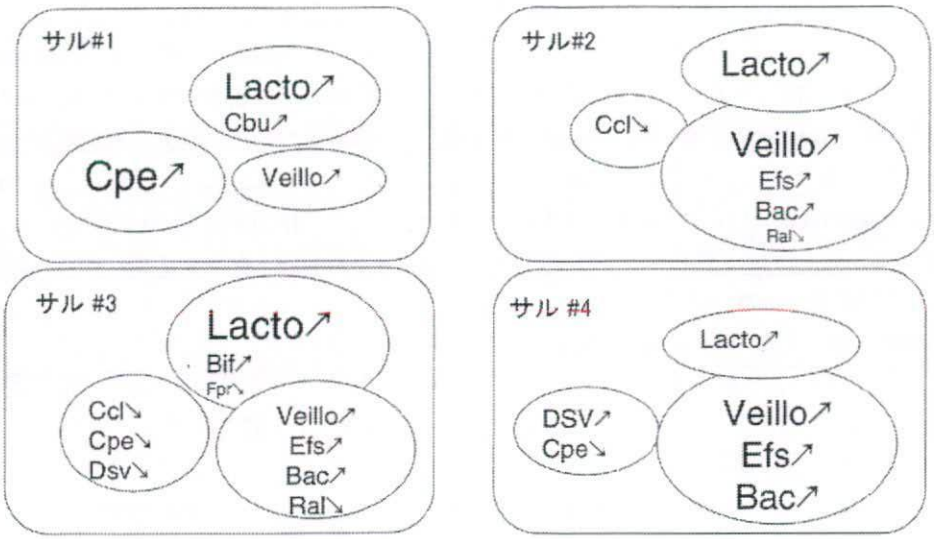

図7. イヌリンの腸内細菌叢への影響（3倍以上の増加：↗、1/3以下の減少：↘）
視覚的にイメージし易いように、細菌の増減の度合いに応じて字の大きさを変えてある。

動について紹介する。

4-2-2. 方法

メスカニクイザル4頭より卵巣除去し、閉経モデルを作成した。馴化期間後、ヒトの日常的摂取量に換算した量のイヌリン（2.0 g/head/day）を3ヶ月間毎日経口投与し、経時的に糞便を採取した。「4-1.ダイズ成分の評価試験」と同様に、糞便サンプル中の代表的な腸内細菌のDNA量をリアルタイムPCR法で定量測定した（表1）。

4-2-3. 結果と考察

イヌリン投与前の糞便中細菌DNA量には個体によるばらつきが見られた。特に今回着目すべき細菌について、表2に各個体の投与前細菌DNA量を示した。サル#1のように、もともとBacteroidesのような日和見菌が多く、BifidobacteriaやLactobacilliのような乳酸菌が少ない個体がいるのがわかった。

3ヶ月のイヌリン連続経口投与の結果、これまで報告されていたように、Bifidobacteriaの増加は今回の実験でも認められ、個体によって投与開始後1ヶ月、あるいは2ヶ月後にピークがあった。しかしながら3ヶ月間の投与終了時点では、Bifidobacteriaの量は結果的に殆ど投与前のレベルに戻っていた。イヌリン投与開始前と3ヶ月連続投与後の腸内細菌叢を比較し、最終的に増減が認められた細菌をまとめると図7のようになる。全ての個体においてLactobacilliとVeillonellaの増加が見られた。これまで、ヒトの短期摂取実験や*in vitro*実験ではLactobacilli

表2. イヌリン投与前の糞便中細菌DNA相対量

プライマー略号	*Uniに対する比×10^8			
	サル#1	サル#2	サル#3	サル#4
Bif	33	1479	105	109
Lacto	562	561022	8972	37327
Bac	2172286	308488	80043	4377
Veillo	4	112	86	828
Cpe	211	25420	55373	32625

*：今回特に着目した細菌の特異的プライマーで検出したDNA量を、ユニバーサルプライマーによるDNA量で割ったもの。見やすくするために、10^8倍してある。

の増加よりもBifidobacteriaの増加が大きいことが報告されていたが[24, 25]、そこでは投与（実験）期間が短かったために、長期摂取の影響が捉えられなかったのかもしれない。また、老化に伴い、BifidobacteriaよりもLactobacilliが多くなるという報告[26]と一致して、今回使用したサルモデルはもともとLactobacilliの方が多かったことから（表2）、我々の試験はヒトにおける「老齢で閉経」という状態をよく近似していると思われる。

Veillonellaは乳酸菌がイヌリンを分解して産生した乳酸を利用して増えた可能性がある。感染症が報告されているためにVeillonellaは有害菌に区分されることが多いが、常在菌としてサルモネラ等の病原菌感染を抑制するとの報告もあり[27]、その性質はまだよくわかっていない。

サルのうち、1頭は有害菌の*Clostridium perfringens*（Cpe）が増加していた。Cpeはグルコースを利用し[28]、酸性環境下でもかなり耐性であるので[29]、イヌリン投与で乳酸菌が増えた状態でも増加する可能性はある。この個体は他個体と比較すると、投与前よりCpeも少なかっ

たがBifidobacteriaやLactobacilliも少なかった（表2）。このような場合、プロバイオティクスとの組合せ投与が腸内細菌叢の改善により有効かもしれず、今後の課題である。これらのことから、機能食品のヒトへの適用において投与前および投与期間中の腸内細菌叢をモニターする意義が示唆された。

5. まとめ

昨今、機能食品の需要・供給がますます増大しつつあるが、機能食品の有効性・安全性については適切な試験を行い、科学的根拠をもって消費者に提供すべきである。サル類はヒトに類似のライフサイクルや生理学的な特性を持つので、機能食品試験においても他の動物には取って代われない有用なモデルとなる。ただ、サルの実験・飼育には大きな費用、労力がかかるので、サルを使える実験施設は限られる。また、試験に使用するサル個体数は制限されるので、科学的にも充分に洗練された試験を行う必然性が出てくる。動物福祉という観点からも、実験デザインの最適化に努め、意義のある試験を行う必要がある。

引用文献

1) 林浩孝, 大野智, 太田康之, 新井隆成, 鈴木信孝, 特定保健用食品の許認可について, 日本補完代替医療学会誌, 4, 103-112 (2007).
2) 保健機能食品制度, 厚生労働省, URL: http://www.mhlw.go.jp/topics/2002/03/tp0313-2a.html
3) 杉山幸丸ら, "サルの百科", データハウス, 1996.
4) L. M. Newman, E. M. Johnson and R. E. Staples, Assessment of the effectiveness of animal developmental toxicity testing for human safety, *Reprod. Toxicol.*, **7**, 359-390 (1993).
5) M. Hirano, S. Nakamura, F. Mitsunaga, M. Okada, K. Shimizu and T. Imamura, Transfer of maternally administered fusogenic liposome-DNA complexes into monkey fetuses in a pregnancy model, *J. Gene Med.*, **4**, 560-566 (2002).
6) National Research Council, "Guide for the care and use of laboratory animals", National Academy Press, Washington, DC, 1996.
7) 中村志帆, 光永総子, 中村伸, サルの安全な取扱いのために－感染リスクの対応手引き, 霊長類研究, **15**, 337-394 (1999).
8) 光永総子, 藤本浩二, 中村伸, Bウイルス (*Cercopithecine herpesvirus* 1) 感染の予防, 緊急対応および治療に関するガイドライン, 霊長類研究, **20**, 147-164 (2004).
9) K. Ohsawa, T. W. Lehenbauer and R. Eberle, *Herpesvirus papio* 2: alternative antigen for use in monkey B virus diagnostic assays, *Lab. Anim. Sci.*, **49**, 605-616 (1999).
10) J. Takano, T. Narita, K. Fujimoto, R. Mukai and A. Yamada, Detection of B virus infection in cynomolgus monkeys by ELISA using simian agent 8 as alternative antigen, *Exp. Anim.*, **50**, 345-347 (2001).
11) F. Mitsunaga, S. Nakamura, T. Hayashi and R. Eberle, Changes in the titer of anti-B virus antibody in captive macaques (*Macaca fuscata*, *M. mulatta*, *M. fascicularis*), *Comp. Med.*, **57**, 120-124 (2007).
12) 中村伸, 光永総子, 山内英典, 岡田真紀, Aram Joeng, 林隆志, Rosalio M. Perez, Romualdo L. Carrasco, 中川博司, ダイズ成分による高脂血症および動脈硬化への予防・治療効果の作用機序：サルモデルを用いた分子病態生理学的研究, 大豆たん白質研究, **6**, 57-62 (2003).
13) 中村伸, 光永総子, 山内英典, Aram Joeng, 林隆志, Joseph Gabriel, ダイズ食品・成分による加齢性機能障害および生活習慣病の予防作用：サルモデルでのゲノム医生物学的研究(I), 大豆たん白質研究, **7**, 13-19 (2004).
14) 中村伸, 光永総子, A-Ram Joeng, 山内英典, 林隆志, Joseph Gebriel, 大豆食品・成分による加齢性機能障害および生活習慣病の予防効果：サルモデルでのゲノム医生物学的研究(II), 大豆たん白質研究, **8**, 1-7 (2005).
15) E. A. Lucas, S. A. Lightfoot, L. J. Hammond, L. Devareddy, D. A. Khalil, B. P. Daggy, Y. Soungdo and B. H. Arjmandi, Soy isoflavones prevent ovariectomy-induced atherosclerotic lesions in Golden Syrian hamster model of postmenopausal hyperlipidemia, *Menopause*, **10**, 314-321 (2003).
16) T. Uesugi, T. Toda, K. Tsuji and H. Ishida, Comparative study on reduction of bone loss and lipid metabolism abnormality in ovariectomized rats by soy isoflavones, daidzin, genistin, and glycitin, *Biol. Pharm. Bull.*, **24**, 368-372 (2001).
17) T. Rinttilä, A. Kassinen, E. Malinen, L. Krogius and A. Palva, Development of an extensive set of 16S rDNA-targeted primers for quantification of pathogenic and indigenous bacteria in faecal samples by real-time PCR, *J. Appl. Microbiol.*, **97**, 1166-1177 (2004).
18) S. Bartosch, A. Fite, G. T. Macfarlane and M. E. McMurdo, Characterization of bacterial communities in feces from healthy elderly volunteers and hospitalized elderly patients by using real-time PCR and effects of antibiotic treatment on the fecal microbiota, *Appl. Environ. Microbiol.*, **70**, 3575-3581 (2004).
19) M. A. Nadkarni, F. E. Martin, N. A. Jacques and N. Hunter, Determination of bacterial load by real-time PCR using a broad-range (universal) probe and primers set, *Microbiology*, **148** (Pt 1), 257-266 (2002).
20) 中村伸, 光永総子, 橋本寛之, 出井早苗, 林隆志, Joseph Gabriel, 大豆食品・成分による閉経性/加齢性機能障害の予防作用：サルモデルでのゲノム医生物学的研究(III), 大豆たん白質研究, **9**, 1-8 (2006).
21) T. M. Calub, A. L. Waterhouse and A. D. French, Conformational analysis of inulobiose by molecular mechanics, *Carbohydr. Res.*, **207**, 221-235 (1990).
22) K. R. Niness, Inulin and oligofructose: What are They?, *J. Nutr.*, **129**, 1402S-1406S (1999).
23) M. B. Roberfroid, Introducing inulin-type fructans, *Br. J. Nutr.*, **93**, S13-25 (2005).
24) B. Kleessen, B. Svkura, H. -J. Zunft and M. Blaut, Effects of

inulin and lactose on fecal microflora, microbial activity, and bowel habit in elderly constipated persons, *Am. J. Clin. Nutr.*, **65**, 1397-1402 (1997).
25) Y. Bouhnik, L. Raskine, K. Champion, C. Andrieux, S. Penven, H. Jacobs and G. Simoneau, Prolonged administration of low-dose inulin stimulates the growth of bifidobacteria in humans, *Nutr. Res.*, **27**, 187-193 (2007).
26) M. J. Hopkins and G. T. Macfarlane, Changes in predominant bacterial populations in human faeces with age and with Clostridium difficile infection, *J. Med. Microbiol.*, **51**, 448-454 (2002).
27) A. Hinton Jr. and M. E. Hume, Antibacterial activity of the metabolic by-products of a Veillonella species and Bacteroides fragilis, *Anaerobe*, **1**, 121-127 (1995).
28) M. Egert, A. A. de Graaf, A. Maathuis, P. de Waard, C. M. Plugge, H. Smidt, N. E. P. Deutz, C. Dijkema, W. M. de Vos and K. Venema, Identification of glucose-fermenting bacteria present in an in vitro model of the human intestine by RNA-stable isotope probing, *FEMS Microbiol. Ecol.*, **60**, 126-135 (2007).
29) P. D. Cotter and C. Hill, Surviving the acid test: Responses of gram-positive bacteria to low pH, *Microbiol. Mol. Biol. Rev.*, **67**, 429-453 (2003).

PROFILE

光永　総子
京都大学霊長類研究所
教務補佐員
獣医学修士

1986年北海道大学大学院獣医学研究科予防治療学専攻修士課程修了、同年京都大学霊長類研究所研修員、1997年同研究支援推進員、2006年NPO法人プライメイト・アゴラ監事。

中村　伸
京都大学霊長類研究所
助教
東京理科大学
客員教授
理学博士

J Med Primatol doi:10.1111/j.1600-0684.2008.00289.x

ORIGINAL ARTICLE

Gene expression profile of Th1 and Th2 cytokines and their receptors in human and nonhuman primates

A. Ram Jeong, Shin Nakamura & Fusako Mitsunaga

Department of Cellular and Molecular Biology, Primate Research Institute, Kyoto University, Inuyama, Aichi, Japan

Keywords
chimpanzee – IFN-γ – IFN-γR – IL-12 – IL-4 – IL-4Rα – macaque –baboon

Correspondence
Shin Nakamura, PhD, Primate Research Institute, Kyoto University, Inuyama, Aichi 484-8506, Japan.
Tel.: +81 568 63 0579;
fax: +81 568 63 0579;
e-mail: snakamur@pri.kyoto-u.ac.jp

Accepted December 29, 2007.

Abstract

Background To date comparative knowledge concerning gene expression profiles of T-helper 1(Th1)/Th2 cytokines and their receptors between human and non-human primates is scarce.

Methods We assessed the gene expression level of both Th1 [interleukin-4(IL-4)] and Th2 [IL-12, interferon- γ(IFN-γ)] cytokines and the receptors (IL-4Rα, IFN-γR1, IFN-γR2) in peripheral blood mononuclear cells (PBMC) from humans, chimpanzee, baboon, and macaque by a quantitative real-time reverse transcriptase-polymerase chain reaction(RT-PCR).

Results The expression level of the IFN-γ gene was markedly lower in humans than that in non-human primates. The IL-4 gene expression was significantly higher, whereas that of IL-12 was distinctly lower, in human/chimpanzee than in baboon/macaque. The IFN-γR2 gene expression was especially higher in the macaque than in the other three primates.

Conclusions These results indicate distinct gene expression of Th1/Th2 cytokines and their receptors in primates. These also suggest characteristic differences in Th1/Th2 immune responses affecting host defense and/or disease susceptibility among these primates.

Introduction

Non-human primates are used as invaluable models for biomedical studies of immune diseases and/or their therapy, such as allergy [33], gene vaccine [16], and infectious disease [22]. To date comparative knowledge concerning gene expression profiles of cytokines and their receptors participating in T-helper 1 and T-helper 2 pathways between human and non-human primates is scarce.

T-helper (Th) cells are divided into two groups, Th type 1 (Th1) and type 2 (Th2) [27]. Th1 cells express/produce Th1 cytokines, interferon-γ(IFN-γ), interleukin-2(IL-2), and tumor necrosis factor-β(TNF-β) which participate in cell-mediated immunity, whereas, Th2 cells produce Th2 cytokines, i.e. IL-4, IL-5, IL-6, IL-10, and IL-13, associated with antibody-mediated immunity [1, 28, 30]. The Th1 and Th2 cells counter-regulate each other through expression/production of their distinctive cytokines, which appear to be a regulatory network for the immune responses mediated by them. IL-12, which is mainly produced by macrophages and dendritic cells, favors the differentiation of Th1 cells and plays a central role in Th1 responses [17, 23, 40]. In this study, we examined the gene expression profile of cytokines (IL-4, IL-12, IFN-γ) and the receptors of two of them (IL-4Rα, IFN-γR1, IFN-γR2) in humans and in three non-human primates (chimpanzee, baboon, macaque), which are widely used in biomedical research. We found several characteristic expression profiles: a distinct difference in expression of IL-4 and IL-12 genes between hominoids (human, chimpanzee) and monkeys (macaque, baboon), the suppressed expression of the IFN-γ gene in humans compared with its expression in non-human primates, and the elevated expression of both IFN-γR1 and IFN-γR2 genes in the macaque.

Materials and methods

Collection of blood samples

Heparinized venous blood samples were obtained from human volunteers (n = 6), chimpanzees (*Pan troglodytes*, n = 4), baboons (*Papio hamadryas*, n = 3), and macaques (*Macaca fascicularis*, n = 3). All the non-human primates used were normal adults raised at the Kyoto University Primate Research Institute (KUPRI) in accordance with the guidelines of the KUPRI for the Care and Use of Laboratory Primates. This institutional guideline is based on the 'Guide for the Care and Use of Laboratory Animals' by the US National Research Council [5, 29].

Preparation of PBMC

Blood samples were centrifuged at 1000 *g* for 15 minutes at room temperature to remove plasma. The cellular fraction was suspended in the same volume of sterile phosphate-buffered saline (PBS). The suspensions of non-human primate and human cells were then mixed with one-fifth volume of 5% Dextran T-2000 and T-500, respectively, and then incubated at 37°C for 30 minutes to separate the erythrocytes. The supernatant obtained, i.e. the leukocyte fraction, was overlaid on a Ficoll-Conray solution (density, 1.077) and centrifuged at 400 *g* for 30 minutes at room temperature. Peripheral blood mononuclear cells(PBMC) accumulated on the top of the Ficoll-Conray layer, and the PBMC fraction was washed twice with cold sterile PBS to remove contaminating platelets. All solutions for PBMC preparation were pyrogen-free.

RNA isolation and reverse transcription

Total RNA was extracted from PBMC by using an RNeasy micro kit (Qiagen, Santa Clarita, CA, USA) according to the manufacturer's protocol in conjunction with on-column DNase treatment (RNase-Free DNase Set, Qiagen). The yield and purity of the obtained RNA were determined spectrophotometrically at 260 and 280 nm. cDNA was synthesized from 1 μg of total RNA by reverse transcription with AMV reverse transcriptase XL (Takara Bio, Otsu, Japan) and primed with oligo (dT)12–18 primer (Invitrogen, Carlsbad, CA, USA).

Real-time PCR for gene expression analysis of cytokines and their receptors

Real-time polymerase chain reaction(PCR) was performed with the ABI Prism 7700 Sequence Detector System (Applied Biosystems, Foster City, CA, USA) using an SYBR detection system. PCR primers for cytokine (IL-4, IL-12, IFN-γ), receptor (IL-4Rα, IFN-γR1, IFN-γR2), and GAPDH genes are listed in Table 1. PCR conditions were as follows: briefly, PCR proceeded in a 10-μl reaction mixture containing 5 μl of 2x SYBR Premix Ex Taq (Takara Bio, Otsu, Japan), 0.2 μl of 50x ROX Reference Dye (Takara Bio), 0.2 μl of a mixture of forward and reverse primers (initial concentration of 10 μM for each), 1 μl of the reverse transcriptase(RT) product corresponding to 40 ng of RNA, and 3.6 μl of distilled water. Samples were pre-incubated for 10 s at 95°C, and then subjected to 40 cycles of amplification at 95°C for 5 s for denaturing and at 63°C for 30 s for annealing-extension. A dissociation curve was generated at the end of the PCR cycles to verify that a single gene product had been amplified. GAPDH was chosen as an internal control for each RT product, and the standard curve for GAPDH cDNA within 10^5 to $10^{10} \times 15$ copies was generated to quantify the control GAPDH mRNA level. The semi-quantitative assessment of the mRNA of interest was done by dividing with the expression level of the internal GAPDH and hence expressed as the so-called 'relative expression to GAPDH'. All assays were conducted in duplicate, and the mean value was used as the gene expression level. For interspecies comparison among primates the relative expression of each target gene was calculated vs. that of the human corresponding gene. All PCR amplicons were subjected to sequence analysis with ABI Genetic Analyzer 310 and their sizes (bp) estimated from the sequence data.

Results

As to the relative gene expressions of IL-4 and IL-12, a distinct difference in the expression level of these genes was observed between human/chimpanzee (hominoid) and baboon/macaque (monkey). As shown in Fig. 1A, the expression level of the IL-4 gene in hominoids was markedly higher than that in monkeys; i.e. the human IL-4 gene expression was 89- and 30-fold higher than that of the baboon and macaque genes, respectively. In contrast, the expression level of the IL-12 gene was significantly lower in the hominoids than that in the monkeys (Fig. 1B). Interestingly, the expression level of the human IFN-γ gene was clearly distinct from that for non-human primates including the chimpanzee (Fig. 1C), being 12–24 times lower.

Figure 2 shows the relative gene expression levels of the cytokine receptors examined, i.e. IL-4Rα, INF-

Table 1 Sequences of each primer and annealing site of target gene

	Forward	Reverse	Reference	PCR product (bp)*
IL-4 primer	5′-CGAAACTCTGAACAGCCTCACAGAG-3′	5′-TCAGCTCGAACACTTTGAATATTTCTCTCTCAT-3′	L26027	
Human	·A····T·····	·····	NM_000589	358
Chimpanzee	·A····T·····	·····	AY130260	358
Cynomolgus	·····	·····T····	AB000515	358
IL-4Rα primer	5′-TGACCTGGAGCAACCCGTATC-3′	5′-GAAGGGCTCCCTGTAGGAGT-3′	NM_000418/AY459192	
Human	·····	·····	NM_000418	266
Chimpanzee	·····	·····	XM_001135957	266
Cynomolgus	·····	·····	AY459192	266
IL-12 primer	5′-AGCAGCTGGTCATCTCTTGGTT-3′	5′-CCAGCATCTCCAAACTCTTTGA-3′	**	
Human	····T·····	·····	AF180563	247
Chimpanzee	····T·····	·····	XM_527101	247
***Rhesus	·····	·····	U19841	247
IFN-γ primer	5′-TGCAGAGCCAAATTGTCTCCTTTTAC-3′	5′-GGGATGCTCTTCGACCTCGAAA-3′	NM_000619/D89985/NM_001032905	
Human	·····	·····	NM_000619	294
Chimpanzee	·····	·····	XM_001151968	294
Cynomolgus	·····	·····	D89985	291
IFN-γR1 primer	5′-GAGACGAGCAGGAAGTCGATT-3′	5′-ACTGGAATCACTAACTGGCACT-3′	NM_000416(for Forward)/AF227551 (for Reverse)	
Human	·····	·····G·····	NM_000416	157
Chimpanzee	·····	·····G·····	XM_001171493	157
Cynomolgus	····A·····	·····	AB173721	157
IFN-γR2 primer	5′-AGCACCCGAAGATTCGCCT-3′	5′-GCTCAGCTCGAAGGCGTAGA-3′	NM_005534	
Human	·····	·····	NM_005534	255
Chimpanzee	·····	·····	XM_525461	255
***Rhesus	·····	·····A··C···	XM_001091864	255
GAPDH primer	5′-CCATGGAGAAGGCTGGGG-3′	5′-CAAAGTTGTCATGGATGACC-3′	NM_002046	
Human	·····	·····	NM_002046	246
Chimpanzee	····T·····	·····	XR_019963	246
Cynomolgus	···C·····	·····	AB220454	246

*Size of PCR product was estimated from its sequence analysis.
**Hofmann-Lehmann R *et al.* AIDS Res Hum Retroviruses 2002; 18:627–39.
***Rhesus macaque sequences were reffered because of little data of cynomolgus macaque ones.

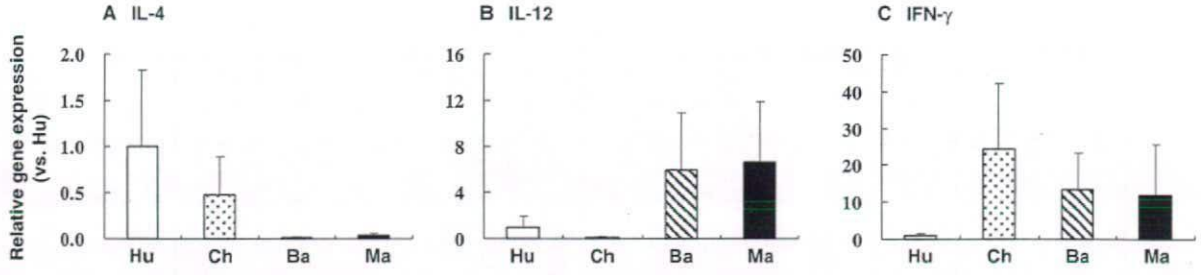

Fig. 1 Relative gene expression levels of Th1/Th2 cytokines in human (Hu; □), chimpanzee (Ch; ▨), baboon (Ba; ▩), and macaque (Ma; ■); preparation of peripheral blood mononuclear cells. IL-4 (A), IL-12 (B), and IFN-γ (C) gene expression levels are shown as a ratio to those of GAPDH. Values are mean ± SD relative to the levels of humans as controls, who were arbitrarily assigned a value of 1.

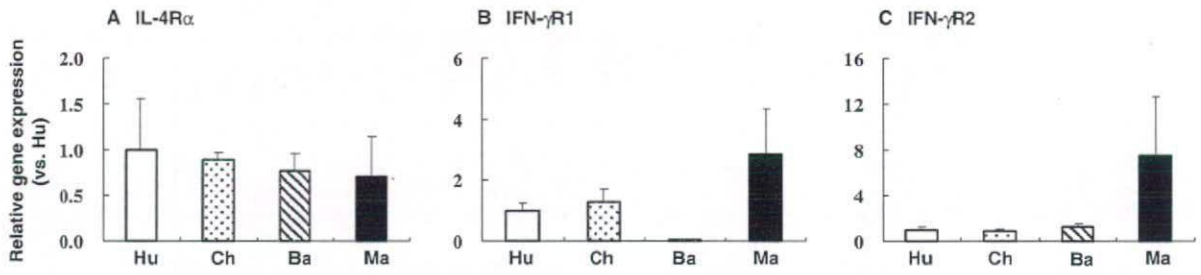

Fig. 2 Relative gene expression levels of Th1/Th2 cytokine receptors in humans (Hu;□), chimpanzee (Ch;▨), baboon (Ba;▩) and macaque (Ma;■) PBMC. IL-4Rα (A), IFN-γR1 (B), and IFN-γR2 (C) gene expression levels are shown as a ratio to those of GAPDH. Values are mean ± SD relative to the levels of humans as controls, who were arbitrarily assigned a value of 1.

γR1, and INF-γR2. Among primates, unique gene expression levels of both IFN-γ receptors, INF-γR1 and INF-γR2, were observed in the macaque, whose expression levels of IFN-γR1 and IFN-γR2 genes were approximately three- and eight-fold, respectively, higher than those of humans or other non-human primates (Fig. 2B,C). The IFN-γR1 gene was expressed at an exceptionally low level in the baboon, being 37-, 48-, and 107-fold lower than its level in humans, chimpanzee, and macaque, respectively (Fig. 2B). The level of IL-4Rα gene expression appeared to be little different among the primates examined here (Fig. 2A).

Discussion

Comparative studies of the physiological status of the immune system, especially Th1 and Th2 cytokines and their receptors, in human and non-human primates, give us invaluable information to perform immune-related biomedical examinations using non-human models. The gene expression profile on steady-state mRNA level in immune tissues or cells is a powerful genomics to assess some self-defense systems in physiological and/or pathological conditions in human and non-human primates [13, 24, 37]. The comparative studies of protein level using antibodies are limited to cross-related species [25, 42], as there is much more difference in binding affinity or reactivity between target proteins and antibodies than that of RNA/DNA sequences. For this reason it is hard to find available antibody to examine quantitative analysis of target cytokines and/or their receptors between humans and monkey.

In this line we performed interspecies comparison on gene expression of cytokines and their receptors in unstimulated PBMC from humans, chimpanzee, baboon, and macaque. Gene expression profiling using unstimulated PBMC gives us information concerning potential homeostasis of Th1 and Th2 cytokines and their receptors under physiological conditions. These are also essential to know the *in vivo* counter-balance between Th1 and Th2 response in primates, while studies of stimulated PBMC provided more insights [3, 7].

Our new findings indicate a distinct difference in the expression level of Th1 (IL-12) and Th2 cytokine (IL-4) genes between hominoids (human, chimpanzee) and monkeys (baboon, macaque) and IFN-γ gene within the hominoids. Higher gene expression of both IFN-γ

receptors, IFN-γ R1 and IFN-γ R2, was also observed in the macaque. In this study, each PCR primer was designed based on the DNA sequence of the target human and/or macaque gene. On sequence comparison of IL-4 gene around both primer binding sites, cynomolgus macaque had one mismatch near the 3′-end of the reverse primer (Table 1), while no mismatch in the sequence of rhesus macaque was observed. Using the IL-4 primers we obtained almost the same level of IL-4 gene expression between both macaques (data not shown), indicating that the apparent lower expression level of IL-4 gene in cynomolgus was not a result of PCR efficiency but the amount of its mRNA. Additionally, the nucleotide sequences of examined cytokine and receptor genes had very high homology with 93% to 100% among the four species (data not shown). Thus, the apparent differences in the gene expression level among primates are attributable not to the differential efficiency of the PCR reaction but to actual differences in the level of their steady-state mRNAs.

Regarding the expression level of the IL-4 gene, it appeared to be higher in hominoids than in monkeys, whereas that of the IL-12 gene was markedly lower in the hominoids than in the monkeys. Although we have no direct evidence to account for these differences yet, it was reported earlier that IL-4 downregulated the transcription of the IL-12 gene by reducing the promoter activity of this gene as well as destabilizing IL-12 mRNA [35]. Those findings suggest that IL-4 regulates IL-12 gene expression at both transcriptional and post-transcriptional steps. Such regulation is a plausible reason for the very low level of IL-12 gene expression in hominoids, which showed a very high expression level of their IL-4 gene.

A marked difference in IFN-γ gene expression was found between humans and chimpanzee. Its expression in humans was distinctly lower than that in chimpanzees as well as in baboons and macaques. It is well known that intestinal parasite infections affect Th1 or Th2 immune responses by regulating the expression of the genes encoding cytokines [2, 6, 32, 36]. Protozoan parasites are a potent stimulator of Th1 responses and of gene expression of the Th1 cytokine IFN-γ [12, 21, 31]. Previous studies of helminth (S.Nakamura, unpublished data) and protozoal [38, 39] infections in chimpanzees and macaques (maybe in baboons also) indicated very strong and frequent infection in these non-human primates. This poor hygienic condition with high parasite infection of non-human primates is clearly different from that of the Japanese subjects examined here. Moreover, in pervious study it was reported that African people under poor hygienic conditions had higher Th1 cytokine (IFN-γ) levels than that of people in western countries [26]. The same trend was observed when comparising IFN-γ gene expression in PBMC of normal subject(s) belonging to a developing country and those of Japan (S. N. unpublished data). Probably this difference in hygiene is a main factor responsible for the distinctly lower gene expression of IFN-γ in humans than in non-human primates, though details of the mechanism regulating its gene expression remain to be clarified.

The IFN-γ receptor consists of four subunit chains (two chains of ligand-binding subunit: IFN-γR1 and two chains of signal-transducing subunit: IFN-γR2) [34]. IFN-γR2 appears to be a limiting factor in the IFN-γ signal pathway, as the gene expression of IFN-γR1 is ubiquitous during the cell cycle whereas that of IFN-γR2 is tightly regulated in accordance with cellular differentiation or activation [4, 8, 9, 41]. Interestingly, unique higher gene expression of both IFN-γ receptor subunit chains, IFN-γR1 and IFN-γR2, was observed in the macaque. As we have little data concerning the mechanism regulating this unique gene expression of IFN-γ receptors in the macaque yet, further studies on it are undoubtedly needed. This previously unknown finding should prompt comparative studies on the IFN-γ signaling pathway in macaques, as they are widely used as a primate model for biomedical research in Th1 immunity-associated vaccine development and/or gene therapy.

Humans and chimpanzee had similar characteristics in gene expression profiles of some cytokines and their receptors. Especially in terms of the gene expression of IL-4 and IL-12, they were clearly distinct from monkeys. In recent studies [18, 43] some evolutionary considerations concerning neutral, positive, or negative selection on gene expression among primates were claimed. A genome-wide effect of positive selection was suggested in gene expression of immune system in primates [18]. These give an insight into the occurrence of a selective event in the gene expression of the cytokines during evolution of the hominoid lineage. This event would be caused by transcriptional and/or post-transcriptional regulation for cytokine gene expression. Bostik *et al.* reported distinct sequence differences in the promoter region of cytokine genes among humans, macaque and mangabey monkey [10]. Insertion of repeat sequences such as *Alu* or *ERV* is known to regulate gene expression [14, 15]. Epigenetic events such as DNA methylation or histone acetylation are also known to affect transcriptional regulation of gene expression [19, 20]. Non-cording (nc) RNA, especially micro-RNA, participates in post-transcriptional gene regulation [11]. These genome-wide effects could contribute

J Med Primatol **37** (2008) 290–296

to the differently regulated expression of hominoid cytokine genes, although little has been reported about these repeat sequences, epigenetic event, or ncRNA in primate cytokine genes.

In conclusion, we have shown the characteristic differences in the gene expression profile of Th1/Th2 cytokines and their receptors among primates, which imply especially that humans are distinct from non-human primates. These differences suggest the species-specific nature implicated in the Th1/Th2 immune responses affecting host defense and/or disease susceptibility among these primates, which should be carefully considered in biomedical researches using non-human primates as experimental models instead of humans. Additionally, our SYBR Green real-time RT-PCR assay used here serves as a useful method to assess primate cytokines and their receptors in a homogenous and reproducible manner.

Acknowledgments

We would like to thank H. Yamauchi and H. Hashimoto for their technical assistance. This study was supported partly by a NEXT Grant-in-Aid for the 21st Century COE Program (A2 to Kyoto University) to SN.

References

1 Abbas AK, Murphy KM, Sher A: Functional diversity of helper T lymphocytes. *Nature* 1996; **383**:787–93.
2 Alexander J, Satoskar AR, Russell DG: *Leishmania* species: models of intracellular parasitism. *J Cell Sci* 1999; **18**(Pt 18):2993–3002.
3 Arredondo J, Xusheng Lü F, Villinger F, McChesney MB, McGhee JR, Miller CJ: Antigen-dependent cytokine mRNA expression by individual rhesus macaque T helper cells by flow cytometry. *Cell Immunol* 2000; **201**:94–108.
4 Bach EA, Aguet M, Schreiber RD: The IFN gamma receptor: a paradigm for cytokine receptor signaling. *Annu Rev Immunol* 1997; **15**:563–91.
5 Bayne K: Developing guidelines on the care and use of animals. *Ann N Y Acad Sci* 1998; **862**:105–10.
6 Bentwich Z, Weisman Z, Moroz C, Bar-Yehuda S, Kalinkovich A: Immune dysregulation in Ethiopian immigrants in Israel: relevance to helminth infections? *Clin Exp Immunol* 1996; **103**:239–43.
7 Benveniste O, Vaslin B, Villinger F, Le Grand R, Ansari AA, Dormont D: Cytokine mRNA levels in unmanipulated (ex vivo) and in vitro stimulated monkey PBMCs using a semi-quantitative RT-PCR and high sensitivity fluorescence-based detection strategy. *Cytokine* 1996; **8**:32–41.
8 Bernabei P, Coccia EM, Rigamonti L, Bosticardo M, Forni G, Pestka S, Krause CD, Battistini A, Novelli F: Interferon-gamma receptor 2 expression as the deciding factor in human T, B, and myeloid cell proliferation or death. *J Leukoc Biol* 2001; **70**:950–60.
9 Bernabei P, Bosticardo M, Losana G, Regis G, Di Paola F, De Angelis S, Giovarelli M, Novelli F: IGF-1 down-regulates IFN-gamma R2 chain surface expression and desensitizes IFN-gamma/STAT-1 signaling in human T lymphocytes. *Blood* 2003; **102**:2933–9.
10 Bostik P, Watkins M, Villinger F, Ansari AA: Genetic analysis of cytokine promoters in nonhuman primates: implications for Th1/Th2 profile characteristics and SIV disease pathogenesis. *Clin Dev Immunol* 2004; **11**:35–44.
11 Bushati N, Cohen SM: microRNA functions. *Annu Rev Cell Dev Biol* 2007; **23**:175–205.
12 Denkers EY, Gazzinelli RT: Regulation and function of T-cell-mediated immunity during *Toxoplasma gondii* infection. *Clin Microbiol Rev* 1998; **11**:569–88.
13 Dillman JF III, Phillips CS: Comparison of non-human primate and human whole blood tissue gene expression profiles. *Toxicol Sci* 2005; **87**:306–14.
14 Dunn CA, Romanish MT, Gutierrez LE, van de Lagemaat LN, Mager DL: Transcription of two human genes from a bidirectional endogenous retrovirus promoter. *Gene* 2006; **366**:335–42.
15 Häsler J, Strub K: Alu elements as regulators of gene expression. *Nucleic Acids Res* 2006; **34**:5491–7.
16 Hirano M, Nakamura S, Mitsunaga F, Okada M, Shimizu K, Ueda M, Bennett A, Eberle R: Efficacy of a B virus gD DNA vaccine for induction of humoral and cellular immune responses in Japanese macaques. *Vaccine* 2002; **20**:2523–32.
17 Hsieh CS, Macatonia SE, Tripp CS, Wolf SF, O'Garra A, Murphy KM: Development of TH1 CD4+ T cells through IL-12 produced by *Listeria*-induced macrophages. *Science* 1993; **260**:547–9.
18 Khaitovich P, Enard W, Lachmann M, Pääbo S: Evolution of primate gene expression. *Nat Rev Genet* 2006; 7:693–702.
19 Klose RJ, Bird AP: Genomic DNA methylation: the mark and its mediators. *Trends Biochem Sci* 2006; **31**:89–97.
20 Kuo MH, Allis CD: Roles of histone acetyltransferases and deacetylases in gene regulation. *Bioessays* 1998; **20**:615–26.
21 Louis J, Himmelrich H, Parra-Lopez C, Tacchini-Cottier F, Launois P: Regulation of protective immunity against *Leishmania major* in mice. *Curr Opin Immunol* 1998; **10**:459–64.
22 Lukashevich IS, Tikhonov I, Rodas JD, Zapata JC, Yang Y, Djavani M, Salvato MS: Arenavirus-mediated liver pathology: acute lymphocytic choriomeningitis virus infection of rhesus macaques is characterized by

high-level interleukin-6 expression and hepatocyte proliferation. *J Virol* 2003; **77**:1727–37.
23 Macatonia SE, Hosken NA, Litton M, Vieira P, Hsieh CS, Culpepper JA, Wysocka M, Trinchieri G, Murphy KM, O'Garra A: Dendritic cells produce IL-12 and direct the development of Th1 cells from naive CD4+ T cells. *J Immunol* 1995; **154**:5071–9.
24 Magness CL, Fellin PC, Thomas MJ, Korth MJ, Agy MB, Proll SC, Fitzgibbon M, Scherer CA, Miner DG, Katze MG, Iadonato SP: Analysis of the Macaca mulatta transcriptome and the sequence divergence between Macaca and human. *Genome Biol* 2005; **6**:R60.
25 Mäkitalo B, Andersson M, Arestrom I, Karlén K, Villinger F, Ansari A, Paulie S, Thorstensson R, Ahlborg N: ELISpot and ELISA analysis of spontaneous, mitogen-induced and antigen-specific cytokine production in cynomolgus and rhesus macaques. *J Immunol Methods* 2002; **270**:85–97.
26 Malhotra I, Ouma J, Wamachi A, Kioko J, Mungai P, Omollo A, Elson L, Koech D, Kazura JW, King CL: In utero exposure to helminth and mycobacterial antigens generates cytokine responses similar to that observed in adults. *J Clin Invest* 1997; **99**:1759–66.
27 Mosmann TR, Coffman RL: TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. *Annu Rev Immunol* 1989; **7**: 145–73.
28 Mosmann TR, Sad S: The expanding universe of T-cell subsets: Th1, Th2 and more. *Immunol Today* 1996; **17**:138–46.
29 National Research Council: Guide for the Care and Use of Laboratory Animals. Washington, DC: National Academy Press, 1996.
30 Paul WE, Seder RA: Lymphocyte responses and cytokines. *Cell* 1994; **76**:241–51.
31 Reiner SL, Locksley RM: The regulation of immunity to *Leishmania major*. *Annu Rev Immunol* 1995; **13**:151–77.
32 Sacks D, Sher A: Evasion of innate immunity by parasitic protozoa. *Nat Immunol* 2002; **3**:1041–7.
33 Schelegle ES, Gershwin LJ, Miller LA, Fanucchi MV, Van Winkle LS, Gerriets JP, Walby WF, Omlor AM, Buckpitt AR, Tarkington BK, Wong VJ, Joad JP, Pinkerton KB, Wu R, Evans MJ, Hyde DM, Plopper CG: Allergic asthma induced in rhesus monkeys by house dust mite (*Dermatophagoides farinae*). *Am J Pathol* 2001; **158**:333–41.
34 Schroder K, Hertzog PJ, Ravasi T, Hume DA: Interferon-gamma: an overview of signals, mechanisms and functions. *J Leukoc Biol* 2004; **75**:163–89.
35 Seegmuller I, Hacker H, Wagner H: IL-4 regulates IL-12 p40 expression post-transcriptionally as well as via a promoter-based mechanism. *Eur J Immunol* 2003; **33**:428–33.
36 Sher A, Coffman RL: Regulation of immunity to parasites by T cells and T cell-derived cytokines. *Annu Rev Immunol* 1992; **10**:385–409.
37 Sui Y, Potula R, Pinson D, Adany I, Li Z, Day J, Buch E, Segebrecht J, Villinger F, Liu Z, Huang M, Narayan O, Buch S: Microarray analysis of cytokine and chemokine genes in the brains of macaques with SHIV-encephalitis. *J Med Primatol Methods* 2003; **32**:229–39.
38 Tachibana H, Cheng XJ, Kobayashi S, Fujita Y, Udono T: *Entamoeba dispar*, but not *E. histolytica*, detected in a colony of chimpanzees in Japan. *Parasitol Res* 2000; **86**:536–41.
39 Tachibana H, Cheng XJ, Kobayashi S, Matsubayashi N, Gotoh S, Matsubayashi K: High prevalence of infection with *Entamoeba dispar*, but not *E. histolytica*, in captive macaques. *Parasitol Res* 2001; **87**:14–7.
40 Trinchieri G: Interleukin-12 and the regulation of innate resistance and adaptive immunity. *Nat Rev Immunol* 2003; **3**:133–46.
41 Valente G, Ozmen L, Novelli F, Geuna M, Palestro G, Forni G, Garotta G: Distribution of interferon-gamma receptor in human tissues. *Eur J Immunol* 1992; **22**:2403–12.
42 Villinger F, Brar SS, Mayne A, Chikkala N, Ansari AA: Comparative sequence analysis of cytokine genes from human and nonhuman primates. *J Immunol* 1995; **155**:3946–54.
43 Whitehead A, Crawford DL: Variation within and among species in gene expression: raw material for evolution. *Mol Ecol* 2006; **15**:1197–211.

J Med Primatol **37** (2008) 290–296 © 2008 The Authors
Journal compilation © 2008 Blackwell Munksgaard