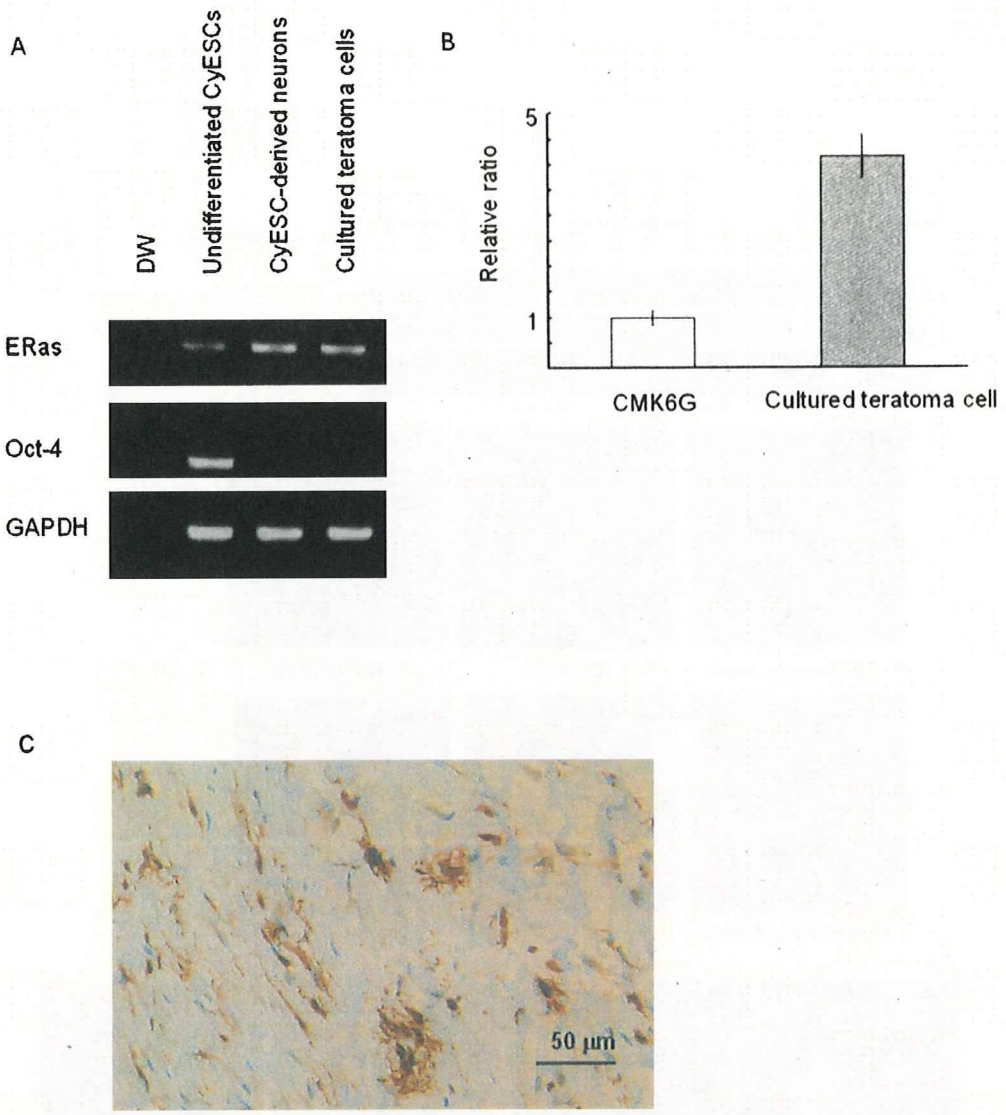

Figure 3. Cynomolgus ES cell-derived cells express ERas but do not form tumors in vivo. (A) The ERas gene was expressed in cynomolgus ES cell-derived neurons and teratoma cells as assessed by RT-PCR, whereas the Oct-4 gene was expressed in neither. DW indicates no template in the reaction. RT-PCR of the GAPDH sequence is also shown as an internal control. (B) The ERas gene expression level was nearly five times higher in the cultured teratoma cells than in the undifferentiated cynomolgus ES cells as assessed by quantitative RT-PCR. The gene expression level was adjusted using the internal control GAPDH. (C) The cultured teratoma cells were transplanted into the thigh muscles of NOG mice and examined for tumorigenicity in vivo after 2.5 months. Staining of the specimen with anti-GFP is shown. Although the transplanted cell progeny (GFP positive, brown) were detected, no tumor was observed.

tomas in all NOG mice. Taken together, cynomolgus ES cell-derived differentiated progeny and teratoma cells also express the ERas gene, but do not produce tumors in vivo.

ERas Overexpression in Cynomolgus Cells

To examine whether the cynomolgus ERas contributes to cell proliferation, we transfected cynomolgus stromal cells with a plamid expressing the cynomolgus or mouse ERas, EGFP, and puromycin resistance genes. Transfectants were obtained by treatment with puromycin and more than 90% of the cells expressed EGFP (Fig. 4A). These transfectants did not show significant morphological changes. Quantitative RT-PCR showed that the transfected cells expressed approximately 1000 times more ERas than undifferentiated cynomolgus ES

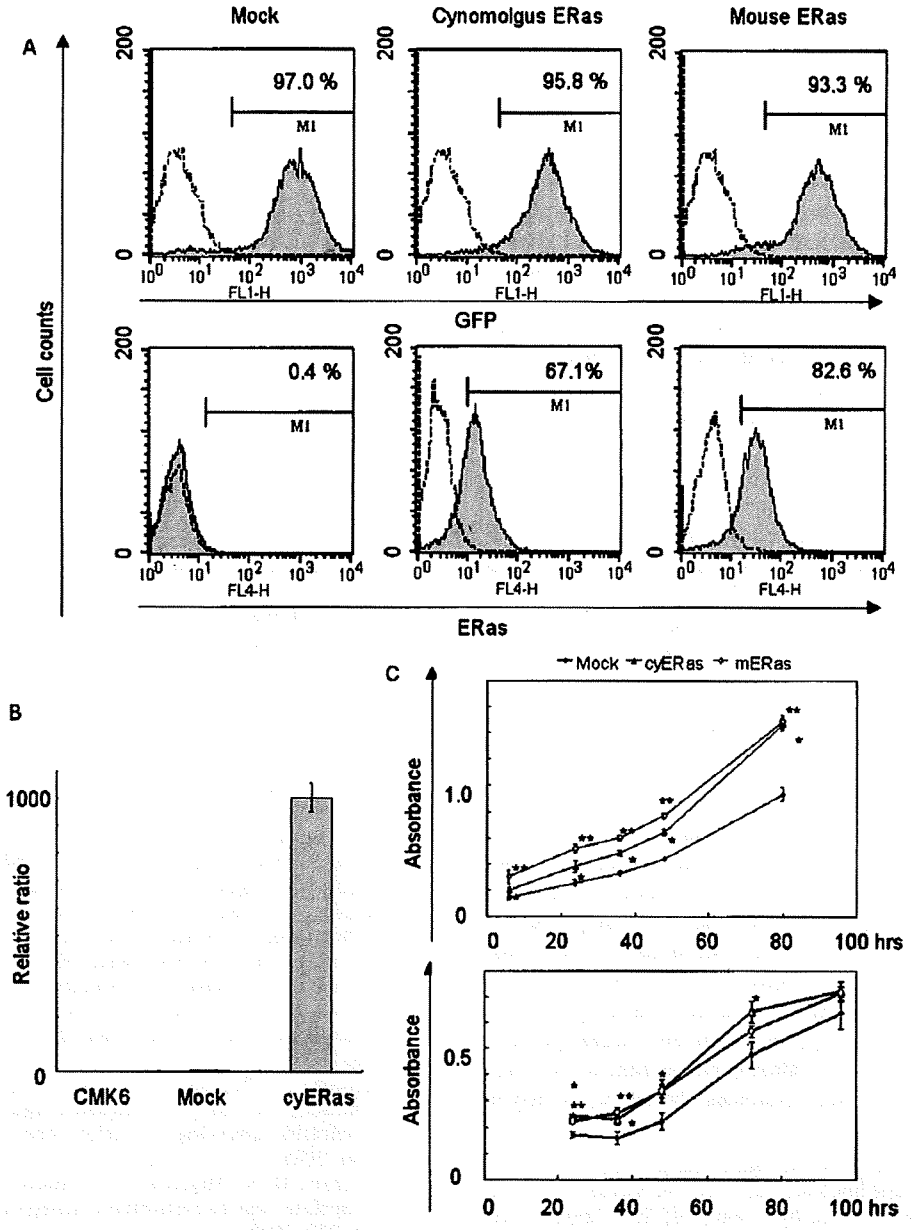

Figure 4. Overexpression of ERas does not promote cell proliferation but improves plating efficiency. (A) The plasmids expressing the cynomolgus or mouse ERas, EGFP, and puromycin resistance genes were transfected into cynomolgus stromal cells. After puromycin selection, more than 90% of cells expressed EGFP (upper). The ERas expression was also detected by flow cytometry (lower). (B) The level of cynomolgus ERas gene expression was 1000 times higher in the cynomolgus ERas-transfected cells (cyERas) than in naive cynomolgus ES cells (CMK6) or mock-transfected cells by quantitative RT-PCR. The gene expression levels were adjusted using the internal control GAPDH. (C) The mock-, cynomolgus ERas (cyERas)-, and mouse ERas (mERas)-transfected cells were plated at 5×10^3 per well and total cell numbers were measured after 6, 24, 36, 48, and 80 h of incubation (upper). The mock-, cyERas-, and mERas-transfected cells were plated at 2×10^3 per well and proliferating cell numbers were measured after 24, 36, 48, 72, and 96 h of incubation (lower). The cyERas- and mERas-transfected cells showed larger total cell or proliferating cell numbers after plating than the mock-transfected cells, but did not show any more rapid proliferation thereafter. Statistical differences with the *t*-test are indicated: $*p < 0.01$ for the cyERas- versus mock-transfected cells, $**p < 0.01$ for the mERas- versus mock-transfected cells.

cells (Fig. 4B). These cells expressing either cynomolgus or mouse ERas showed larger total cell numbers or proliferating cell numbers after plating than the mock-transfected cells, but did not show any more rapid proliferation thereafter (Fig. 4C). Thus, cynomolgus ERas improves plating efficiency but does not promote cell proliferation, even when it is expressed at high levels.

In this report, we showed that the cynomolgus ERas gene is expressed in cynomolgus ES cells and tissues. Its expression pattern is quite different from that of mouse ERas, which is not expressed in mouse ES cell-derived differentiated progeny or mouse tissues. Although cynomolgus ERas improved the plating efficiency when overexpressed, its expression did not promote cell proliferation or induce tumor formation in vivo (Figs. 3C, 4C). Thus, cynomolgus ERas might only suppress the apoptosis of cynomolgus cells (5). Because the formation of teratomas is one of the greatest obstacles to the clinical application of human ES cells (3,8,18), it is important to elucidate whether the ERas gene expressed in cynomolgus ES cells is related to teratoma development in vivo in order to tell whether nonhuman primate models are really suitable for preclinical research. From our study, it is at least suggested that cynomolgus ES cells are more similar to human than mouse ES cells in that ERas does not contribute to the formation of teratomas in vivo.

To date, the pluripotent marker Oct-4 has been used to predict the formation of teratomas (4) and the removal of Oct-4-positive cells from ES cell-derived progenitor preparations is reported to prevent teratomas from developing posttransplant (2). However, Oct-4-negative immature cells are also reported to contribute to the formation of teratomas (6). Therefore, although Oct-4 could be used to predict whether teratomas develop to some extent, it does not regulate the developmental process. For future clinical applications, the mechanism by which primate ES cells form teratomas should be studied in more detail.

ACKNOWLEDGMENTS: We thank Naomi Takino for technical assistance and Shuh-hei Fujishiro for helpful discussion. This study was supported by grants (JMS 21st Century COE Program, High-tech Research Center Program, and KAKENHI) from the Ministry of Education, Culture, Sports, Science and Technology of Japan as well as grants (KAKENHI) from the Ministry of Health, Labor and Welfare of Japan.

REFERENCES

1. Bhattacharya, B.; Miura, T.; Brandenberger, R.; Mejido, J.; Luo, Y.; Yang, A. X.; Joshi, B. H.; Ginis, I.; Thies, R. S.; Amit, M.; Lyons, I.; Condie, B. G.; Itskovitz-Eldor, J.; Rao, M. S.; Puri, R. K. Gene expression in human embryonic stem cell lines: unique molecular signature. Blood 103:2956–2964; 2004.
2. Bieberich, E.; Silva, J.; Wang, G.; Krishnamurthy, K.; Condie, B. G. Selective apoptosis of pluripotent mouse and human stem cells by novel ceramide analogues prevents teratoma formation and enriches for neural precursors in ES cell-derived neural transplants. J. Cell Biol. 167:723–734; 2004.
3. Bjorklund, L. M.; Sanchez-Pernaute, R.; Chung, S.; Andersson, T.; Chen, I. Y.; McNaught, K. S.; Brownell, A. L.; Jenkins, B. G.; Wahlestedt, C.; Kim, K. S.; Isacson, O. Embryonic stem cells develop into functional dopaminergic neurons after transplantation in a Parkinson rat model. Proc. Natl. Acad. Sci. USA 99:2344–2349; 2002.
4. Campbell, P. A.; Perez-Iratxeta, C.; Andrade-Navarro, M. A.; Rudnicki, M. A. Oct4 targets regulatory nodes to modulate stem cell function. PLoS ONE 2:e553; 2007.
5. Cox, A. D.; Der, C. J. The dark side of Ras: Regulation of apoptosis. Oncogene 22:8999–9006; 2003.
6. Dihne, M.; Bernreuther, C.; Hagel, C.; Wesche, K. O.; Schachner, M. Embryonic stem cell-derived neuronally committed precursor cells with reduced teratoma formation after transplantation into the lesioned adult mouse brain. Stem Cells 24:1458–1466; 2006.
7. Erdo, F.; Buhrle, C.; Blunk, J.; Hoehn, M.; Xia, Y.; Fleischmann, B.; Focking, M.; Kustermann, E.; Kolossov, E.; Hescheler, J.; Hossmann, K. A.; Trapp, T. Host-dependent tumorigenesis of embryonic stem cell transplantation in experimental stroke. J. Cereb. Blood Flow Metab. 23: 780–785; 2003.
8. Fujikawa, T.; Oh, S. H.; Pi, L.; Hatch, H. M.; Shupe, T.; Petersen, B. E. Teratoma formation leads to failure of treatment for type I diabetes using embryonic stem cell-derived insulin-producing cells. Am. J. Pathol. 166:1781–1791; 2005.
9. Hasegawa, K.; Fujioka, T.; Nakamura, Y.; Nakatsuji, N.; Suemori, H. A method for the selection of human embryonic stem cell sublines with high replating efficiency after single-cell dissociation. Stem Cells 24:2649–2660; 2006.
10. Heins, N.; Englund, M. C.; Sjoblom, C.; Dahl, U.; Tonning, A.; Bergh, C.; Lindahl, A.; Hanson, C.; Semb, H. Derivation, characterization, and differentiation of human embryonic stem cells. Stem Cells 22:367–376; 2004.
11. Hematti, P.; Obrtlikova, P.; Kaufman, D. S. Nonhuman primate embryonic stem cells as a preclinical model for hematopoietic and vascular repair. Exp. Hematol. 33:980–986; 2005.
12. Kameda, T.; Thomson, J. A. Human ERas gene has an upstream premature polyadenylation signal that results in a truncated, noncoding transcript. Stem Cells 23:1535–1540; 2005.
13. Kaufman, D. S.; Thomson, J. A. Human ES cells—haematopoiesis and transplantation strategies. J. Anat. 200: 243–248; 2002.
14. Kishi, Y.; Tanaka, Y.; Shibata, H.; Nakamura, S.; Takeuchi, K.; Masuda, S.; Ikeda, T.; Muramatsu, S.; Hanazono, Y. Variation in the incidence of teratomas after transplantation of nonhuman primate ES cells into immunodeficient mice. Cell Transplant. 17(9):1095–1102; 2008.
15. Nakayama, T.; Momoki-Soga, T.; Inoue, N. Astrocyte-derived factors instruct differentiation of embryonic stem cells into neurons. Neurosci. Res. 46:241–249; 2003.
16. Nakayama, T.; Momoki-Soga, T.; Yamaguchi, K.; Inoue, N. Efficient production of neural stem cells and neurons from embryonic stem cells. Neuroreport 15:487–491; 2004.
17. Rao, M. Conserved and divergent paths that regulate self-

renewal in mouse and human embryonic stem cells. Dev. Biol. 275:269–286; 2004.
18. Shibata, H.; Ageyama, N.; Tanaka, Y.; Kishi, Y.; Sasaki, K.; Nakamura, S.; Muramatsu, S.; Hayashi, S.; Kitano, Y.; Terao, K.; Hanazono, Y. Improved safety of hematopoietic transplantation with monkey embryonic stem cells in the allogeneic setting. Stem Cells 24:1450–1457; 2006.
19. Suemori, H.; Tada, T.; Torii, R.; Hosoi, Y.; Kobayashi, K.; Imahie, H.; Kondo, Y.; Iritani, A.; Nakatsuji, N. Establishment of embryonic stem cell lines from cynomolgus monkey blastocysts produced by IVF or ICSI. Dev. Dyn. 222:273–279; 2001.
20. Takada, T.; Suzuki, Y.; Kondo, Y.; Kadota, N.; Kobayashi, K.; Nito, S.; Kimura, H.; Torii, R. Monkey embryonic stem cell lines expressing green fluorescent protein. Cell Transplant. 11:631–635; 2002.
21. Takagi, Y.; Takahashi, J.; Saiki, H.; Morizane, A.; Hayashi, T.; Kishi, Y.; Fukuda, H.; Okamoto, Y.; Koyanagi, M.; Ideguchi, M.; Hayashi, H.; Imazato, T.; Kawasaki, H.; Suemori, H.; Omachi, S.; Iida, H.; Itoh, N.; Nakatsuji, N.; Sasai, Y.; Hashimoto, N. Dopaminergic neurons generated from monkey embryonic stem cells function in a Parkinson primate model. J. Clin. Invest. 115:102–109; 2005.
22. Takahashi, K.; Mitsui, K.; Yamanaka, S. Role of ERas in promoting tumour-like properties in mouse embryonic stem cells. Nature 423:541–545; 2003.
23. Tanaka, Y.; Nakamura, S.; Shibata, H.; Kishi, Y.; Ikeda, T.; Masuda, S.; Sasaki, K.; Abe, T.; Hayashi, S.; Kitano, Y.; Nagao, Y.; Hanazono, Y. Sustained macroscopic engraftment of cynomolgus embryonic stem cells in xenogeneic large animals after in utero transplantation. Stem Cells Dev. 17:367–382; 2008.
24. Thomson, J. A.; Kalishman, J.; Golos, T. G.; Durning, M.; Harris, C. P.; Becker, R. A.; Hearn, J. P. Isolation of a primate embryonic stem cell line. Proc. Natl. Acad. Sci. USA 92:7844–7848; 1995.
25. Wolf, D. P. Nonhuman primate embryonic stem cells: An underutilized resource. Regen. Med. 3:129–131; 2008.

ELSEVIER

Experimental Hematology

Experimental Hematology 2009;37:1250–1257

Cotransplantation with MSCs improves engraftment of HSCs after autologous intra-bone marrow transplantation in nonhuman primates

Shigeo Masuda[a], Naohide Ageyama[b], Hiroaki Shibata[b], Yoko Obara[c], Tamako Ikeda[a], Kengo Takeuchi[d], Yasuji Ueda[e], Keiya Ozawa[c], and Yutaka Hanazono[a]

[a]*Division of Regenerative Medicine, Center for Molecular Medicine, Jichi Medical University, Tochigi, Japan;* [b]*Tsukuba Primate Research Center, National Institute of Biomedical Innovation, Ibaraki, Japan;* [c]*Division of Hematology, Department of Medicine, Jichi Medical University, Tochigi, Japan;* [d]*Division of Pathology, The Cancer Institute, Tokyo, Japan;* [e]*Department of Gene Therapy, Graduate School of Medicine, Chiba University, Chiba, Japan*

(Received 18 June 2009; revised 21 July 2009; accepted 22 July 2009)

Objective. **Hematopoietic stem cells (HSCs) reside in the osteoblastic niche, which consists of osteoblasts. Mesenchymal stromal cells (MSCs) have an ability to differentiate into osteoblasts. Here, using nonhuman primates, we investigated the effects of cotransplantation with MSCs on the engraftment of HSCs after autologous intra-bone marrow transplantation.**

Materials and Methods. **From three cynomolgus monkeys, CD34-positive cells (as HSCs) and MSCs were obtained. The former were divided into two equal aliquots and each aliquot was genetically marked with a distinctive retroviral vector to track the in vivo fate. Each HSC aliquot with or without MSCs was autologously injected into the bone marrow (BM) cavity of right or left side, enabling the comparison of in vivo fates of the two HSC grafts in the same body.**

Results. **In the three monkeys, $CD34^+$ cells transplanted with MSCs engrafted 4.4, 6.0, and 1.6 times more efficiently than $CD34^+$ cells alone, as assessed by BM colony polymerase chain reaction. In addition, virtually all marked cells detected in the peripheral blood were derived from the cotransplantation aliquots. Notably, colony-forming units derived from the cotransplantation aliquots were frequently detected in BM distant sites from the injection site, implying that cotransplantation with MSCs also restored the ability of gene-marked HSCs to migrate and achieve homing in the distant BM.**

Conclusion. **Cotransplantation with MSCs would improve the efficacy of transplantation of gene-modified HSCs in primates, with enhanced engraftment in BM as well as increased chimerism in peripheral blood through migration and homing.** **© 2009 ISEH - Society for Hematology and Stem Cells. Published by Elsevier Inc.**

Hematopoietic stem cells (HSCs) have been shown to reside in the hematopoietic niche, such as the osteoblastic or vascular niche [1,2]. The osteoblastic niche is provided by bone marrow (BM) osteoblasts, which are derived from mesenchymal stromal cells (MSCs) [3–5]. Although conditioning, such as total body irradiation or administration of busulfan, would be required for successful engraftment of transplanted HSCs, the treatment may destroy the osteoblastic niche, hampering engraftment [6]. If an osteoblastic niche could be generated through cotransplantation with MSCs, the engraftment of HSCs would be enhanced; however, MSCs cannot home to or engraft in BM when transplanted via vessels [7]. On the other hand, the direct transplantation of HSCs into a BM cavity, namely intra-bone marrow transplantation (iBMT), improves engraftment of HSCs compared with intravascular transplantation [8–13]. In fact, one clinical trial of iBMT has been published recently [14] that demonstrates early donor engraftment

Offprint requests to: Yutaka Hanazono, M.D., Ph.D., Division of Regenerative Medicine, Center for Molecular Medicine, Jichi Medical University, 3311-1 Yakushiji, Shimotsuke-shi, Tochigi 329-0498, Japan; E-mail: hanazono@jichi.ac.jp

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.exphem.2009.07.008.

0301-472X/09 $–see front matter.
doi: 10.1016/j.exphem.2009.07.008

after allogeneic iBMT, suggesting safety and efficacy of iBMT. Previous studies have demonstrated that cotransplantation with MSCs improves engraftment of HSCs in mice [15–17], especially after iBMT [18]. However, neither systematic human nor large animal's studies have been conducted to evaluate the efficacy of cotransplantation with MSCs.

To assess the efficacy of cotransplantation with MSCs in nonhuman primates, we applied hemi-iBMT in cynomolgus monkeys; i.e., the BM on one side (right or left) of the body was transplanted with HSCs together with MSCs and the other side was transplanted with HSCs alone. We genetically marked HSCs before transplantation with two distinct retroviral vectors to identify transplanted cells derived from the two HSC aliquots and to compare their in vivo fates [19,20]. This hemi-iBMT method combined with the dual genetic marking technique enables us to evaluate the results in the same body, comparing the outcomes of cells of interest with that of control cells; thus, there is no need to consider the variation in results among monkeys. Here we show that cotransplantation with MSCs improves engraftment of HSCs after iBMT in nonhuman primates.

Materials and methods

Animals

Cynomolgus macaques (*Macaca fascicularis*) were housed and handled in accordance with the Rules for Care and Management at the Tsukuba Primate Research Center (Ibaraki, Japan) and with the Guiding Principles for Animal Experiments using Non-Human Primates formulated by the Primate Society of Japan. The protocol of the experimental procedures was approved by the Animal Welfare and Animal Care Committee of the National Institute of Biomedical Innovation (Osaka, Japan).

Isolation of cynomolgus MSCs and CD34$^+$ cells

BM cells were collected by aspiration from the iliac bone of cynomolgus monkeys and processed as described previously [13,21]. From the harvested cells, the nucleated cell fraction was obtained after red blood cell lysis with ACK buffer (155 mM NH_4Cl, 10 mM $KHCO_3$, and 0.1 mM ethylenediamine tetraacetic acid; Wako, Osaka, Japan). MSCs were isolated by plastic adherence for 1 hour, and cultured for 2 to 3 weeks (i.e., three to four passages) in Dulbecco's modified Eagle's medium supplemented with 20% fetal bovine serum and penicillin/streptomycin at 37°C with 5% CO_2. After isolation of adherent cells, nonadherent cells were enriched for CD34$^+$ cells by magnet beads conjugated with anti-human CD34 (clone 561; Dynal, Lake Success, NY, USA) which cross-reacts with cynomolgus CD34 [21]. The purity of CD34$^+$ cells ranged from 90% to 95%, as assessed with another anti-human CD34 (clone 563; Pharmingen, San Diego, CA, USA) which cross-reacts with cynomolgus CD34 [21]. Mean enrichment after the selection of CD34$^+$ cells was 84-fold in terms of colony-forming units (CFUs).

Retroviral transduction

We used G1Na and LNL6 retroviral vectors expressing the neomycin resistance gene (*neo^R*) [19,20]. Titers of the viral supernatants used in the present study were both 1×10^6 particles/μL, as assessed by Retrovirus Titer Set (for real-time polymerase chain reaction [PCR]) according to manufacturer's instruction (Takara, Shiga, Japan). CD34$^+$ cells were cultured at starting concentrations of 1 to 5×10^5 cells/mL in vector supernatant (frozen-and-thawed once) of G1Na or LNL6. Four-day transduction (one supernatant transduction per day) with recombinant human stem cell factor (SCF), recombinant human Fms-like tyrosine kinase 3 ligand (Flt3-ligand) (R&D Systems, Minneapolis, MI, USA), and recombinant human thrombopoietin (Kirin, Tokyo, Japan) each at 100 ng/mL in dishes coated with 20 μg/cm^2 of RetroNectin (Takara, Shiga, Japan) was conducted as described previously [13]. After 4-day transduction, cells were washed, frozen, and stocked until transplantation.

iBMT

Before transplantation, conditioning was performed; either total body irradiation (550 cGy×2) in the first monkey examined, or administration of busulfan (Busulfex) in the second (8 mg/kg×2) and third (10 mg/kg×1) monkey. After conditioning, monkeys were anesthetized and two needles were inserted into both ends of the femur or humerus [13,22]. A syringe containing 50 mL heparin-added saline was connected to one needle and an empty syringe was connected to the other. Normal saline was irrigated gently from one syringe to another through the marrow cavity twice to remove BM cells physically. Then, gene-marked CD34$^+$ cells with or without MSCs were suspended in 500 μL phosphate-buffered saline (PBS) containing 50% autologous serum, and injected into the marrow cavity.

Sampling of BM cells and peripheral blood cells

One or two months after transplantation, peripheral blood (PB) and BM cells were taken to assess the in vivo fate of two aliquots. PB cells were collected routinely post-transplantation. To harvest BM cells, monkeys were sacrificed, the ilium marrow was aspirated using BM needles, and the limb marrow was taken by irrigation with PBS.

Clonogenic hematopoietic progenitor assays

Cells were plated in a 35-mm Petri dish in 1 mL MethoCult GF$^+$ H4435 (StemCell Technologies, Vancouver, BC, Canada). After incubation for 14 days at 37°C with 5% CO_2, colonies containing >50 cells were counted using an inverted light microscope, and plucked for PCR as described here. Experiments were conducted in triplicate.

PCR

All the procedures were followed in detail as described previously [13]. From PB nucleated cells, genomic DNA was extracted using the QIAamp DNA Blood Mini Kit (Qiagen, Chatsworth, CA, USA). From BM colonies, genomic DNA was extracted as follows; well-separated, individual colonies at day 14 were plucked into 50 μL distilled water, and digested with 20 μg/mL proteinase K (Takara, Shiga, Japan) at 56°C overnight, followed by 99°C for 10 minutes. For the semi-quantitative PCR of PB samples, DNA (50 ng) was amplified in triplicate with *neo^R*-specific primers for both G1Na and LNL6 (5′-TCCAT CATGGCTGATGCAATGCGGC-3′ and 5′-GATAGAAGGCGAT-GCGCTGCGAATCG-3′). The final sizes of the PCR products were 435 base pairs (bp) for both G1Na and LNL6. For PCR of BM colonies, the outer primer set for both G1Na and LNL6 vector was 5′-GGCCAGACTGTTACCACTCC-3′ and 5′-CAGT CATAGCCGAATAGCCTCT-3′, and the inner primer set for

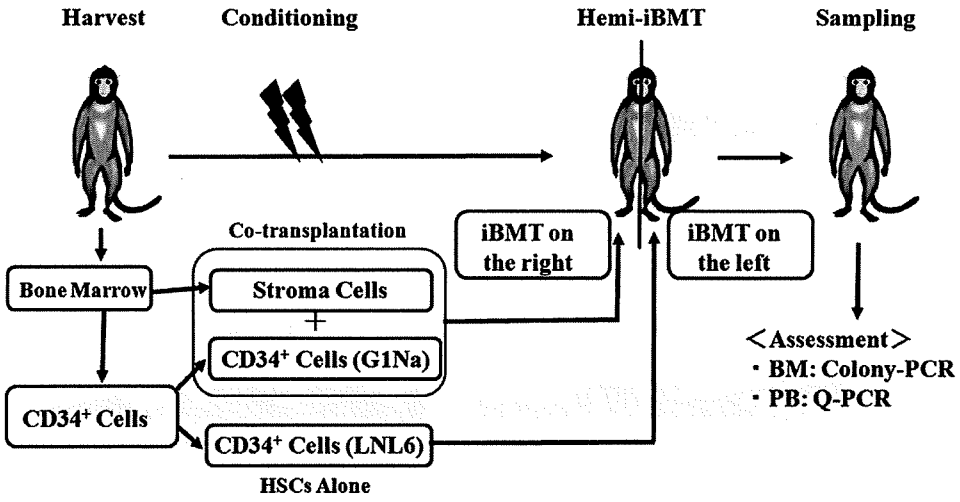

Figure 1. Study design using cynomolgus monkeys. Autologous bone marrow (BM) cells were harvested and separated into two populations, either adherent stromal cells or nonadherent cells. From the nonadherent cells, $CD34^+$ cells were purified (referred to as hematopoietic stem cells [HSCs]), and divided into two equal aliquots. Each aliquot was genetically marked with a distinctive neo^R-retroviral vector, G1Na or LNL6. The stromal cells (referred to as mesenchymal stromal cells [MSCs]) were expanded ex vivo, and then transplanted together with HSCs as a cotransplantation aliquot into the right side of the BM cavity after conditioning. The other aliquot of HSCs was transplanted alone into the left side of the BM cavity of the same body, namely hemi intra-bone marrow transplantation (iBMT). After transplantation, BM and peripheral blood (PB) cells were taken and subjected to colony and semi-quantitative polymerase chain reaction (Q-PCR), respectively.

both G1Na and LNL6 vector was 5′-CGGATCGCTCACAAC CAGTC-3′ and 5′-AGAACCTGCGTGCAATCCATC-3′. The final sizes of the nested PCR products were 455 and 439 bp for G1Na and LNL6, respectively. The final PCR products were separated on 2% agarose gels, or analyzed by capillary electrophoresis (HAD-GT12 System; Qiagen), which allows distinguishing of the sizes of DNA products (16-bp difference) in high resolution.

Statistics

Statistical analyses were performed using Student's *t*-test. A p value <0.05 was considered statistically significant.

Results

Improved engraftment of HSCs after cotransplantation with MSCs

We examined whether gene-marked $CD34^+$ cells with MSCs would engraft more efficiently than those without MSCs after iBMT in a cynomolgus autologous transplantation model. We performed hemitransplantation (Fig. 1); the concept has been explained in the introduction. $CD34^+$ cells were isolated from three monkeys and divided into two equal aliquots. Each aliquot was transduced with the neo^R-expressing retroviral vector G1Na or LNL6. The mean transduction efficiency was 74.0% with no marked differences between the two vectors (Table 1). We injected the transduced $CD34^+$ cells with or without MSCs directly into the BM cavity after gently irrigating the cavity with saline. The procedure was safely conducted without pulmonary embolism or infection of BM. After iBMT, we compared the engraftment of the two aliquots by BM colony PCR; that is, we plated BM cells in methylcellulose medium and examined resulting colonies for the provirus by PCR. Schematic representation of the study design is shown in Figure 1.

Table 1. Ex vivo transduction and transplantation

Animal (ID no.)	Sex/Age (y)/ body weight (kg)	Groups of hemi-iBMT	Vectors for marking	No. of infused $CD34^+$ cells/kg	Fraction of provirus-positive CFUs in infused $CD34^+$ cells (%)	No. of infused MSCs/kg
Monkey 1 (H025)	Male/5/3.5	Cotransplant*	G1Na	4.1×10^6	7/12 (58.3)	3.0×10^6
		HSC alone	LNL6	3.3×10^6	6/11 (54.5)	0
Monkey 2 (H030)	Male/8/6.5	Cotransplant*	LNL6	3.4×10^5	23/33 (69.7)	7.7×10^5
		HSC alone	G1Na	2.8×10^5	27/30 (90.0)	0
Monkey 3 (H033)	Male/8/6.2	Cotransplant*	G1Na	6.6×10^4	11/13 (78.6)	5.2×10^5
		HSC alone	LNL6	7.1×10^4	13/14 (92.9)	0

CFUs=colony-forming units; HSC=hematopoietic stem cell; iBMT=intra-bone marrow transplantation; MSCs=mesenchymal stromal cells.
*Cotransplant means transplantation of HSCs combined with MSCs.

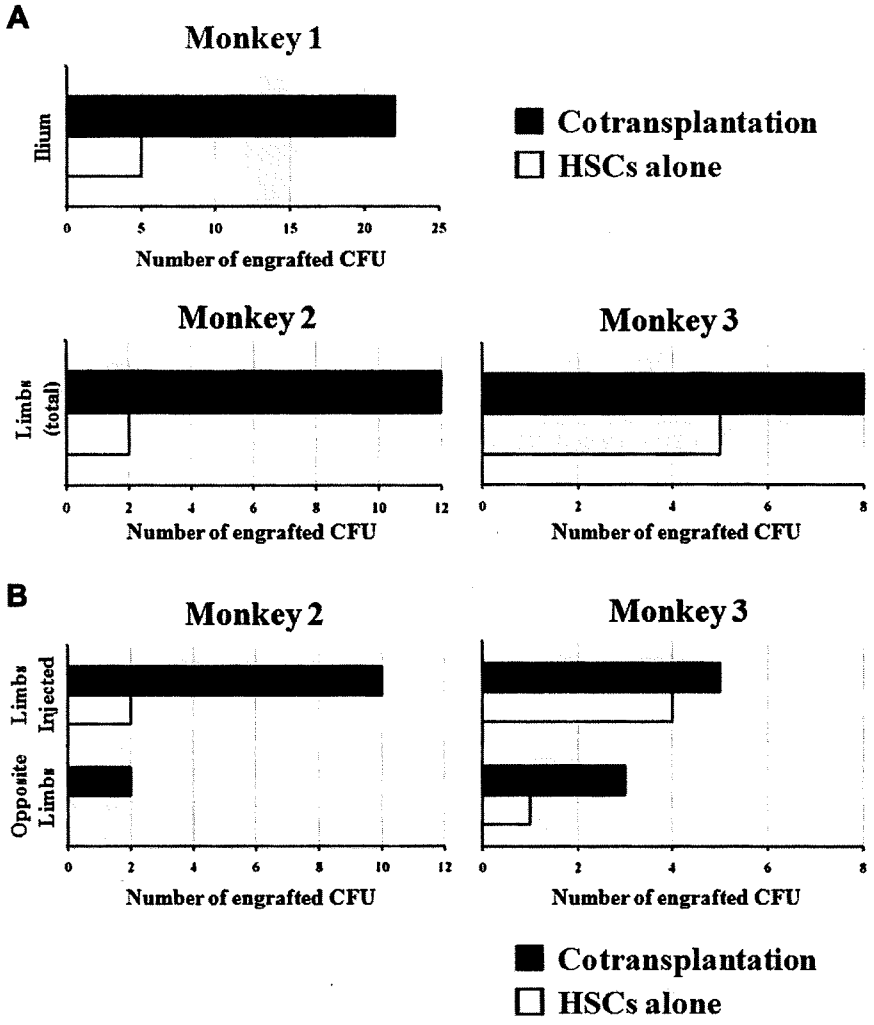

Figure 2. Improved engraftment of hematopoietic stem cells (HSCs) after cotransplantation with mesenchymal stromal cells (MSCs). **(A)** Efficacy of cotransplantation with MSCs. The numbers of engrafted gene-marked colony-forming units (CFUs) are shown, either derived from the cotransplantation aliquots (black columns) or from the HSC-alone aliquots (white columns). After sacrificing the animals, BM cells were harvested from the ilium in Monkey 1 and from the limbs (humerus and femur of both sides) in Monkeys 2 and 3. Total numbers of CFUs marked with G1Na or LNL6 from the marrow samples of each animal are indicated. **(B)** The engraftment site of gene-marked CFUs. Numbers of gene-marked CFUs detected in the limbs of either the injection or opposite side are separately shown for each aliquot.

In the first monkey examined (Monkey 1), one HSC aliquot, genetically marked with G1Na, was transplanted into the right side together with MSCs, while another HSC aliquot, genetically marked with LNL6, was transplanted into the left side without MSCs. At day 46 after transplantation, the cotransplantation aliquot engrafted 4.4 times more efficiently than the HSC-alone aliquot, as assessed by colony PCR of the ilium marrow that was neutral from both sides (47.8% vs 10.9%, respectively) (Table 2 and Fig. 2A). In the second monkey (Monkey 2), hemi-iBMT was conducted with the switching of vectors to exclude the possibility of a vector-associated bias. The cotransplantation aliquot again engrafted more efficiently (6.0 times) than the HSC-alone aliquot, as assessed by colony PCR of marrow from the four limbs at day 39 after transplantation (6.0% vs 1.0%, respectively) (Table 2 and Fig. 2A). In the third monkey (Monkey 3), the cotransplantation aliquot engrafted more efficiently than the HSC-alone aliquot at day 56 after transplantation, although the difference in this monkey was not as significant, as assessed by colony PCR (7.1% vs 4.5%, respectively) (Table 2 and Fig. 2A). These results suggest that cotransplantation with MSCs improves engraftment of HSCs in nonhuman primates.

In addition, in two of the three animals (Monkeys 2 and 3), CFUs derived from the cotransplantation aliquots were detected in the BM of the opposite side (Fig. 2B), implying that the transduced $CD34^+$ cells injected into the BM might migrate and achieve homing to the distant BM.

Table 2. In vivo transduction levels post-transplantation with or without MSCs

Animal (ID no.)	Groups of hemi-iBMT	Site of iBMT	Vectors for marking	Sampling of BM* (post-transplantation)	In vivo marking (% of provirus-positive colonies)	Fold increase in engrafted CFUs†
Monkey 1 (H025)	Cotransplant	Right humerus/femur	G1Na	Ilium (day 46)	22/46 (47.8)	4.4
	HSC alone	Left humerus/femur	LNL6		5/46 (10.9)	
Monkey 2 (H030)	Cotransplant	Right humerus/femur	LNL6	Limbs (day 39)	12/192 (6.0)	6.0
	HSC alone	Left humerus/femur	G1Na		2/192 (1.0)	
Monkey 3 (H033)	Cotransplant	Right humerus/femur	G1Na	Limbs (day 56)	8/112 (7.1)	1.6
	HSC alone	Left humerus/femur	LNL6		5/112 (4.5)	

BM=bone marrow; iBMT=intra-bone marrow transplantation; MSCs=mesenchymal stromal cells.
*Bone marrow cells were taken from the ilium and/or limbs (humerus and femur on both sides).
†Fold increase in engrafted colony-forming units (CFUs) was calculated by dividing the number of CFUs derived from cotransplantation aliquots by that from hematopoietic stem cell (HSC)-alone aliquots.

Origin of marked cells in PB

Although there were no marked cells detected in the PB in Monkey 1, 2.0% and 0.4% of the PB cells were marked in Monkeys 2 and 3 on days 33 and 35 post-transplantation, respectively, as assessed by semiquantitative PCR (Fig. 3A). With capillary electrophoresis, which can detect a 16-bp difference between G1Na and LNL6, these cells were proven to be marked with the vector for the cotransplant aliquot (Fig. 3B). In all monkeys examined, cells derived from the HSC-alone aliquots were barely detected in the PB. These results clearly indicate that cotransplantation with MSCs enhances transplant-derived chimerism in the PB.

Mechanism of improvements by MSCs

Previous studies demonstrated that, in human-mouse xenograft models, transplanted human MSCs, after their differentiation into osteoblasts, osteocytes, and endothelial cells, appeared to be involved in the maintenance of human hematopoiesis through two ways: via their physical interaction with primitive hematopoietic cells [18] and via factors they secrete, such as stromal derived factor-1 (SDF-1) [18], which have been shown to regulate the proliferation and survival of hematopoietic stem and progenitor cells [12,23–25]. To assess the former possibility, we retrovirally marked MSCs with the β-galactosidase gene before transplantation in Monkey 3, but could not detect LacZ-positive cells in the BM (data not shown), possibly due to the low expression level or immune clearance of LacZ. As grounds of the latter possibility, it has been reported that the expression of hematopoiesis-supporting cytokines including SCF, SDF-1, and angiopoietin-1 was upregulated in preadipocytes during differentiation from MSCs [26]. Contrary to our expectations, we found the reverse tendency: decreased concentrations of these cytokines in the BM transplanted with HSCs and MSCs as compared to those with HSCs alone, albeit not at significant levels (SDF-1α, $p=0.21$; SCF, $p=0.46$; angiopoietin-1; $p=0.39$; Supplementary Figure E1 (online only, available at www.exphem.org). It is possible to speculate that poor engraftment with HSCs alone might stimulate compensatory signaling pathways to upregulate the expression of these hematopoiesis-supporting cytokines.

Discussion

Clinical trials to intravenously coinfuse MSCs have already been reported both in the autologous [27] and allogeneic [28,29] settings; however, they aimed at feasibility and safety, and are not controlled studies. We obtained the favorable results in terms of engraftment of HSCs in the controlled autologous setting in monkeys.

Although the migration of HSCs post-transplantation has already been demonstrated in mouse syngeneic iBMT, human-mouse xeno-iBMT, and monkey auto-iBMT models [8,10–13], it has been reported that ex vivo cultured HSCs lose their capacity for migration and homing [30]. However, there was still a residual capacity for migration in ex vivo manipulated HSCs without cotransplantation of MSCs in our study (Fig. 2B). After cotransplantation with MSCs, the ex vivo manipulated HSCs began to migrate to the distant BM much more frequently, suggesting that cotransplanted MSCs restored the properties of HSCs for migration and homing, at least in part. Moreover, the efficacy of MSCs was much more marked in the PB rather than in the BM, implying that MSCs might have some effects on the mobilization steps.

Our setting of hemitransplantation with dual genetic marking allows tracking and comparison of two HSC grafts with or without MSCs in the same monkeys, and thus it is meritorious for evaluation of migration and homing as described here. It is also useful, considering that there are large individual differences in outbred monkeys, unlike mice. Individual differences in monkeys often overwhelm differences in experimental data. In fact, the differences in the engraftment post-transplantation among Monkeys 1 to 3 (up to 10-fold) are apparently larger than the differences in the engraftment between the two HSC grafts (up to 6.0-fold) (Table 2).

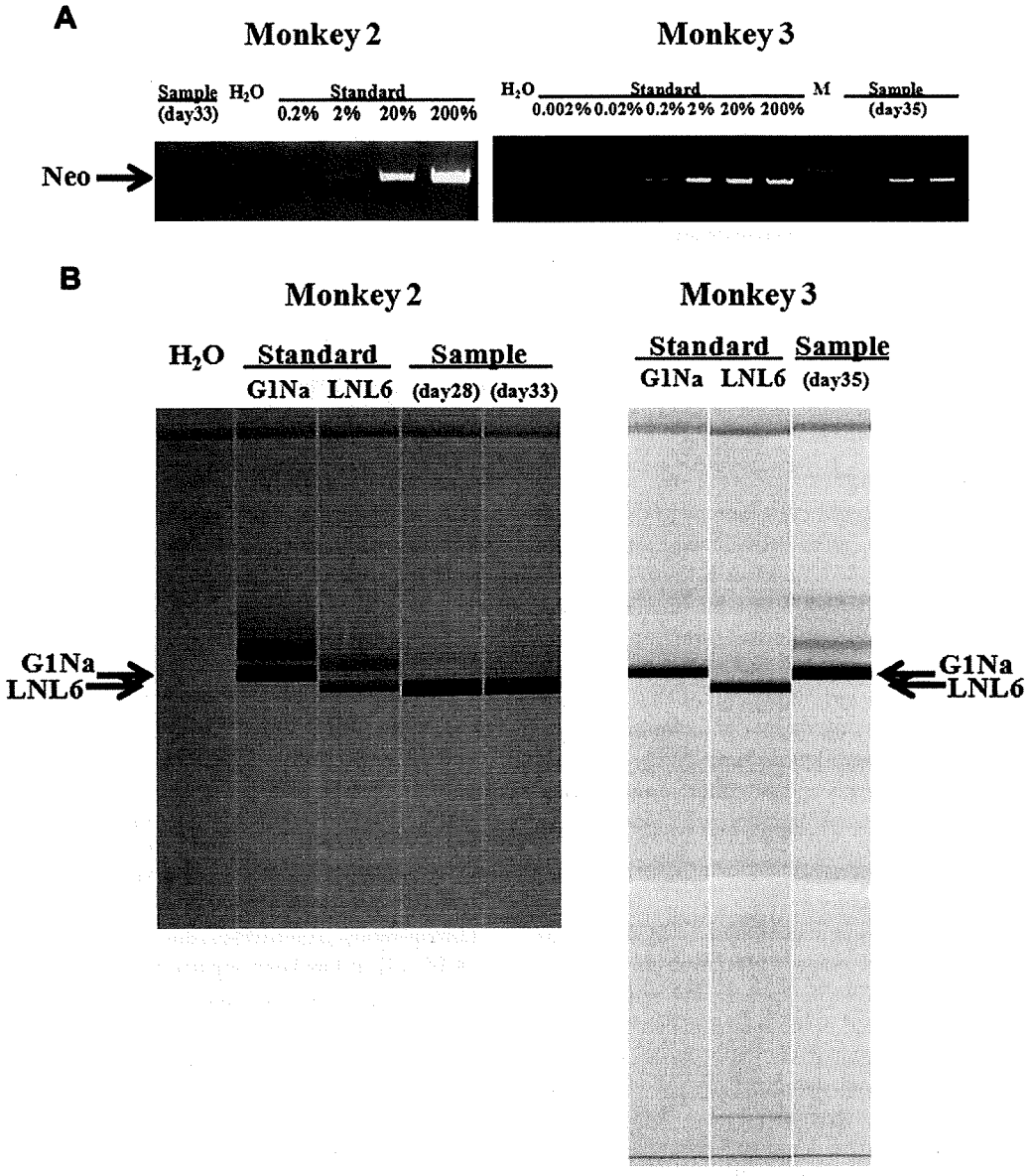

Figure 3. Contribution of transplant-derived cells to the peripheral blood (PB). (**A**) Polymerase chain reaction (PCR) products separated on agarose gels. The genomic DNA extracted from the PB of Monkey 2 on day 33 and Monkey 3 on day 35 post-transplantation was subjected to semiquantitative PCR for the vector *neo*^{R} sequence. *Neo*^{R}-marked cells were detected in the PB after transplantation. Positive controls corresponding to 0.002%, 0.02%, 0.2%, 2%, 20%, and 200% of marked cells were included. (**B**) Capillary electrophoresis of PCR products. The PCR products in (**A**) were subjected to capillary electrophoresis with positive controls. This technique enables us to distinguish G1Na and LNL6 markings with only a 16-bp difference. Upper and lower bands denote G1Na and LNL6, respectively. The detected bands in the PB were all derived from the cotransplantation aliquots.

Current literature on the subject of coinfusion of MSCs with HSCs in an allogeneic setting suggests that enhanced engraftment is achieved via dampening of the immune response against donor HSCs [28,29]. Such a mechanism cannot be formally ruled out, even in our autologous setting, because transplanted HSCs were genetically modified to express a foreign gene (*neo*^{R}).

Although the efficacy of cotransplantation with MSCs was suggested, the overall rate of HSCs engraftment in this study appears to be low, as is the case in other studies using nonhuman primates [31,32]. Further improvements should be needed to apply these methods to human trials. We did not follow the animals long-term in the present study. We would like to see whether or not cotransplantation with MSCs brings better engraftment. Thus, we euthanized monkeys at 1 to 2 months post-transplantation to harvest all the ilium and limbs marrow and we could not follow them longer-term. Therefore, the present results will reflect the behavior of short-term hematopoietic repopulating cells, and longer follow-up (at least 180 day) should be needed to

evaluate the long-term efficacy of cotransplantation with MSCs. There also remains a concern over safety. Because we used ex vivo expanded MSCs, there is a possibility of causing tumors, such as osteosarcoma. Longer-term studies are needed to assess safety as well.

Acknowledgments

We thank Y. Katakai (The Corporation for Production and Research of Laboratory Primates [Ibaraki, Japan]) for animal care and Y. Furukawa (Jichi Medical University [Tochigi, Japan]) for technical help. G1Na and LNL6 were kindly provided by C. E. Dunbar (Hematology Branch, National Heart, Lung, and Blood Institute [Bethesda, MD, USA], National Institutes of Health [Bethesda, MD, USA]). We acknowledge Kirin's supply of thrombopoietin. This study was supported by the Japanese government grants to Y.H. (JMS 21st Century COE Program, High-tech Research Center Program, and KAKENHI from the Ministry of Education, Culture, Sports, Science and Technology of Japan [Tokyo, Japan], as well as KAKENHI from the Ministry of Health, Labor and Welfare of Japan [Tokyo, Japan]). This work was also in part supported by the funding to S.M. (KAKENHI for Young Scientists from the Ministry of Education, Culture, Sports, Science and Technology of Japan, Mitsubishi Pharma Research Foundation [Osaka, Japan], Kanae Foundation for the Promotion of Medical Science [Tokyo, Japan], Aichi Cancer Research Foundation [Aichi, Japan], and Takeda Science Foundation [Osaka, Japan]).

Conflict of Interest

No financial interest/relationships with financial interest relating to the topic of this article have been declared.

References

1. Calvi LM, Adams GB, Weibrecht KW, et al. Osteoblastic cells regulate the haematopoietic stem cell niche. Nature. 2003;425:841–846.
2. Zhang J, Niu C, Ye L, et al. Identification of the haematopoietic stem cell niche and control of the niche size. Nature. 2003;425:836–841.
3. Prockop DJ. Marrow stromal cells as stem cells for nonhematopoietic tissues. Science. 1997;276:71–74.
4. Pittenger MF, Mackay AM, Beck SC, et al. Multilineage potential of adult human mesenchymal stem cells. Science. 1999;284:143–147.
5. Ozawa K, Sato K, Oh I, et al. Cell and gene therapy using mesenchymal stem cells (MSCs). J Autoimmun. 2008;30:121–127.
6. Plett PA, Frankovitz SM, Orschell-Traycoff CM. In vivo trafficking, cell cycle activity, and engraftment potential of phenotypically defined primitive hematopoietic cells after transplantation into irradiated or nonirradiated recipients. Blood. 2002;100:3545–3552.
7. Wynn RF, Hart CA, Corradi-Perini C, et al. A small proportion of mesenchymal stem cells strongly expresses functionally active CXCR4 receptor capable of promoting migration to bone marrow. Blood. 2004;104:2643–2645.
8. Zhong JF, Zhan Y, Anderson WF, et al. Murine hematopoietic stem cell distribution and proliferation in ablated and nonablated bone marrow transplantation. Blood. 2002;100:3521–3526.
9. Nakamura K, Inaba M, Sugiura K, et al. Enhancement of allogeneic hematopoietic stem cell engraftment and prevention of GVHD by intra-bone marrow transplantation plus donor lymphocyte infusion. Stem Cells. 2004;22:125–134.
10. Wang J, Kimura T, Asada R, et al. SCID-repopulating cell activity of human cord blood-derived CD34- cells assured by intra-bone marrow injection. Blood. 2003;101:2924–2931.
11. Mazurier F, Doedens M, Gan OI, et al. Rapid myeloerythroid repopulation after intrafemoral transplantation of NOD-SCID mice reveals a new class of human stem cells. Nat Med. 2003;9:959–963.
12. Yahata T, Ando K, Sato T, et al. A highly sensitive strategy for SCID-repopulating cell assay by direct injection of primitive human hematopoietic cells into NOD/SCID mice bone marrow. Blood. 2003;101:2905–2913.
13. Ueda K, Hanazono Y, Shibata H, et al. High-level in vivo gene marking after gene-modified autologous hematopoietic stem cell transplantation without marrow conditioning in nonhuman primates. Mol Ther. 2004;10:469–477.
14. Frassoni F, Gualandi F, Podestà M, et al. Direct intrabone transplant of unrelated cord-blood cells in acute leukaemia: a phase I/II study. Lancet Oncol. 2008;9:831–839.
15. Noort WA, Kruisselbrink AB, in 't Anker PS, et al. Mesenchymal stem cells promote engraftment of human umbilical cord blood-derived CD34(+) cells in NOD/SCID mice. Exp Hematol. 2002;30:870–878.
16. in 't Anker PS, Noort WA, Kruisselbrink AB, et al. Nonexpanded primary lung and bone marrowderived mesenchymal cells promote the engraftment of umbilical cord blood-derived CD34(+) cells in NOD/SCID mice. Exp Hematol. 2003;31:881–889.
17. Bensidhoum M, Chapel A, Francois S, et al. Homing of in vitro expanded Stro-1$^-$ or Stro-1$^+$ human mesenchymal stem cells into the NOD/SCID mouse and their role in supporting human CD34 cell engraftment. Blood. 2004;103:3313–3319.
18. Muguruma Y, Yahata T, Miyatake H, et al. Reconstitution of the functional human hematopoietic microenvironment derived from human mesenchymal stem cells in the murine bone marrow compartment. Blood. 2006;107:1878–1887.
19. Heim DA, Hanazono Y, Giri N, et al. Introduction of a xenogeneic gene via hematopoietic stem cells leads to specific tolerance in a rhesus monkey model. Mol Ther. 2000;1:533–544.
20. Tisdale JF, Hanazono Y, Sellers SE, et al. Ex vivo expansion of genetically marked rhesus peripheral blood progenitor cells results in diminished long-term repopulating ability. Blood. 1998;92:1131–1141.
21. Shibata H, Hanazono Y, Ageyama N, et al. Collection and analysis of hematopoietic progenitor cells from cynomolgus macaques (Macaca fascicularis): assessment of cross-reacting monoclonal antibodies. Am J Primatol. 2003;61:3–12.
22. Kushida T, Inaba M, Ikebukuro K, et al. Comparison of bone marrow cells harvested from various bones of cynomolgus monkeys at various ages by perfusion or aspiration methods: a preclinical study for human BMT. Stem Cells. 2002;20:155–162.
23. Lataillade JJ, Clay D, Dupuy C, et al. Chemokine SDF-1 enhances circulating CD34(+) cell proliferation in synergy with cytokines: possible role in progenitor survival. Blood. 2000;95:756–768.
24. Lataillade JJ, Clay D, Bourin P, et al. Stromal cell derived factor 1 regulates primitive hematopoiesis by suppressing apoptosis and by promoting G(0)/G(1) transition in CD34(+) cells: evidence for an autocrine/paracrine mechanism. Blood. 2002;99:1117–1129.
25. Cashman J, Clark-Lewis I, Eaves A, Eaves C. Stromal-derived factor 1 inhibits the cycling of very primitive human hematopoietic cells in vitro and in NOD/SCID mice. Blood. 2002;99:792–799.
26. Oh I, Ozaki M, Miyazato A, et al. Screening of genes responsible for differentiation of mouse mesenchymal stromal cells by DNA microarray analysis of C3H10T1/2 and C3H10T1/2-derived cell lines. Cytotherapy. 2007;9:80–90.
27. Koç ON, Gerson SL, Cooper BW, et al. Rapid hematopoietic recovery after coinfusion of autologous-blood stem cells and culture-expanded marrow mesenchymal stem cells in advanced breast cancer patients receiving high-dose chemotherapy. J Clin Oncol. 2000;18:307–316.
28. Lazarus HM, Koc ON, Devine SM, et al. Cotransplantation of HLA-identical sibling culture-expanded mesenchymal stem cells and

hematopoietic stem cells in hematologic malignancy patients. Biol Blood Marrow Transplant. 2005;11:389–398.
29. Ball LM, Bernardo ME, Roelofs H, et al. Cotransplantation of ex vivo expanded mesenchymal stem cells accelerates lymphocyte recovery and may reduce the risk of graft failure in haploidentical hematopoietic stem-cell transplantation. Blood. 2007;110:2764–2767.
30. Yamamura K, Ohishi K, Masuya M, et al. Ex vivo culture of human cord blood hematopoietic stem/progenitor cells adversely influences their distribution to other bone marrow compartments after intrabone marrow transplantation. Stem Cells. 2008;26:543–549.
31. Larochelle A, Choi U, Shou Y, et al. In vivo selection of hematopoietic progenitor cells and temozolomide dose intensification in rhesus macaques through lentiviral transduction with a drug resistance gene. J Clin Invest. 2009;119:1952–1963.
32. Zhang XB, Beard BC, Beebe K, Storer B, Humphries RK, Kiem HP. Differential effects of HOXB4 on nonhuman primate short- and long-term repopulating cells. PLoS Med. 2006;3:e173.

A Rice-Based Oral Cholera Vaccine Induces Macaque-Specific Systemic Neutralizing Antibodies but Does Not Influence Pre-Existing Intestinal Immunity[1]

Tomonori Nochi,* Yoshikazu Yuki,* Yuko Katakai,† Hiroaki Shibata,‡ Daisuke Tokuhara,* Mio Mejima,* Shiho Kurokawa,* Yuko Takahashi,* Ushio Nakanishi,* Fumiko Ono,† Hitomi Mimuro,§ Chihiro Sasakawa,§ Fumio Takaiwa,¶ Keiji Terao,‡ and Hiroshi Kiyono[2]*

We previously showed that oral immunization of mice with a rice-based vaccine expressing cholera toxin (CT) B subunit (MucoRice-CT-B) induced CT-specific immune responses with toxin-neutralizing activity in both systemic and mucosal compartments. In this study, we examined whether the vaccine can induce CT-specific Ab responses in nonhuman primates. Orally administered MucoRice-CT-B induced high levels of CT-neutralizing serum IgG Abs in the three cynomolgus macaques we immunized. Although the Ab level gradually decreased, detectable levels were maintained for at least 6 mo, and high titers were rapidly recovered after an oral booster dose of the rice-based vaccine. In contrast, no serum IgE Abs against rice storage protein were induced even after multiple immunizations. Additionally, before immunization the macaques harbored intestinal secretory IgA (SIgA) Abs that reacted with both CT and homologous heat-labile enterotoxin produced by enterotoxigenic *Escherichia coli* and had toxin-neutralizing activity. The SIgA Abs were present in macaques 1 mo to 29 years old, and the level was not enhanced after oral vaccination with MucoRice-CT-B or after subsequent oral administration of the native form of CT. These results show that oral MucoRice-CT-B can effectively induce CT-specific, neutralizing, serum IgG Ab responses even in the presence of pre-existing CT- and heat-labile enterotoxin-reactive intestinal SIgA Abs in nonhuman primates. ***The Journal of Immunology,*** **2009, 183: 6538–6544.**

Seven distinct cholera pandemics have occurred since 1817 (1). The first six originated from the Indian subcontinent, whereas the last arose on the island of Sulawesi in Indonesia in 1961 and is still spreading throughout the world (1). These pandemics were all caused by oral infection with *Vibrio cholerae* O1 biotype El Tor; however, a non-O1 serogroup, now categorized as O139, recently appeared and caused a large epidemic of cholera in India and Bangladesh (2). A recent report on cholera in the weekly epidemiological record of the World Health Organization showed that the number of cholera cases dramatically increased in 2006 (236,896 cases, including 6,311 deaths) because of several major outbreaks (3).

Currently, three oral cholera vaccines, Dukoral, Orochol, and the Vietnamese vaccine, have been developed for public use (4). Dukoral, the most widely used cholera vaccine, especially in Europe, consists of four types of inactivated *V. cholerae* O1 plus recombinant cholera toxin (CT)[3] B subunit (CT-B; 5, 6). Orochol contains live attenuated CVD 103-HgR derived from the classical *V. cholerae* Inaba strain with 94% deletion of the toxic activity (7, 8). The Vietnamese vaccine contains inactivated forms of both *V. cholerae* O1 and O139 (9, 10). The primary reason for choosing an oral vaccine against cholera is that oral vaccines induce Ag-specific immune responses in both systemic and mucosal compartments, thereby providing two layers of protective immunity (11–13). Despite the efficacy of these three vaccines, their requirement for "cold-chain" maintenance for preservation is a major concern for their use in the field, especially in developing countries (14). Owing to this difficulty, the development of a "cold-chain-free" oral vaccine is needed (15, 16).

To overcome this concern, we have turned to a foreign protein expression system that uses rice as a vaccine production platform, because rice seeds can be preserved for long periods at ambient temperatures (17). Oral immunization with a rice-based oral vaccine expressing CT-B, named MucoRice-CT-B, successfully induced protective immunity in both systemic and intestinal tissues

*Division of Mucosal Immunology, §Division of Bacterial Infection, Department of Microbiology and Immunology, Institute of Medical Science, University of Tokyo, Japan; †Corporation for Production and Research of Laboratory Primates, Japan; ‡Tsukuba Primate Research Center, National Institute of Biomedical Innovation, Japan; and ¶Plant Biotechnology Department, National Institute of Agrobiological Sciences, Japan

Received for publication May 11, 2009. Accepted for publication September 3, 2009.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported by grants from the "Development of Fundamental Technologies for Production of High-Value Materials Using Transgenic Plants" project of the Ministry of Economy, Trade and Industry (to H.K.); the Ministry of Education, Culture, Sports, Science and Technology, and the Ministry of Health and Labour (to H.K. and Y.Y.); GATES Grand Challenges Explorations (to H.K.); the Global COE Program "Center of Education and Research for the Advanced Genome-Based Medicine: For Personalized Medicine and the Control of Worldwide Infectious Diseases" (to H.K.); Research on Vaccine of Next Generation of The Ministry of Health, Labour and Welfare (to H.K. and K.T.); Research Fellowship of the Japan Society for the Promotion of Science (to T.N.); and the Research and Development Program for New Bio-industry Initiatives of the Bio-oriented Technology Research Advancement Institution (to Y.Y.).

[2] Address correspondence and reprint requests to Dr. Hiroshi Kiyono, Division of Mucosal Immunology, Department of Microbiology and Immunology, Institute of Medical Science, University of Tokyo, 4–6-1 Shirokanedai, Minato-ku, Tokyo, Japan. E-mail address: kiyono@ims.u-tokyo.ac.jp

[3] Abbreviations used in this paper: CT, cholera toxin; CT-B, cholera toxin B subunit; PB, protein body; SIgA, secretory IgA Ab; LT, heat-labile enterotoxin; LT-B, heat-labile enterotoxin B subunit; WT, wild type; RT, room temperature; DC, dendritic cell.

www.jimmunol.org/cgi/doi/10.4049/jimmunol.0901480

in mice without coadministration of whole-cell *V. cholerae* or mucosal adjuvant, and its immunogenicity was maintained for over 1.5 years in storage at room temperature (17). Another advantage to using the rice expression system for the development of oral vaccines is that the rice seeds possess unique protein storage organelles, the protein bodies (PBs; 18, 19). In particular, the endoplasmic reticulum-derived PB that deposits prolamins, PB-I, is not susceptible to digestive enzymes, and thus can survive in the harsh environment of the gastrointestinal tract (18, 19). The use of an endosperm-specific promoter and a signal peptide in MucoRice-CT-B causes CT-B to be expressed and to accumulate in PBs, making the CT-B highly resistant to digestive enzymes and thus giving it mucosal immunogenicity that induces serum IgG and intestinal secretory IgA Abs (SIgA), which protect against CT (17).

Before testing MucoRice-CT-B in human studies, we designed experiments to assess its immunogenicity in nonhuman primates. As it did in mice (17), it successfully induced CT-protective serum IgG Ab responses in cynomolgus macaques. However, to our surprise, the macaques also had pre-existing CT-reactive intestinal SIgA Abs, which appeared to be maximally expressed without immunization. This provided an opportunity to explore the effects of pre-existing intestinal immunity on the potential use of MucoRice-CT-B as a new-generation oral cholera vaccine in humans.

Materials and Methods

Nonhuman primates

We used serum and fecal extracts from 26 randomly selected, untreated cynomolgus macaques (*Macaca fascicularis*, 1 mo to 29 years old; 6 male, 20 female) bred and housed in two different environments in the Tsukuba Primate Research Center (n = 22, Ibaraki, Japan) and Hamry Company (n = 4, Ibaraki, Japan) to examine whether Abs against CT-B and heat-labile enterotoxin (LT)-B were present before immunization. All other experiments, including the study of MucoRice-CT-B immunization, were performed at the Tsukuba Primate Research Center with four additional cynomolgus macaques (each 5 years old; female). All animal experiments were approved by the Animal Care and Use Committee of the Institute of Medical Science at the University of Tokyo and the Tsukuba Primate Research Center at the National Institute of Biomedical Innovation.

Immunization

MucoRice-CT-B was generated as described previously (17). In brief, the codon-optimized *CTB* gene was inserted into a binary vector (pGPTV-35S-HPT), and the plasmid was transformed into rice (*Oryza sativa* L. cv. Kitaake). After harvest, the seeds were first ground to a fine powder in a Multibeads shocker (Yasui Kikai). Three cynomolgus macaques (no. 001, no. 002, and no. 003) were orally immunized with 667 mg of powdered MucoRice-CT-B, containing 1 mg of CT-B, and one macaque (no.004) was given the same amount of powdered nontransgenic wild-type (WT) rice. The rice powder was suspended in 5 ml of physiologic saline and administered on five occasions at 2-wk intervals under ketamine anesthesia. Six months after the last immunization, the macaques were orally boosted with the same amount of MucoRice-CT-B or WT rice. Finally, to follow up the Ag-specific Ab responses including pre-existing CT-reactive SIgA, 100 μg of CT dissolved in PBS was given orally to all four macaques on three occasions at 2-wk intervals.

Sample collection and gel filtration chromatography

Serum and fecal extracts were collected from the four macaques before immunization; 1 wk after each immunization; and 2, 4, and 6 mo after the last oral immunization with MucoRice-CT-B (Fig. 2). The feces were suspended (20% w/v) in cold PBS containing Complete Protease Inhibitor Cocktail (Roche) and 0.1% sodium azide. After centrifugation, the supernatant was filtered through a 0.45-μm filter (Pall Corporation) and stored at −80°C before use. A 1-ml aliquot of each fecal extract was separated by gel filtration chromatography on a Sephacryl S-500 (GE Healthcare) column (1.5 × 50 cm, Bio-Rad). Each 2-ml fraction collected was used in the CT-specific ELISA and toxin-neutralizing GM1-ELISA. Bovine IgM (Sigma-Aldrich; MW: 90 kDa) and β-lactoalbumin (Sigma-Aldrich; MW: 18.4 kDa) were used as molecular standards for the gel filtration chromatography.

ELISA

The Ag-specific Ab responses were analyzed by ELISA as described previously (17), with some modifications. In brief, 5 μg/ml CT (List Biologic Laboratories), recombinant CT-B, or recombinant LT-B prepared in our laboratory (20) or 20 μg/ml rice storage protein extracted with 0.01% Triton X-100 was used to coat 96-well plates overnight at 4°C. Two-fold serial dilutions of samples were blocked with 1% BSA, added to the plates, and incubated for 2 h at room temperature (RT). For the CT-specific analysis, the samples were then treated with HRP-conjugated goat anti-monkey IgG (Nordic Immunological Laboratory) or HRP-conjugated goat anti-monkey IgA (Cortex Biochem), each diluted 1/1,000, or HRP-conjugated anti-human IgE cross-reacting with monkey IgE (Serotec) diluted 1/10,000, for 1 h at RT. Because our recent and separate murine study showed that free form of GM1 ganglioside in fecal extracts affected the in vitro toxin-neutralizing assay, it was also important to address the presence or absence of GM1 ganglioside in gel-filtrated fecal extracts. The samples were thus also treated with rabbit anti-GM1 ganglioside (Calbiochem) diluted 1/1,000 for 2 h at RT, followed by an HRP-conjugated anti-rabbit IgG (Southern Biotechnology Associates) diluted 1/4,000 for 1 h at RT. The reaction was developed by using TMB Substrate (XPL), and end-point titers were expressed as the reciprocal $\log_2$ of the last dilution that gave an OD_{450} of 0.1 greater than the negative control.

Western blotting

Extracts of rice were prepared with sample buffer containing 2% (w/v) SDS, 8 M urea, 5% (v/v) 2-ME, 50 mM Tris HCl (pH 6.8), and 20% (v/v) glycerol as described previously (17). The rice extracts and CT-B were subjected to SDS-PAGE in a NuPAGE 12% Bis-Tris Gel (Invitrogen) before being transferred to a polyvinylidene difluoride membrane (Millipore). After blocking with 5% skim milk (Wako), the membranes were treated for 1 h at RT with serum diluted 1/500 or undiluted fecal extract obtained before immunization or after the booster dose, followed, respectively, by HRP-conjugated anti-monkey IgG (Nordic Immunological Laboratory) or HRP-conjugated anti-monkey IgA (Cortex Biochem), each diluted 1/500, for 1 h at RT. After washes, the reactions were developed with 3,3-diaminobenzidine substrate (Vector).

Neutralizing assay

A neutralizing assay was performed by using a GM1-ELISA as described previously (17), with some modifications. In brief, serum (10%, v/v) or gel-filtered fecal extract (50%, v/v) was pretreated with CT (50 ng/ml final concentration) for 1 h at RT and then incubated in 96-well plates coated with monosialoganglioside GM1 (5 μg/ml, Sigma-Aldrich) for 1 h at RT. After washes, the plates were incubated with an HRP-conjugated rabbit anti-CT-B Ab (500 ng/ml) prepared in our laboratory (17) for 1 h at RT, and the reaction was detected by using TMB substrate. The inhibitory effect of serum against the binding of CT to GM1 ganglioside was determined by comparison to CT treated with PBS (positive control).

Results

Unimmunized cynomolgus macaques have intestinal SIgA Abs reactive to CT and LT

The cynomolgus macaques used in this study had been bred in a conventional environment and not in a specific pathogen-free environment. Therefore, before immunizing them with MucoRice-CT-B, we first examined whether they already possessed Abs against CT in the sera and fecal extracts. The fecal and serum samples obtained from 22 randomly selected macaques aged from 1 mo to 29 years old had very few to no CT-B–specific Abs in serum (Fig. 1*A*), as expected, because the quarantine record of these animals did not indicate any *V. cholerae* infection (data not shown). However, all of the fecal extracts unexpectedly contained CT-B–reactive intestinal SIgA Abs (Fig. 1*B*). Because CT possesses high homology to LT (21), we next examined whether the intestinal SIgA Abs present in the fecal extracts reacted with LT-B. Although the serum samples did not show any LT-B–reactive IgG Abs (Fig. 1*C*; similar to the reactivity against CT-B), all of the macaques had LT-B–reactive SIgA Abs in their feces (Fig. 1*D*).

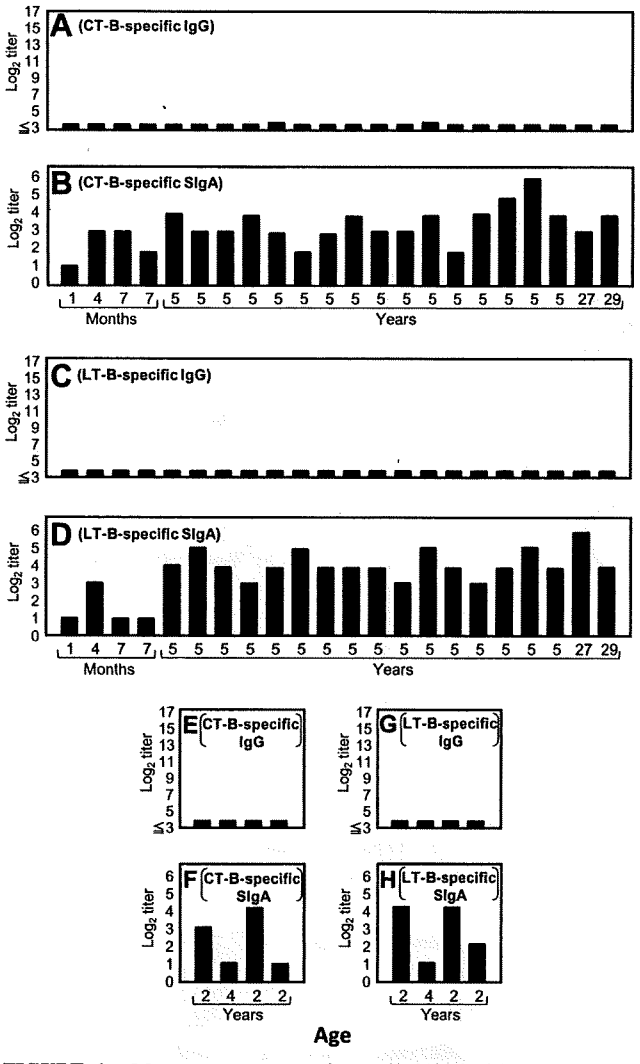

FIGURE 1. Macaques spontaneously acquire intestinal SIgA but not serum IgG Abs specific for CT-B and LT-B. CT-B–specific (*A*, *B*, *E*, *F*) and LT-B–specific (*C*, *D*, *G*, *H*) immune responses in serum (*A*, *C*, *E*, *G*) and fecal extracts (*B*, *D*, *F*, *H*) of 22 randomly selected macaques (*A–D*) and 4 additional macaques housed at a different facility (*E–H*) were examined by ELISA.

To examine whether cynomolgus macaques bred in different housing conditions also had CT-B– and LT-B–reactive SIgA Abs, we randomly selected four additional macaques housed in a different facility. These macaques were 2 to 4 years old, with no record of *V. cholerae* infection. All of these additional macaques also possessed CT-B– and LT-B–reactive intestinal SIgA Abs in their feces (Fig. 1, *F* and *H*) but not serum IgG Abs (Fig. 1, *E* and *G*). Taken together, our results show that macaques acquire CT-B– and LT-B–reactive SIgA Abs in their gastrointestinal immune system under the conventional environment.

Oral immunization of cynomolgus macaques with MucoRice-CT-B induces CT-specific serum IgG Ab responses

To test the immunogenicity of the rice-based vaccine in macaques, we orally immunized three macaques with MucoRice-CT-B, and gave one other macaque nontransgenic WT rice. Five doses of MucoRice-CT-B were given orally at 2-wk intervals as the primary immunization and a booster was given 6 mo after the last immunization (Fig. 2). Serum IgG and intestinal SIgA Abs were measured before immunization and after each dose. Similar to the

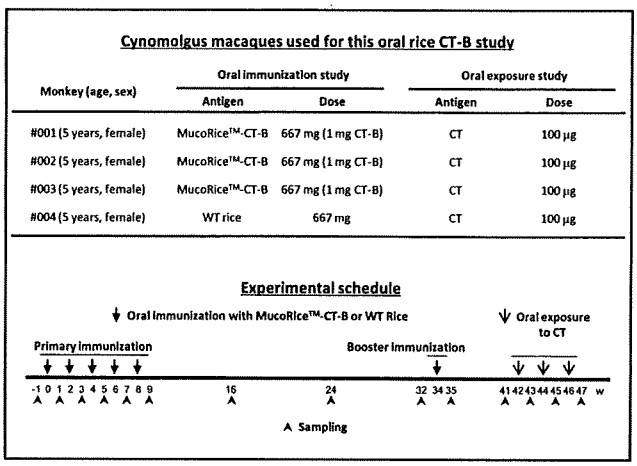

FIGURE 2. Schedule of oral immunization with MucoRice-CT-B. Macaques were orally immunized with 667 mg of MucoRice-CT-B, containing 1 mg of CT-B, or the same amount of wild-type (WT) rice on five occasions at 2-wk intervals. Six months after the fifth immunization, the macaques were given boosters of MucoRice-CT-B or WT rice. All macaques were subsequently given 100 μg of CT orally on three occasions at 2-wk intervals.

26 unimmunized macaques used in the initial study, these four macaques also had pre-existing CT-reactive SIgA Abs with toxin-neutralizing activity but did not have serum IgG Abs (Figs. 3*A* and 5*A*). The aim of this study was to examine whether oral MucoRice-CT-B could induce Ag-specific immune responses in nonhuman primates, which are closer to humans than the rodents used in our previous study (17). After two to three doses of the primary immunization, the levels of Ag-specific serum IgG, but not serum IgA, increased in all macaques immunized with MucoRice-CT-B, but not in the control macaque (Fig. 3*A*). Among the three immunized macaques, no. 001 maintained a high titer of CT-specific Ab responses for more than 6 mo (Fig. 3*A*). Although the Ab levels gradually decreased in the other two macaques after the final immunization, they continuously exceeded the detection limit for 6 mo (Fig. 3*A*). When these macaques were given an oral booster dose of the rice-based vaccine 6 mo after the last immunization, the levels of CT-specific serum IgG Abs immediately recovered to titers higher than those observed after the initial immunization (Fig. 3*A*). These results indicate that MucoRice-CT-B is a potent oral vaccine that is capable of both inducing long-term Ag-specific systemic immunity and eliciting oral booster activity in nonhuman primates.

CT-specific serum IgG Abs induced by MucoRice-CT-B possess toxin-neutralizing activity

To determine the ability of the CT-specific serum IgG Abs induced by oral immunization with MucoRice-CT-B to protect against the toxin, we performed an in vitro neutralizing assay by using a GM1-ELISA, a standard assay for demonstrating the neutralizing activity of CT-specific Abs (17, 22). When CT was preincubated with serum and assayed, the binding of CT to its receptor, GM1 ganglioside, was inhibited by sera from all of the immunized macaques at a level corresponding to the toxin-specific Ab titer, whereas the serum obtained from the control macaque did not show any inhibitory effect (Fig. 3*B*). Although serum from the macaque with the highest Ab responses (no. 001) also showed more neutralizing activity than the sera from the other two immunized macaques, the activity of the sera from these two macaques dramatically increased after the oral booster dose (Fig. 3*B*). Taken

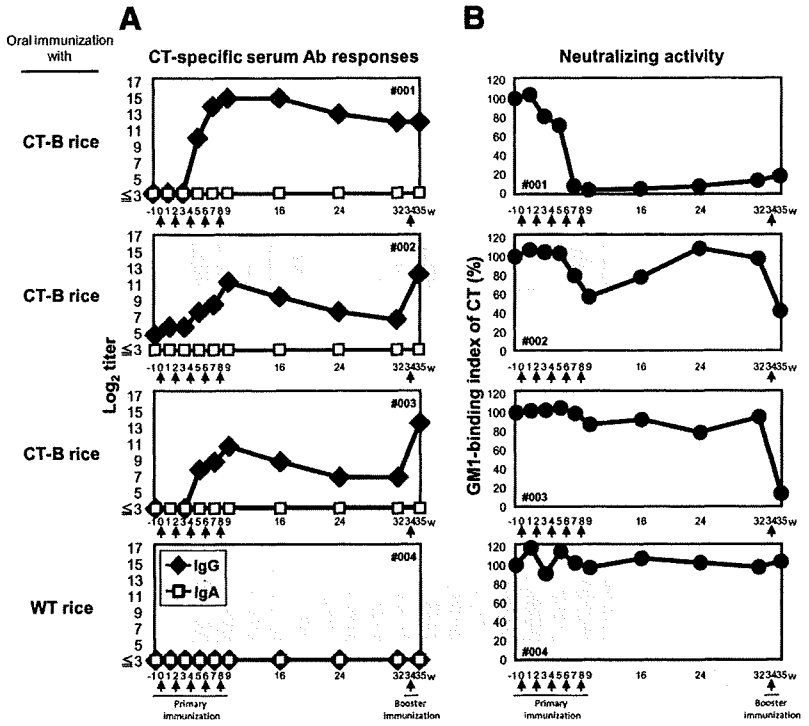

FIGURE 3. Oral vaccination with MucoRice-CT-B induces CT-specific serum IgG Abs with toxin-neutralizing activity. Oral MucoRice-CT-B but not WT rice effectively induced CT-specific serum IgG but not serum IgA Abs for at least 6 mo after the fifth immunization (*A*). Although the titer gradually decreased in two immunized macaques, it rapidly recovered after an oral booster immunization with MucoRice-CT-B (*A*). The serum collected from immunized macaques but not the control macaque inhibited the binding of CT to GM1 ganglioside at a level corresponding to the Ab titer (*B*). The CT-neutralizing activity of the two macaques with decreasing Ab titers after the primary immunization series was dramatically increased after the first oral booster dose (*B*). w = week.

together, these results indicate that oral immunization with MucoRice-CT-B can induce Ag-specific serum IgG Abs that have potential protective activity in nonhuman primates.

Oral immunization with MucoRice-CT-B does not induce IgE Ab responses to rice storage protein

To assess whether oral immunization with MucoRice-CT-B could induce a rice allergy, we examined rice storage protein-specific serum IgE and IgG Ab levels before and during the vaccination study. Rice storage protein-specific serum IgE Abs were barely detected before immunization and were not above the limit of detection after the macaques were orally immunized with the rice-based vaccine or WT rice (Fig. 4*A*). Similarly, all four macaques possessed low levels of rice storage protein-specific serum IgG Abs before immunization, but these levels were not elevated after vaccination (Fig. 4*B*). A subsequent Western blot analysis confirmed that the reactivity of serum IgG Abs against rice storage proteins prolamin and glutelin did not change between the preimmunization and post booster measurements, whereas the reactivity of Abs against CT-B did increase after vaccination (Fig. 4*C*). Taken together, these results suggest that oral MucoRice-CT-B can safely induce protective immunity without causing undesired immune responses.

Oral immunization with MucoRice-CT-B does not increase CT-reactive intestinal SIgA Abs from pre-existing levels

We next assessed whether oral immunization with the rice-based vaccine would increase the spontaneously acquired CT-reactive intestinal SIgA Abs in fecal extracts. Despite the induction of high titers of CT-specific serum IgG Abs, the pre-existing CT-reactive intestinal SIgA Ab titers did not increase even after multiple oral doses of the vaccine (Fig. 5*A*). The booster immunization 6 mo after the last immunization also did not influence the level of CT-reactive intestinal SIgA Abs (Fig. 5*A*). Similarly, a Western blot analysis showed that the reactivity of the SIgA Abs against CT-B did not change from preimmunization levels, even after the booster vaccination (Fig. 5*B*). These findings suggest that oral vaccination with MucoRice-CT-B cannot modulate the pre-existing CT-reactive intestinal SIgA Ab responses.

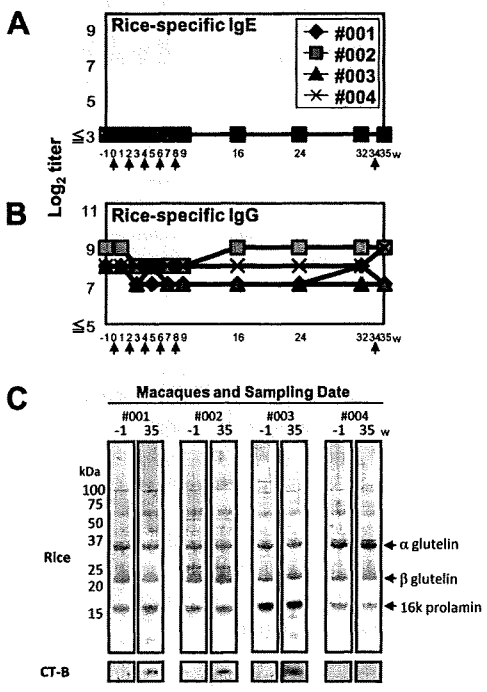

FIGURE 4. Oral immunization with MucoRice-CT-B does not induce rice storage protein-specific immune IgE Ab responses. Very low levels of serum IgE Abs specific for rice storage proteins were detected in each of the macaques orally immunized with MucoRice-CT-B or WT rice (*A*). In addition, rice storage protein-specific serum IgG Ab levels did not increase after multiple vaccinations (*B*). A Western blot analysis also showed that levels of serum IgG Abs to rice storage proteins prolamin and glutelin did not change during the vaccination period, but Abs against CT-B did increase (*C*). w = week.

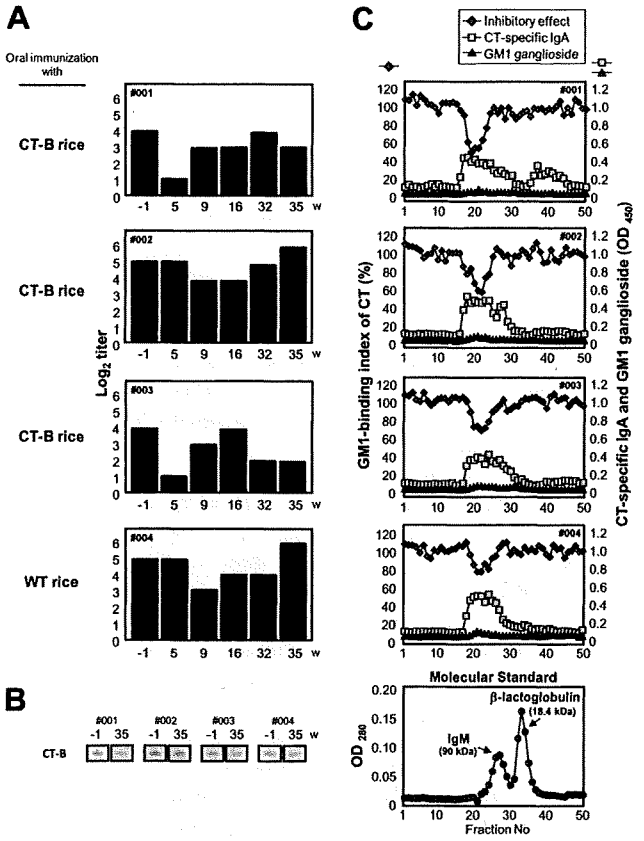

FIGURE 5. Oral immunization with MucoRice-CT-B does not increase spontaneously acquired CT-reactive intestinal SIgA Ab responses, but these SIgA Abs possess toxin-neutralizing activity. Unlike the CT-specific serum IgG Ab responses, CT-specific SIgA Ab responses were not enhanced by oral immunization with MucoRice-CT-B (*A*). Western blot analysis of feces also showed that the reactivity of SIgA Abs to CT-B did not change even after boosting with MucoRice-CT-B (*B*). Fecal extracts collected from the immunized (no. 001, no. 002, and no. 003) and control (no. 004) macaques separated by gel chromatography showed a CT-specific SIgA Ab fraction that corresponded with the toxin-neutralizing activity (inhibitory effect), but did not show a CT-reactive GM1 ganglioside-containing fraction (*C*). The inhibitory effect was calculated in comparison to the control (PBS added instead of sample). Bovine IgM and β-lactoalbumin were used as molecular standards for the gel filtration chromatography (*C*). w = week.

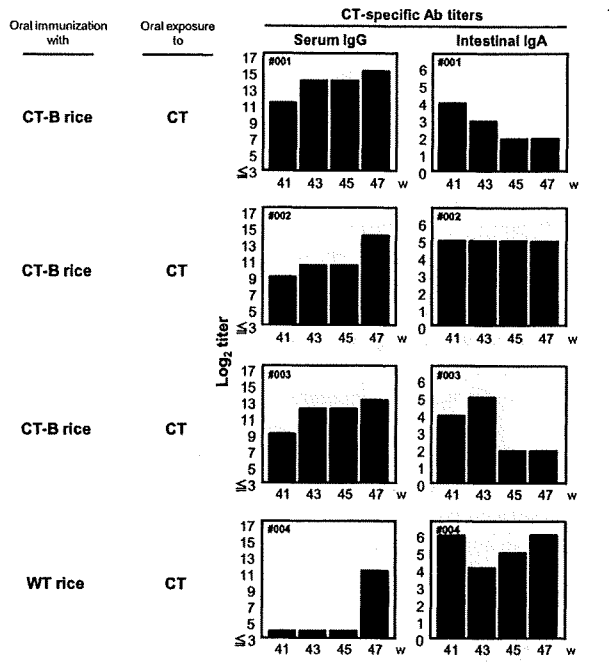

FIGURE 6. Oral administration of CT enhances serum IgG but not CT-reactive intestinal SIgA Ab responses. CT-specific serum IgG Ab responses were sharply increased in the control macaque (no. 004) and showed a further tendency to increase in three macaques previously immunized with MucoRice-CT-B (no. 001, no. 002, and no. 003). In contrast, CT-reactive intestinal SIgA Ab responses did not change consistently in any of the macaques after oral doses of CT. w = week.

Pre-existing CT-reactive intestinal SIgA Abs acquired in a conventional environment possess toxin-neutralizing activity

We recently showed that fecal extracts obtained from naive mice and mice immunized with MucoRice-CT-B contained equivalent levels of abundant, high-molecular mass, CT-reactive GM1 ganglioside derived from dead intestinal epithelial cells; this ganglioside possessed neutralizing activity in vitro but not in vivo (D. Tokuhara, Y. Yuki, T. Nochi, T. Kodama, M. Mejima, S. Kurokawa, Y. Takahashi, M. Nanno, F. Takaiwa, T. Honda, et al., in preparation). To examine whether the pre-existing CT-reactive intestinal SIgA Abs can neutralize the binding of CT to GM1 ganglioside, we first used gel filtration chromatography to separate SIgA Abs from the high-molecular mass form of GM1 ganglioside in the fecal extracts obtained after the final immunization, then assayed the collected fractions by CT- and GM1 ganglioside-specific ELISAs and an in vitro neutralizing assay. However, unlike our observation in the fecal extracts obtained from mice, we did not observe the released GM1 ganglioside in the expected molecular mass fractions obtained from immunized or control macaques (Fig. 5*C*). In contrast, CT-specific intestinal SIgA Abs were observed in the expected fractions of all macaques (Fig. 5*C*), which consisted of the fractions containing polymeric form of the total IgA (data not shown). The weak inhibitory signals were also detected by CT-specific ELISA in some low-molecular mass fractions (fractions 36 to 44) from one immunized macaque (no. 001; Fig. 5*B*). However, these fractions did not contain polymeric or dimeric forms of total IgA (data not shown), suggesting that these signals could be due to nonspecific reactivity of the secondary Ab used in this study. Most importantly, the inhibition of the binding of CT to GM1 ganglioside was observed only in fractions containing the CT-reactive intestinal SIgA Abs (Fig. 5*C*). These results support that the macaques had spontaneously acquired CT-reactive intestinal SIgA Abs possessing toxin-neutralizing activity.

CT-reactive SIgA Ab responses were not affected by oral administration of the native form of CT

Because the primary and booster oral immunization with MucoRice-CT-B did not influence the level of pre-existing CT-reactive intestinal SIgA Abs, we next administered the native form of CT to all macaques to test whether this potent Ag, which is highly immunogenic and possesses strong adjuvant activity (23) in addition to its toxic effects, would alter the SIgA Ab levels (Fig. 2). CT-specific serum IgG Ab responses dramatically increased in the control macaque and showed further increases in the three macaques previously immunized with MucoRice-CT-B (Fig. 6). However, CT-reactive SIgA Ab responses were not consistently altered in either the control or the experimental macaques even after three oral doses of CT (Fig. 6). These results suggest that the pre-existing CT-reactive intestinal humoral immunity that had developed in the conventional housing environment may have already reached immunological plateau levels.

Discussion

A major benefit of oral vaccines is that they induce protective immunity in both the systemic compartment and the aerodigestive mucosa (13). One of most important roles of the mucosal immune system is to elicit Ag-specific IgA Ab production in mucosal tissues and simultaneously to assist in the induction of Ag-specific systemic Ab responses (11). In fact, oral vaccination of cynomolgus macaques with MucoRice-CT-B effectively induced Ag-specific serum IgG Ab responses with toxin-neutralizing activity. In addition, a booster dose of the vaccine enhanced the Ag-specific Ab responses. However, to our surprise, because the macaques already had pre-existing CT-reactive intestinal SIgA Abs and probably permanently maintained them at maximum levels, these SIgA Ab levels were not increased by oral administration of MucoRice-CT-B or even by oral administration of the native form of CT. Considering their housing conditions, it is not likely that the macaques were naturally exposed to *V. cholerae*, and their medical records showed no evidence of *V. cholerae* infection. Although we do not have any definitive explanation of how the macaques may have spontaneously acquired CT-reactive SIgA Abs, CT and LT have high homology (21), and the CT-reactive intestinal SIgA Abs also cross-reacted with LT. It is therefore reasonable to consider that they had been infected by LT-producing enterotoxigenic *Escherichia coli* or homologous unknown bacteria, which may be capable of producing a CT- or LT-like molecule.

In contrast to the pre-existing CT-reactive intestinal SIgA Abs, few or no CT-specific serum IgG Abs were detectable in macaques of any age before oral immunization. The dendritic cells (DCs) in Peyer's patches and isolated lymphoid follicles can retain commensal microbiota sampled by M cells, thereby facilitating the induction of intestinal SIgA Ab responses specific for commensal flora-derived Ags (24). In contrast, commensal-specific immune responses are not induced in the systemic compartments, such as the spleen, because the mesenteric lymph nodes confine the circulation of intestinal commensal-derived Ags to DCs (24). Similar to the commensal microbiota-induced Ag-specific SIgA Ab responses, naturally infecting enterotoxigenic *E. coli* may not be pathogenic for macaques but may still spontaneously stimulate the gastrointestinal (but not systemic) immune system and induce local Ag-specific SIgA Ab production in the intestine. In contrast, the mechanisms that induce the acquired systemic immune system to respond to mucosa-derived Ags may be totally different from those spontaneously acquired mucosal Ab families, including the pre-existing CT-reactive intestinal SIgA Abs, because we recently showed in a separate study that oral immunization of Peyer's patch-deficient mice with the rice-based vaccine induces normal CT-specific serum IgG Ab responses (D. Tokuhara, Y. Yuki, T. Nochi, T. Kodama, M. Mejima, S. Kurokawa, Y. Takahashi, M. Nanno, F. Takaiwa, T. Honda, et al., in preparation). In this regard, it is known that intestinal DCs in the lamina propria directly take up luminal Ags by extending their dendrites (25, 26), and villous M cells also participate in sampling external Ags (27). Thus, another possible explanation for our current results is that MucoRice-CT-B is directly taken up by intestinal DCs and/or villous M cells even in the presence of pre-existing CT-reactive intestinal SIgA Abs, and therefore induces Ag-specific systemic IgG Ab responses through Peyer's patch-independent immunity. Although we do not have any direct evidence to support this hypothesis, it is worth testing in a future study.

IgA is the most abundant Ig produced in our body (11), especially in mucosal tissues, and the production of intestinal IgA is initiated shortly after birth in response to the colonization of commensal microbiota in the gastrointestinal tract (28). However, because the intestinal microbial composition of SIgA-lacking $pIgR^{-/-}$ mice is not completely different from that seen in WT mice (29), the precise immunological role of naturally occurring SIgA Abs is still obscure. $pIgR^{-/-}$ mice are more susceptible to *Salmonella typhimurium* infection than WT mice because they lack naturally occurring bacteria-reactive SIgA Abs (30), suggesting that these SIgA Abs may contribute to the formation of the first protective barrier against mucosal pathogens. It should be noted that macaques are not susceptible to *V. cholerae*, and oral challenge with *V. cholerae* does not cause any cholera symptoms, such as diarrhea (31). In our study, we also found that the macaques did not have diarrhea even after the oral administration of CT (data not shown). Taken together with the previous findings (31), our results suggest that spontaneously acquired CT-reactive intestinal SIgA Abs may play a pivotal role in protecting against *V. cholerae* infection in macaques.

An epidemiological study of 62,285 volunteers in Bangladesh showed that oral vaccination with 1×10^{11} killed *V. cholerae* plus 1 mg of CT-B elicited a 26% reduction in diarrhea for 1 year after the vaccination (32). Similarly, one of three macaques immunized with MucoRice-CT-B retained CT-specific long-term protective immunity in the serum for at least 6 mo after the final vaccination without a booster immunization. Although the Ab level gradually decreased in the other two immunized macaques, it remained above the detection limit, and high titers were rapidly recovered after oral boosting with the rice-based vaccine. These results indicate that oral immunization with MucoRice-CT-B is a suitable strategy not only for inducing long-term immunity, in this case over several months, but also for boosting immunity in nonhuman primates.

Another important revelation of this study is that only 667 mg of MucoRice-CT-B, which contains 1 mg of CT-B and is equivalent to approximately 30 seeds, was sufficient to induce CT-specific serum IgG Ab responses in macaques in our mouse study, we used more than 50 mg of MucoRice-CT-B, containing 75 μg of CT-B, to induce Ag-specific immune responses in mice, even though the body weight of mice is 1/150 that of macaques (17). These facts suggest that MucoRice-CT-B will be effective as a new form of oral vaccine. At same time, we also realize that five oral doses at 2-wk intervals is not a practical schedule for vaccination in the field. Because the present study was the first opportunity to demonstrate whether orally administered MucoRice-CT-B can induce Ag-specific Ab responses in limited numbers of macaques, we chose to use an excessive immunization schedule and therefore could not precisely elucidate the minimum effective dose and frequency of oral MucoRice-CT-B. To address this important issue, we are designing a new series of experiments to test the minimum dose and frequency of oral MucoRice-CT-B that can successfully induce Ag-specific immunity.

In addition, it was interesting to note that macaques harbored rice storage protein-specific IgG Abs in serum obtained before immunization (Fig. 4, *B* and *C*). The response was most likely induced by their dietary chow, which contained small amounts of rice-derived materials. However, it is important to emphasize that the pre-existing rice-specific serum IgG Abs did not increase even after multiple oral immunizations with the rice-based vaccine, and there was no evidence of induction of rice-specific IgE Ab responses (Fig. 4*A*). These results suggest that oral immunization with MucoRice-CT-B did not lead to undesired allergic immune responses even when rice-specific Abs were present in the host. Thus, we conclude that MucoRice-CT-B is a safe, immunogenic oral cholera vaccine for nonhuman primates and should be studied in humans for its possible use as a new-generation cold-chain- and needle/syringe-free vaccine.

Acknowledgments

We thank F. Adachi at the Institute of Medical Science in the University of Tokyo for her technical support; Dr. T. Shimizu at Hamry Company, for providing the feces of macaques; and Rohto Pharmaceutical Company, and Nippon Paper Group for their contributions.

Disclosures

The authors have no financial conflict of interest.

References

1. Faruque, S. M., M. J. Albert, and J. J. Mekalanos. 1998. Epidemiology, genetics, and ecology of toxigenic *Vibrio cholerae*. *Microbiol. Mol. Biol. Rev.* 62: 1301–1314.
2. Calain, P., J. P. Chaine, E. Johnson, M. L. Hawley, M. J. O'Leary, H. Oshitani, and C. L. Chaignat. 2004. Can oral cholera vaccination play a role in controlling a cholera outbreak? *Vaccine* 22: 2444–2451.
3. 2007. Weekly epidemiological record. *World Health Organization* 82: 273–284.
4. Lopez, A. L., J. D. Clemens, J. Deen, and L. Jodar. 2008. Cholera vaccines for the developing world. *Hum. Vaccin.* 4: 165–169.
5. Clemens, J. D., D. A. Sack, J. R. Harris, J. Chakraborty, M. R. Khan, B. F. Stanton, B. A. Kay, M. U. Khan, M. Yunus, W. Atkinson, et al. 1986. Field trial of oral cholera vaccines in Bangladesh. *Lancet* 2: 124–127.
6. Sanchez, J. L., B. Vasquez, R. E. Begue, R. Meza, G. Castellares, C. Cabezas, D. M. Watts, A. M. Svennerholm, J. C. Sadoff, and D. N. Taylor. 1994. Protective efficacy of oral whole-cell/recombinant-B-subunit cholera vaccine in Peruvian military recruits. *Lancet* 344: 1273–1276.
7. Kotloff, K. L., S. S. Wasserman, S. O'Donnell, G. A. Losonsky, S. J. Cryz, and M. M. Levine. 1992. Safety and immunogenicity in North Americans of a single dose of live oral cholera vaccine CVD 103-HgR: results of a randomized, placebo-controlled, double-blind crossover trial. *Infect. Immun.* 60: 4430–4432.
8. Cryz, S. J., Jr., M. M. Levine, G. Losonsky, J. B. Kaper, and B. Althaus. 1992. Safety and immunogenicity of a booster dose of *Vibrio cholerae* CVD 103-HgR live oral cholera vaccine in Swiss adults. *Infect. Immun.* 60: 3916–3917.
9. Trach, D. D., J. D. Clemens, N. T. Ke, H. T. Thuy, N. D. Son, D. G. Canh, P. V. Hang, and M. R. Rao. 1997. Field trial of a locally produced, killed, oral cholera vaccine in Vietnam. *Lancet* 349: 231–235.
10. Thiem, V. D., J. L. Deen, L. von Seidlein, G. Canh do, D. D. Anh, J. K. Park, M. Ali, M. C. Danovaro-Holliday, N. D. Son, N. T. Hoa, et al. 2006. Long-term effectiveness against cholera of oral killed whole-cell vaccine produced in Vietnam. *Vaccine* 24: 4297–4303.
11. Kiyono, H., J. Kunisawa, J. R. McGhee, and J. Mestechy. 2008. The mucosal immune system In *Fundamental Immunolology,* 5th Ed. W. E. Paul, ed., Lippincott Williams and Wilkins, Philadelphia, PA, pp. 983–1030.
12. Kunisawa, J., T. Nochi, and H. Kiyono. 2008. Immunological commonalities and distinctions between airway and digestive immunity. *Trends Immunol.* 29: 505–513.
13. Holmgren, J., and C. Czerkinsky. 2005. Mucosal immunity and vaccines. *Nat. Med.* 11: S45–S53.
14. Das, P. 2004. Revolutionary vaccine technology breaks the cold chain. *Lancet Infect. Dis.* 4: 719.
15. Varmus, H., R. Klausner, E. Zerhouni, T. Acharya, A. S. Daar, and P. A. Singer. 2003. Public health: grand challenges in global health. *Science* 302: 398–399.
16. Giudice, E. L., and J. D. Campbell. 2006. Needle-free vaccine delivery. *Adv. Drug Deliv. Rev.* 58: 68–89.
17. Nochi, T., H. Takagi, Y. Yuki, L. Yang, T. Masumura, M. Mejima, U. Nakanishi, A. Matsumura, A. Uozumi, T. Hiroi, et al. 2007. Rice-based mucosal vaccine as a global strategy for cold-chain- and needle-free vaccination. *Proc. Natl. Acad. Sci. USA* 104: 10986–10991.
18. Yamagata, H., and K. Tanaka. 1986. The site of synthesis and accumulation of rice storage proteins. *Plant Cell Physiol.* 27: 135–145.
19. Katsube, T., N. Kurisaka, M. Ogawa, N. Maruyama, R. Ohtsuka, S. Utsumi, and F. Takaiwa. 1999. Accumulation of soybean glycinin and its assembly with the glutelins in rice. *Plant Physiol.* 120: 1063–1074.
20. Yuki, Y., Y. Byun, M. Fujita, W. Izutani, T. Suzuki, S. Udaka, K. Fujihashi, J. R. McGhee, and H. Kiyono. 2001. Production of a recombinant hybrid molecule of cholera toxin-B-subunit and proteolipid-protein-peptide for the treatment of experimental encephalomyelitis. *Biotechnol. Bioeng.* 74: 62–69.
21. Dallas, W. S., and S. Falkow. 1980. Amino acid sequence homology between cholera toxin and *Escherichia coli* heat-labile toxin. *Nature* 288: 499–501.
22. Arakawa, T., D. K. Chang, W. H. Langridge. 1998. Efficacy of a food plant-based oral cholera toxin B subunit vaccine. *Nat. Biotech.* 16: 292–297.
23. Kubota, M., C. J. Miller, K. Imaoka, S. Kawabata, K. Fujihashi, J. R. McGhee, and H. Kiyono. 1997. Oral immunization with simian immunodeficiency virus p55gag and cholera toxin elicits both mucosal IgA and systemic IgG immune responses in nonhuman primates. *J. Immunol.* 158: 5321–5329.
24. Macpherson, A. J., and T. Uhr. 2004. Induction of protective IgA by intestinal dendritic cells carrying commensal bacteria. *Science* 303: 1662–1665.
25. Rescigno, M., M. Urbano, B. Valzasina, M. Francolini, G. Rotta, R. Bonasio, F. Granucci, J. P. Kraehenbuhl, and P. Ricciardi-Castagnoli. 2001. Dendritic cells express tight junction proteins and penetrate gut epithelial monolayers to sample bacteria. *Nat. Immunol.* 2: 361–367.
26. Niess, J. H., S. Brand, X. Gu, L. Landsman, S. Jung, B. A. McCormick, J. M. Vyas, M. Boes, H. L. Ploegh, J. G. Fox, et al. 2005. CX3CR1-mediated dendritic cell access to the intestinal lumen and bacterial clearance. *Science* 307: 254–258.
27. Jang, M. H., M. N. Kweon, K. Iwatani, M. Yamamoto, K. Terahara, C. Sasakawa, T. Suzuki, T. Nochi, Y. Yokota, P. D. Rennert, et al. 2004. Intestinal villous M cells: an antigen entry site in the mucosal epithelium. *Proc. Natl. Acad. Sci. USA* 101: 6110–6115.
28. Macpherson, A. J., K. D. McCoy, F. E. Johansen, and P. Brandtzaeg. 2008. The immune geography of IgA induction and function. *Mucosal. Immunol.* 1: 11–22.
29. Sait, L., M. Galic, R. A. Strugnell, and P. H. Janssen. 2003. Secretory antibodies do not affect the composition of the bacterial microbiota in the terminal ileum of 10-week-old mice. *Appl. Environ. Microbiol.* 69: 2100–2109.
30. Wijburg, O. L., T. K. Uren, K. Simpfendorfer, F. E. Johansen, P. Brandtzaeg, and R. A. Strugnell. 2006. Innate secretory antibodies protect against natural *Salmonella typhimurium* infection. *J. Exp. Med.* 203: 21–26.
31. Ivanoff, B., J. L. Tayot, O. Creach, and R. Fontanges. 1978. Experimental cholera in monkeys. *Bull. Soc. Pathol. Exot. Filiales* 71: 240–248.
32. Clemens, J. D., D. A. Sack, J. R. Harris, J. Chakraborty, M. R. Khan, B. F. Stanton, M. Ali, F. Ahmed, M. Yunus, B. A. Kay, et al. 1988. Impact of B subunit killed whole-cell and killed whole-cell-only oral vaccines against cholera upon treated diarrhoeal illness and mortality in an area endemic for cholera. *Lancet* 1: 1375–1379.

OPEN ACCESS Freely available online

PLoS one

A High-Speed Congenic Strategy Using First-Wave Male Germ Cells

Narumi Ogonuki[1], Kimiko Inoue[1,2], Michiko Hirose[1], Ikuo Miura[1], Keiji Mochida[1], Takahiro Sato[3,5], Nathan Mise[1], Kazuyuki Mekada[1], Atsushi Yoshiki[1], Kuniya Abe[1], Hiroki Kurihara[3], Shigeharu Wakana[1], Atsuo Ogura[1,2,4]*

1 RIKEN BioResource Center, Tsukuba, Ibaraki, Japan, 2 Graduate School of Life and Environmental Science, University of Tsukuba, Tsukuba, Ibaraki, Japan, 3 Department of Physiological Chemistry and Metabolism, Graduate School of Medicine, The University of Tokyo, Bunkyo-ku, Tokyo, Japan, 4 Center for Disease Biology and Integrative Medicine, Graduate School of Medicine, The University of Tokyo, Bunkyo-ku, Tokyo, Japan, 5 Tsukuba Safety Assessment Laboratories, Banyu Pharmaceutical Company Limited, Tsukuba, Ibaraki, Japan

Abstract

Background: In laboratory mice and rats, congenic breeding is essential for analyzing the genes of interest on specific genetic backgrounds and for analyzing quantitative trait loci. However, in theory it takes about 3–4 years to achieve a strain carrying about 99% of the recipient genome at the tenth backcrossing (N10). Even with marker-assisted selection, the so-called 'speed congenic strategy', it takes more than a year at N4 or N5.

Methodology/Principal Findings: Here we describe a new high-speed congenic system using round spermatids retrieved from immature males (22–25 days of age). We applied the technique to three genetically modified strains of mice: transgenic (TG), knockin (KI) and *N*-ethyl-*N*-nitrosourea (ENU)-induced mutants. The donor mice had mixed genetic backgrounds of C57BL/6 (B6):DBA/2 or B6:129 strains. At each generation, males used for backcrossing were selected based on polymorphic marker analysis and their round spermatids were injected into B6 strain oocytes. Backcrossing was repeated until N4 or N5. For the TG and ENU-mutant strains, the N5 generation was achieved on days 188 and 190 and the proportion of B6-homozygous loci was 100% (74 markers) and 97.7% (172/176 markers), respectively. For the KI strain, N4 was achieved on day 151, all the 86 markers being B6-homozygous as early as on day 106 at N3. The carrier males at the final generation were all fertile and propagated the modified genes. Thus, three congenic strains were established through rapid generation turnover between 41 and 44 days.

Conclusions/Significance: This new high-speed breeding strategy enables us to produce congenic strains within about half a year. It should provide the fastest protocol for precise definition of the phenotypic effects of genes of interest on desired genetic backgrounds.

Citation: Ogonuki N, Inoue K, Hirose M, Miura I, Mochida K, et al. (2009) A High-Speed Congenic Strategy Using First-Wave Male Germ Cells. PLoS ONE 4(3): e4943. doi:10.1371/journal.pone.0004943

Editor: Simon Melov, Buck Institute for Age Research, United States of America

Received December 11, 2008; **Accepted** February 19, 2009; **Published** March 31, 2009

Funding: This work was supported by KAKENHI (20062012 [A.O.] and 19300151 [A.O.]). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: ogura@rtc.riken.go.jp

Introduction

For nearly 30 years, the genetic manipulation of laboratory mice has contributed substantially to the development of many fields in medical research and mammalian biology. Remarkably, by genetically altering the mouse genome with single nucleotide precision, it is now possible to create mice with desired genetic modifications to assess gene function in healthy animals and in animal models for human diseases. One major issue associated with mouse genetic engineering is that the biological function of engineered genes can vary with their genetic background [1–3]. This often raises serious concerns, because transgenic (TG) or knockout (or knockin, KI) mice are generated in strains that have historically been selected for the ease and convenience of generating the TG or knockout strain, rather than phenotypic characterization of the mutation itself. For example, most embryonic stem (ES) cell lines used for knockout experiments are derived from the 129 strain. Unfortunately, however, this strain has significant biological limitations that interfere with the phenotypic analysis of a target mutation. It consists of a diverse and complex family of substrains [4] and many of these have an atypical brain structure [5]. Therefore, for facilitating definition of transgene or gene-targeted effects over a given genetic background, the engineered gene should be introduced from the donor strain into the desired recipient strain.

The classical protocol for such purpose is congenic breeding: serially backcrossing the gene donor to the recipient strain accompanied by selection for progeny carrying the desired gene in each backcross generation. This protocol calls for 10 backcross generations (N10), followed by an intercross (F1) to produce founders that are homozygous for the desired gene (theoretically more than 99% of the genome) [6]. Although the strategy is