences in haematopoeitic death among inbred strains of mice. In. Comparative Cellular and Species Radiosensitivity. Bond P, Sugawara S. eds. Igakushoin, Tokyo, 1969:20–30.
10 Cherwinski HM, Schumacher JH, Brown KD, Mosmann TR. Two types of mouse helper T cell clone. III. Further differences in lymphokine synthesis between Th1 and Th2 clones revealed by RNA hybridization, functionally monospecific bioassays, and monoclonal antibodies. J Exp Med 1987; 166:1299–44.
11 Hiroi J, Sengoku T, Morita K et al. Effect of tacrolimus hydrate (FK506) ointment on spontaneous dermatitis in NC/Nga mice. Jpn J Pharmacol 1998; 76:175–83.
12 Kotani M, Matsumoto M, Fujita A et al. Persimmon leaf extract and astragalin inhibit development of dermatitis and IgE elevation in NC/Nga mice. J Allergy Clin Immunol 2000; 106:159–66.
13 Vestergaard C, Yoneyama H, Murai M et al. Overproduction of Th2-specific chemokines in NC/Nga mice exhibiting atopic dermatitis-like lesions. J Clin Invest 1999; 104:1097–105.
14 Grewe M, Gyufko K, Schopf E, Krutmann J. Lesional expression of interferon-gamma in atopic dermatitis. Lancet 1994; 343:25–6.
15 Piccirillo C, Chang Y, Prud'homme GJ. TGF-β_1 somatic gene therapy prevents autoimmune disease in nonobese diabetic mice. J Immunol 1998; 161:3950–6.
16 Kitani A, Fuss IJ, Nakamura K, Schwartz OM, Usui T, Strober W. Treatment of experimental (trinitrobenzene sulfate acid) colitis by intranasal administration of TGF-β_1 plasmid: TGF-β_1-mediated suppression of T helper cell type I responses occurs by IL-10 induction and IL-12 receptor β chain downregulation. J Exp Med 2000; 192:41–52.
17 Letterio JJ, Roberts AB. Regulation of immune responses by TGF-β. Annu Rev Immunol 1998; 16:137–61.
18 Alam R, Forsythe P, Stafford S, Fukuda Y. Transforming growth factor-β abrogates the effects of hemotopoietins on eosinophils and induce their apoptosis. J Exp Med 1994; 179:1041–5.
19 Mekori YA, Metcalfe DD. Transforming growth factor-β prevents stem cell factor-mediated rescue of mast cells from apoptosis after IL-3 deprivation. J Immunol 1994; 153:2194–203.
20 DiChiara MR, Kiely JM, Gimbrone MAJr, Lee ME, Perrella MA, Topper JN. Inhibition of E-selectin gene expression by transforming growth factor-β in endothelial cells involves coactivator integration of Smad and nuclear factor kappa-mediated signals. J Exp Med 2000; 192:695–704.
21 Border WA, Noble NA. Transforming growth factor-beta in tissue fibrosis. New Eng J Med 1994; 331:1286–92.

Differential responses of mast cell Toll-like receptors 2 and 4 in allergy and innate immunity

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Toll-like receptor 2 (TLR2) and TLR4 play important roles in the early innate immune response to microbial challenge. To clarify the functional roles of TLRs 2 and 4 in mast cells, we examined bone marrow–derived mast cells (BMMCs) from TLR2 or TLR4 gene-targeted mice. Peptidoglycan (PGN) from *Staphylococcus aureus* stimulated mast cells in a TLR2-dependent manner to produce TNF-α, IL-4, IL-5, IL-6, and IL-13, but not IL-1β. In contrast, LPS from *Escherichia coli* stimulated mast cells in a TLR4-dependent manner to produce TNF-α, IL-1β, IL-6, and IL-13, but not IL-4 nor IL-5. Furthermore, TLR2- but not TLR4-dependent mast cell stimulation resulted in mast cell degranulation and Ca^{2+} mobilization. In a mast cell–dependent model of acute sepsis, TLR4 deficiency of BMMCs in mice resulted in significantly higher mortality because of defective neutrophil recruitment and production of proinflammatory cytokines in the peritoneal cavity. Intradermal injection of PGN led to increased vasodilatation and inflammation through TLR2-dependent activation of mast cells in the skin. Taken together, these results suggest that direct activation of mast cells via TLR2 or TLR4 by respective microligands contributes to innate and allergic immune responses.

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Introduction

Mast cells are particularly frequent in close proximity to epithelial surfaces where they are strategically located for optimal interaction with the environment and for their putative functions in host defense (1, 2). Mast cells have been viewed primarily as effector cells of anaphylactic reactions but also viewed as important cells for initiation of innate and acquired immune response against various pathogens (3–5). These reactions are mediated by a number of preformed or newly synthesized mediators and cytokines that are secreted upon activation of mast cells (6). In contrast to the well-known activation of mast cells via high-affinity IgE receptors (FcεRI), the mechanisms of direct activation of mast cells by various microorganisms have not been studied precisely.

Recent reports suggest that innate immune responses are also initiated by certain pattern recognition receptors, called Toll-like receptors (TLRs). TLRs comprise a family of mammalian cell-surface proteins that stimulate proinflammatory cytokine gene transcription in response to various microbial ligands (7). A large body of evidence studied in TLR2 and TLR4 gene-targeted mice indicated that TLR4 is a signal transducer of LPS, a component of Gram-negative bacteria (GNB) (8–10), whereas TLR2 appears to mediate responses to lipoteichoic acid, lipopeptides, and peptidoglycan (PGN) from Gram-positive bacteria (GPB) and mycobacteria (11–17). We have previously demonstrated, using LPS-low responder C3H/HeJ mice, that there are expressions of TLRs on mast cells and that mast cell TLR4 mediates innate immune response against GNB (18). However, to date, it has still been undefined whether the recognition of pathogens by each TLR on the mast cell is similar to that on the other leukocytes, what roles are played by TLRs in the function of mast cells, and if there are quantitative or qualitative differences in the mast cell activation by different TLRs. *Staphylococcus aureus*, the best-known GPB, colonizes on more than 90% of atopic eczematous skin, and the number of colonized bacteria and an increased number of activated mast cells are well correlated with the severity of the eczematous lesion (19, 20). Since mast cells express TLR2, it is important to clarify whether the clinical features are dependent on the actual activation of mast cells via TLR2 by GPB.

In this study, we first show that mast cell TLR2 or mast cell TLR4 recognizes the pathogen ligands in a way similar to those of other leukocytes; that is, mast cell TLR2 but not mast cell TLR4 confers GPB component responsiveness, and TLR4 but not TLR2 is sufficient for GNB responsiveness. Second, we observe quantitative and qualitative differences in the activation of mast cells via TLR2 and TLR4 stimulation. TLR2-mediated mast cell