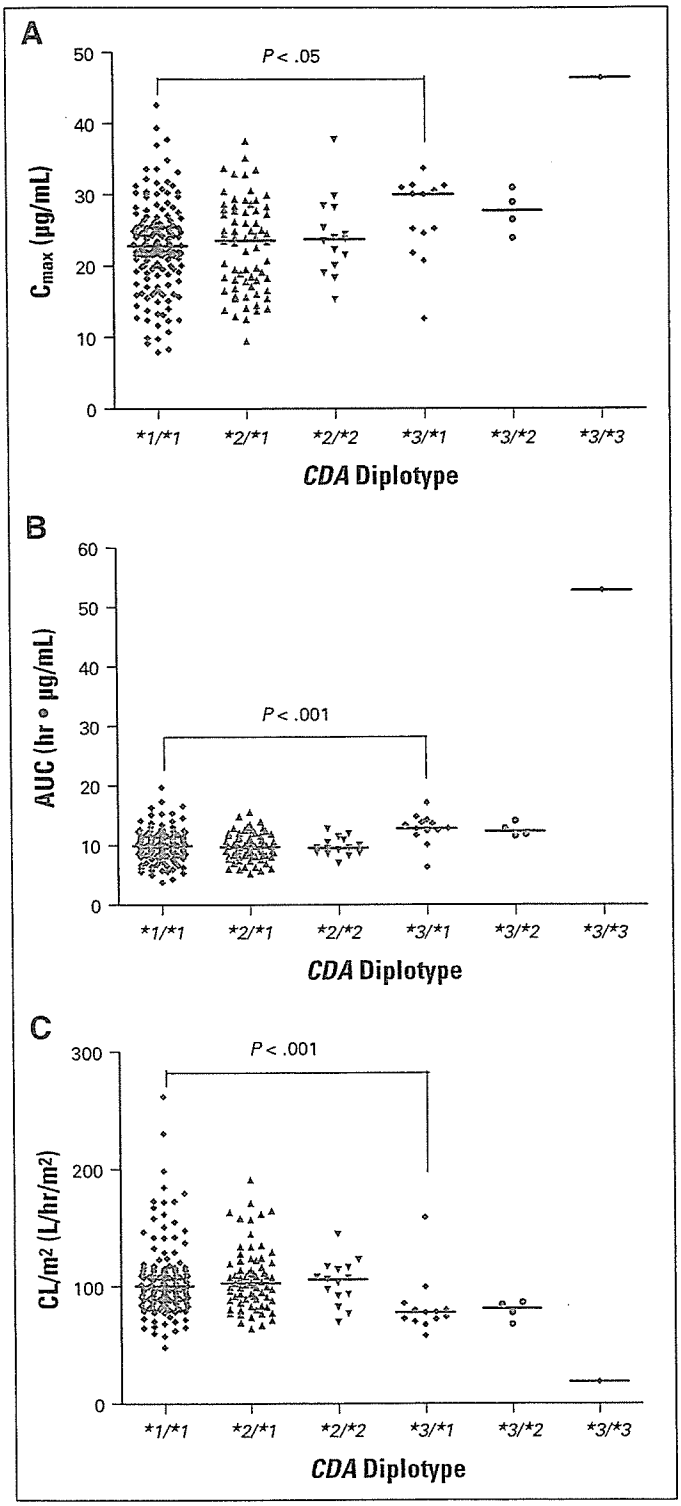

Fig 2. Effects of haplotypes *2 and *3 on the pharmacokinetic parameters of gemcitabine. (A) Peak concentration (C_{max}) and (B) area under the curve (AUC) were corrected assuming that all patients received 1,000 mg/m^2 of gemcitabine. (C) Clearance (CL/m^2). Each point corresponds to an individual patient. The bars denote the median values. *P* values are from Dunn's multiple comparison test.

pharmacokinetics of gemcitabine has not been reported. Plasma CDA activity may be a useful biomarker to screen patients with a markedly decreased metabolic CDA activity such as the patient homozygous for the *3 allele found in our study, who showed extremely low plasma CDA activity. However, a very low contribution of plasma CDA to the total clearance of gemcitabine was reported,[36] and the plasma CDA levels are increased in the inflammatory diseases.[30,40] These may account for the failure in obtaining good correlations between plasma CDA activity and the pharmacokinetic parameters of gemcitabine, as shown in Figure 4.

In conclusion, we analyzed the *CDA* genetic variations and haplotypes in Japanese cancer patients who received gemcitabine. We then investigated the associations between genetic polymorphisms and the pharmacokinetics of gemcitabine or toxicities. Depending on the haplotype *3 harboring 208A, the metabolic clearance of gemcitabine decreased, and AUC and C_{max} values were increased. Moreover, plasma CDA activities correlated well with the *CDA* genotypes. The clinical importance of the SNP 208G>A, especially of homozygotes, should be confirmed by prospective clinical studies because only one homozygous *3 patient was found in this study.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

Although all authors completed the disclosure declaration, the following authors or their immediate family members indicated a financial interest. No conflict exists for drugs or devices used in a study if they are not being evaluated as part of the investigation. For a detailed description of the disclosure categories, or for more information about ASCO's conflict of interest policy, please refer to the Author Disclosure Declaration and the Disclosures of Potential Conflicts of Interest section in Information for Contributors.

Employment: N/A **Leadership:** N/A **Consultant:** N/A **Stock:** N/A **Honoraria:** Nagahiro Saijo, Chugai, AstraZeneca, Bristol-Myers Squibb Co **Research Funds:** Nagahiro Saijo, Bristol-Myers Squibb Co **Testimony:** N/A **Other:** N/A

AUTHOR CONTRIBUTIONS

Conception and design: Nahoko Kaniwa, Shogo Ozawa, Jun-ichi Sawada, Naoyuki Kamatani, Hideki Ueno, Takuji Okusaka, Nagahiro Saijo
Financial support: Jun-ichi Sawada, Teruhiko Yoshida, Nagahiro Saijo
Administrative support: Nahoko Kaniwa, Ryuichi Hasegawa, Yoshiro Saito, Shogo Ozawa, Jun-ichi Sawada, Teruhiko Yoshida, Nagahiro Saijo
Provision of study materials or patients: Keiko Maekawa, Yoshiro Saito, Shogo Ozawa, Junji Furuse, Hiroshi Ishii, Hideki Ueno, Takuji Okusaka
Collection and assembly of data: Emiko Sugiyama, Su-Ryang Kim, Ruri Kikura-Hanajiri, Keiko Maekawa
Data analysis and interpretation: Emiko Sugiyama, Nahoko Kaniwa, Su-Ryang Kim, Yoshiro Saito, Junji Furuse, Hiroshi Ishii, Hideki Ueno, Takuji Okusaka
Manuscript writing: Emiko Sugiyama, Nahoko Kaniwa, Su-Ryang Kim, Hideki Ueno
Final approval of manuscript: Nahoko Kaniwa, Jun-ichi Sawada, Hideki Ueno, Nagahiro Saijo

Table 6. Pharmacokinetic Parameters of Gemcitabine and Plasma CDA Activities in the Patient Groups Categorized According to Diplotypes

Diplotype	Median Gemcitabine PK Parameters				Median CDA Activity (units)		
	No. of Patients	C_{max} (μg/mL)	AUC (hr·μg/mL)	CL/m² (L/hr/m²)	No. of Patients	Gemcitabine	Cytidine
*1/*1	148	22.81	9.96	100.30	63	6.26	5.54
*2/*1	69	23.57	9.71	103.00	25	6.81	5.71
*2/*2	15	23.75	9.57	106.10	14	6.53	6.24
P value*		0.52	0.46	0.99		0.47	0.19
*3/*1	13	30.02	12.83	77.93	13	2.99	3.07
*3/*3	1	46.42	52.86	18.92	1	0.74	1.40
P value†		5.94E-04	6.66E-13	7.77E-04		9.35E-05	2.45E-04

Abbreviations: CDA, cytidine deaminase; C_{max}, peak concentration; AUC, area under the curve; CL/m², clearance.
**P* value of a correlation test among **1/*1*, **1/*2*, and **2/*2*. Multiplicity is adjusted by false-discovery rate.
†*P* value of a correlation test among **1/*1*, **1/*3*, and **3/*3*. Multiplicity is adjusted by false-discovery rate.

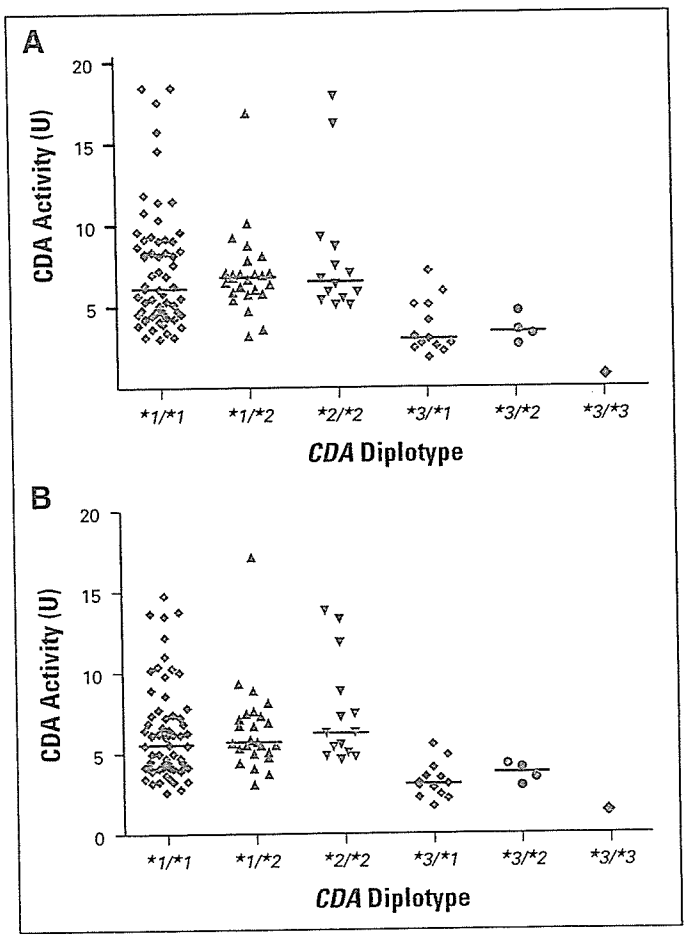

Fig 3. Effects of haplotypes **2* and **3* on plasma cytidine deaminase (CDA) activity toward gemcitabine and cytidine substrates. (A) Gemcitabine was used as a substrate, and (B) cytidine was used as a substrate. Each point corresponds to an individual patient. The bars denote the median values.

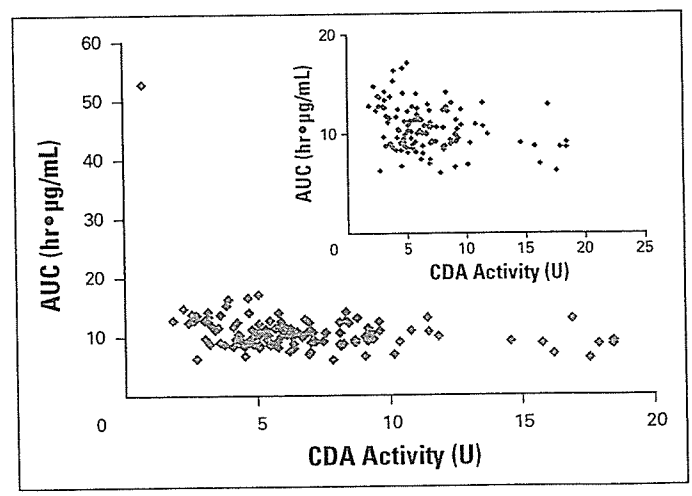

Fig 4. Correlation between plasma area under the curve (AUC) and cytidine deaminase (CDA) activity toward gemcitabine. AUC was corrected assuming that all patients received 1,000 mg/m² of gemcitabine. The inset excludes the data obtained from a homozygous **3* carrier. The correlation coefficient is −0.31 when the homozygous **3* carrier is included and −0.28 when the carrier is excluded.

Table 7. Comparison of Adverse Reaction Incidence and Pharmacokinetic Parameters of Gemcitabine Between Two Patient Groups With and Without Haplotype *3

		Incidence of Neturopenia (nadir)*						
		≥ Grade 3			≥ Grade 4			
Chemotherapy	Genotype	No. of Cases	Total No. of Patients	Probability	No. of Cases	Total No. of Patients	Probability	AUC† (hr·μg/mL)
Monotherapy	*non *3/non *3*	66	167	0.40	8	67	0.05	9.91
	*non *3/*3*	6	10	0.60	1	10	0.10	13.13
	P			0.205			0.514	**0.0017**
With fluorouracil	*non *3/non *3*	3	12	0.25	2	12	0.17	8.11
	*non *3/*3*	2	2	1.00	1	2	0.50	11.98
	P			**0.029**			0.327	0.055
With carboplatin	*non *3/non *3*	9	13	0.69	1	13	0.08	9.87
	*non *3/*3*	3	3	1.00	2	3	0.67	12.48
	P			0.163			**0.033**	**0.031**
With cisplatin	*non *3/non *3*	8	28	0.29	2	28	0.07	9.53
	*non *3/*3*	1	1	1.00	0	1	0.00	11.71
	**3/*3*	1	1	1.00	1	1	1.00	52.86
	P‡			**0.030**			0.128	0.061

Note. No analyses were performed in patients who received gemcitabine with vinorelbine, because only one patient bore the haplotype *3. Boldfacing indicates a statistically significant difference ($P < .05$).
*χ^2-test.
†Kruskal-Wallis test.
‡A *P* value for comparison between *non*3/non*3* and (*non*3/*3* + **3/*3*).

REFERENCES

1. Noble S, Goa KL: Gemcitabine: A review of its pharmacology and clinical potential in non-small cell lung cancer and pancreatic cancer. Drugs 54:447-472, 1997
2. Burris HA III, Moore MJ, Andersen J, et al: Improvements in survival and clinical benefit with gemcitabine as first-line therapy for patients with advanced pancreas cancer: A randomized trial. J Clin Oncol 15:2403-2413, 1997
3. Rauchwerger DR, Firby PS, Hedley DW, et al: Equilibrative-sensitive nucleoside transporter and its role in gemcitabine sensitivity. Cancer Res 60:6075-6079, 2000
4. Mackey JR, Mani RS, Selner M, et al: Functional nucleoside transporters are required for gemcitabine influx and manifestation of toxicity in cancer cell lines. Cancer Res 58:4349-4357, 1998
5. Mackey JR, Yao SY, Smith KM, et al: Gemcitabine transport in xenopus oocytes expressing recombinant plasma membrane mammalian nucleoside transporters. J Natl Cancer Inst 91:1876-1881, 1999
6. Baldwin SA, Yao SY, Hyde RJ, et al: Functional characterization of novel human and mouse equilibrative nucleoside transporters (hENT3 and mENT3) located in intracellular membranes. J Biol Chem 280:15880-15887, 2005
7. Mangravite LM, Badagnani I, Giacomini KM: Nucleoside transporters in the disposition and targeting of nucleoside analogs in the kidney. Eur J Pharmacol 479:269-281, 2003
8. Ritzel MW, Ng AM, Yao SY, et al: Molecular identification and characterization of novel human and mouse concentrative Na+-nucleoside cotransporter proteins (hCNT3 and mCNT3) broadly selective for purine and pyrimidine nucleosides (system cib). J Biol Chem 276:2914-2927, 2001
9. Plunkett W, Huang P, Gandhi V: Preclinical characteristics of gemcitabine. Anticancer Drugs 6:S7-S13, 1995 (suppl 6)
10. Plunkett W, Huang P, Searcy CE, et al: Gemcitabine: Preclinical pharmacology and mechanisms of action. Semin Oncol 23:S3-S15, 1996 (suppl 10)
11. Heinemann V, Xu YZ, Chubb S, et al: Inhibition of ribonucleotide reduction in CCRF-CEM cells by 2',2'-difluorodeoxycytidine. Mol Pharmacol 38:567-572, 1990
12. Kiani A, Kohne CH, Franz T, et al: Pharmacokinetics of gemcitabine in a patient with end-stage renal disease: Effective clearance of its main metabolite by standard hemodialysis treatment. Cancer Chemother Pharmacol 51:266-270, 2003
13. Watanabe S, Uchida T: Expression of cytidine deaminase in human solid tumors and its regulation by 1 alpha, 2, 5-dihydroxyvitamin D3. Biochim Biophys Acta 1312:99-104, 1996
14. Ho DH: Distribution of kinase and deaminase of 1-beta-D-arabinofuranosylcytosine in tissues of man and mouse. Cancer Res 33:2816-2820, 1973
15. Kirch HC, Schroder J, Hoppe H, et al: Recombinant gene products of two natural variants of the human cytidine deaminase gene confer different deamination rates of cytarabine in vitro. Exp Hematol 26:421-425, 1998
16. Schroder JK, Kirch C, Seeber S, et al: Structural and functional analysis of the cytidine deaminase gene in patients with acute myeloid leukaemia. Br J Haematol 103:1096-1103, 1998
17. Gilbert JA, Salavaggione OE, Ji Y, et al: Gemcitabine pharmacogenomics: Cytidine deaminase and deoxycytidylate deaminase gene resequencing and functional genomics. Clin Cancer Res 12:1794-1803, 2006
18. Yue L, Saikawa Y, Ota K, et al: A functional single-nucleotide polymorphism in the human cytidine deaminase gene contributing to ara-C sensitivity. Pharmacogenetics 13:29-38, 2003
19. Aapro MS, Martin C, Hatty S: Gemcitabine: A safety review. Anticancer Drugs 9:191-201, 1998
20. Gallelli L, Nardi M, Prantera T, et al: Retrospective analysis of adverse drug reactions induced by gemcitabine treatment in patients with non-small cell lung cancer. Pharmacol Res 49:259-263, 2004
21. Bokemeyer C, Gerl A, Schoffski P, et al: Gemcitabine in patients with relapsed or cisplatin-refractory testicular cancer. J Clin Oncol 17:512-516, 1999
22. Locker GJ, Wenzel C, Schmidinger M, et al: Unexpected severe myelotoxicity of gemcitabine in pretreated breast cancer patients. Anticancer Drugs 12:209-212, 2001
23. Yonemori K, Ueno H, Okusaka T, et al: Severe drug toxicity associated with a single-nucleotide polymorphism of the cytidine deaminase gene in a Japanese cancer patient treated with gemcitabine plus cisplatin. Clin Cancer Res 11:2620-2624, 2005
24. Colly LP, Peters WG, Richel D, et al: Deoxycytidine kinase and deoxycytidine deaminase values correspond closely to clinical response to cytosine arabinoside remission induction therapy in patients with acute myelogenous leukemia. Semin Oncol 14:S257-S261, 1987 (suppl 1)
25. Steuart CD, Burke PJ: Cytidine deaminase and the development of resistance to arabinosyl cytosine. Nat New Biol 233:109-110, 1971
26. Tattersall MH, Ganeshaguru K, Hoffbrand AV: Mechanisms of resistance of human acute leukaemia cells to cytosine arabinoside. Br J Haematol 27:39-46, 1974
27. Chiba P, Tihan T, Szekeres T, et al: Concordant changes of pyrimidine metabolism in blasts of two cases of acute myeloid leukemia after repeated treatment with ara-C in vivo. Leukemia 4:761-765, 1990
28. Nakamura T, Saito Y, Murayama N, et al: Apparent low frequency of sequence variability within the proximal promoter region of the cytochrome P450(CYP)3A5 gene in established cell lines from Japanese individuals. Biol Pharm Bull 24:954-957, 2001
29. Kitamura Y, Moriguchi M, Kaneko H, et al: Determination of probability distribution of diplotype configuration (diplotype distribution) for each subject from genotypic data using the EM algorithm. Ann Hum Genet 66:183-193, 2002

30. Richards DA, Sherwood RA, Ndebele D, et al: Determination of plasma cytidine deaminase activity by HPLC. Biomed Chromatogr 2:148-151, 1987

31. Benjamini Y, Hochberg Y: Controlling the false discovery rate: A practical and powerful approach to multiple testing. J R Statist Soc Ser B 57:289-300, 1995

32. Bhargava P, Marshall JL, Fried K, et al: Phase I and pharmacokinetic study of two sequences of gemcitabine and docetaxel administered weekly to patients with advanced cancer. Cancer Chemother Pharmacol 48:95-103, 2001

33. Johansson E, Mejlhede N, Neuhard J, et al: Crystal structure of the tetrameric cytidine deaminase from Bacillus subtilis at 2.0: A resolution. Biochemistry 41:2563-2570, 2002

34. Costanzi S, Vincenzetti S, Vita A, et al: Human cytidine deaminase: Understanding the catalytic mechanism. Nucleosides Nucleotides Nucleic Acids 22:1539-1543, 2003

35. Kreis W, Lesser M, Budman DR, et al: Phenotypic analysis of 1-B-D-arabinofuranosylcytosine deamination in patients treated with high doses and correlation with response. Cancer Chemother Pharmacol 30:126-130, 1992

36. Abbruzzese JL, Grunewald R, Weeks EA, et al: A phase I clinical, plasma, and cellular pharmacology study of gemcitabine. J Clin Oncol 9:491-498, 1991

37. Nishida M: Pharmacological and clinical properties of Xeloda (capecitabine), a new oral active derivative of fluoropyrimidine [Japanese]. Nippon Yakurigaku Zasshi 122:549-553, 2003

38. Fukunaga AK, Marsh S, Murry DJ, et al: Identification and analysis of single nucleotide polymorphisms in the gemcitabine pharmacologic pathway. Pharmacogenomics J 4:307-314, 2004

39. Jahns-Streubel G, Reuter C, Auf der Landwehr U, et al: Activity of thymidine kinase and of polymerase alpha as well as activity and gene expression of deoxycytidine deaminase in leukemic blasts are correlated with clinical response in the setting of granulocyte-macrophage colony-stimulating factor-based priming before and during TAD-9 induction therapy in acute myeloid leukemia. Blood 90: 1968-1976, 1997

40. Sherwood RA: The measurement of nucleoside deaminases by high performance liquid chromatography and their use in clinical chemistry. Biomed Chromatogr 5:235-239, 1991

Acknowledgment

We thank Emiko Jimbo, Miho Akimoto, Atsuko Watanabe, Tomoko Chujo, Makiyo Iwamoto, and Mamiko Shimada for assistance in sample collection and management, and Chie Sudo for secretarial assistance.

Regulatory Roles of NKT Cells in the Induction and Maintenance of Cyclophosphamide-Induced Tolerance[1]

Toshiro Iwai,* Yukihiro Tomita,[2]* Shinji Okano,† Ichiro Shimizu,* Yohichi Yasunami,‡ Takashi Kajiwara,* Yasunobu Yoshikai,§ Masaru Taniguchi,‖ Kikuo Nomoto,¶ and Hisataka Yasui*

We have previously reported the sequential mechanisms of cyclophosphamide (CP)-induced tolerance. Permanent acceptance of donor skin graft is readily induced in the MHC-matched and minor Ag-mismatched recipients after treatment with donor spleen cells and CP. In the present study, we have elucidated the roles of NKT cells in CP-induced skin allograft tolerance. BALB/c AnNCrj (H-2^d, Lyt-1.2, and Mls-1^b) wild-type (WT) mice or Vα14 NKT knockout (KO) (BALB/c) mice were used as recipients, and DBA/2 NCrj (H-2^d, Lyt-1.1, and Mls-1^a) mice were used as donors. Recipient mice were primed with 1×10^8 donor SC i.v. on day 0, followed by 200 mg/kg CP i.p. on day 2. Donor mixed chimerism and permanent acceptance of donor skin allografts were observed in the WT recipients. However, donor skin allografts were rejected in NKT KO recipient mice. In addition, the donor reactive Vβ6$^+$ T cells were observed in the thymus of a NKT KO recipient. Reconstruction of NKT cells from WT mice restored the acceptance of donor skin allografts. In addition, donor grafts were partially accepted in the thymectomized NKT KO recipient mice. Furthermore, the tolerogen-specific suppressor cell was observed in thymectomized NKT KO recipient mice, suggesting the generation of regulatory T cells in the absence of NTK cells. Our results suggest that NKT cells are essential for CP-induced tolerance and may have a role in the establishment of mixed chimerism, resulting in clonal deletion of donor-reactive T cells in the recipient thymus. *The Journal of Immunology,* **2006, 177: 8400–8409.**

Natural killer T cells, which are characterized by coexpression of NK cell receptors and a single invariant T cell Ag receptor encoded by *Vα14* and *Jα281* gene segments, have been identified as a novel lymphoid lineage distinct from conventional T cells or NK cells. Although the physiological roles of NKT cells remain obscure, Vα14 NKT cells have been demonstrated to play important roles in tumor immunity (1), autoimmune disease (2), and infectious immunity (3, 4) via the dominant production of Th1 cytokine γ-IFN and Th2 cytokine IL-4. Regarding transplantation immunity, two reports have suggested a regulatory role of NKT cells in both allogeneic and xenogeneic tolerance systems induced by mAbs (5, 6).

Since 1982, we have investigated cyclophosphamide (CP)[3]-induced tolerance that consists of an i.v. injection of 1×10^8 allogeneic spleen cells (SC) (day 0) followed by i.p. administration of 200 mg/kg CP on day 2 (7–18). By using this method, we were able to readily induce long-lasting skin allograft tolerance in most H-2-matched combinations (10–12), but not in fully H-2-mismatched combinations (7, 13). Our previous studies have elucidated the three major mechanisms involved using H-2-compatible, Mls-1^a-disparate combinations and Mls-1^a Ag-reactive Vβ6$^+$ T cells (11–14). The first is the destruction of Ag-stimulated and then proliferating T cells in the periphery by CP treatment. CD4$^+$Vβ6$^+$ T cells proliferated and then disappeared in the periphery of the recipients tolerized to H-2-compatible, Mls-1^a-disparate Ags. The second, at 4–6 wk after the treatments, is the establishment of intrathymic chimerism at both the thymocyte and dendritic cell levels, followed by the clonal deletion of Vβ6$^+$ T cells that begins in the thymus. The third mechanism is the generation of regulatory cells in the late stage of tolerance.

The aim of the present study was to investigate the regulatory role of NKT cells in our CP-induced tolerance system by using Vα14 NKT knockout (KO) mice. Although an essential role for NKT cells in the induction of transplantation tolerance has been suggested in two previous reports (5, 6), the detailed mechanisms have not been clarified. Here, we evaluated the role of NKT cells in our three important mechanisms, i.e., clonal destruction, intrathymic clonal deletion, and generation of regulatory cells. The results clearly showed that NKT cells were essential for CP-induced tolerance through the establishment of intrathymic clonal deletion. Without NKT cell-mediated immunoregulation, however, our results demonstrated that the generation of regulatory cells for the maintenance of tolerance in the late stage of tolerance can occur, in addition to clonal destruction at the early stage.

Materials and Methods

Animals

Inbred mice of the BALB/c AnNCrj (H-2^d, Lyt-1.2, and Mls-1^b) and DBA/2 NCrj (H-2^d, Lyt-1.1, and Mls-1^a) strains were obtained from

*Department of Cardiovascular Surgery and †Division of Pathophysiological and Experimental Pathology, Department of Pathology, Graduate School of Medical Sciencies, Kyushu University, Fukuoka, Japan; ‡Department of Surgery I, Fukuoka University School of Medicine, Fukuoka, Japan; §Department of Infection Control and ¶Department of Immunology, Medical Institute of Bioregulation, Kyushu University, Fukuoka, Japan; and ‖Laboratory for Immune Regulation, RIKEN Research Center for Allergy and Immunology, Yokohama, Japan

Received for publication March 18, 2004. Accepted for publication September 22, 2006.

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 study was supported by a Grant-in-Aid for Scientific Research from the Ministry of Health and Welfare, Japan (to Y.T.). Y.T. was also the recipient of a Surgical Research Foundation Grant from the Japanese Surgical Association.

[2] Address correspondence and reprint requests to Dr. Yukihiro Tomita, Department of Cardiovascular Surgery, Graduate School of Medical Sciences, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan. E-mail address: tomita@heart.med.kyushu-u.ac.jp

[3] Abbreviations used in this paper: CP, cyclophosphamide; αGalCer, α-galactosyl ceramide; BMC, bone marrow cell; Gy, gray; KO, knockout; LMNC, liver mononuclear cell; MST, mean survival time; SC, spleen cell; WBC, white blood cell; WT, wild type.

0022-1767/06/$02.00

Charles River Laboratories. Inbred mice of the B10.D2 SnSlc ($H\text{-}2^d$) strain were obtained from Japan SLC. Jα281 KO (Vα14 NKT KO) mice with a BALB/c background were also used as recipients (1). The recipients were used at 12–16 wk of age. All animals received humane care in compliance with the Guidelines for Animal Experiments of Kyushu University and Law no. 105 and Notification no. 6 of the Japanese government.

Cell preparation

Mice were sacrificed by decapitation. The spleens were collected and kept on ice in RPMI 1640 medium (Invitrogen Life Technologies) supplemented with antibiotics (100 μg/ml penicillin and 100 μg/ml streptomycin). Spleens were disrupted in the medium by pressing spleen fragments between two glass slides. Cell suspensions were filtered through cotton gauze and washed three times with the RPMI 1640 medium. Viable nucleated cells were counted and usually adjusted to 20×10^7/ml.

Conditioning of CP-induced tolerance

A 0.5-ml aliquot containing 1×10^8 SC from DBA/2 mice was injected into the tail vein of recipient BALB/c mice. Two days later, CP (Endoxan; Shionogi) dissolved in PBS at a concentration of 10 mg/ml was injected i.p. at a dose of 200 mg/kg. The day of the injection of DBA/2 SC is referred to as day 0 throughout this report.

Reconstitution of NKT cells in NKT KO mice

We set up two methods to reconstitute NKT cells in NKT KO mice. First, a 0.5-ml aliquot containing 1×10^8 SC from WT mice (containing ~1% NKT cells) was injected into the tail vein of recipient NKT KO mice on day −7. Second, recipient NKT KO mice were irradiated with three gray (Gy) on day −28 and then reconstituted with 1×10^7 SC and 5×10^6 untreated bone marrow cells (BMC) (containing ~0.1–0.4% NKT cells) from WT mice on the same day. The preparation of BMC was performed according to a previous method (19). Briefly, the bone marrow in the femoral and tibial bones was flushed out using a 5-ml syringe with a 26-gauge needle (Terumo).

Skin grafting

Skin grafting was performed using our previously reported procedure (20). Briefly, a square, full-thickness skin graft (1 cm^2) was prepared on the right lateral thoracic wall of the recipient mouse. The graft was fixed to the graft bed with eight interrupted sutures of 5-0 silk thread and covered with protective tape. The first inspection was conducted on the 7th day, followed by daily inspection for 3 wk. Grafts were considered as rejected at the time of complete sloughing or when they formed a dry scar. Survival was expressed as the median survival time and the mean survival time (MST) ± SD.

Thymectomy

Recipients were anesthetized with phenobarbital (Nembutal) at 50 mg/kg administered i.p. After a partial sternotomy, the thymectomy was performed by en bloc excision using two pairs of forceps (21). The absence of thymic tissue was always confirmed when the thymectomized animals were sacrificed, and animals showing the presence of residual thymic tissue were excluded from the analysis.

Flow cytometry

Phenotyping was performed at various times, beginning 2 wk after the injection of SC. Recipients were tail bled and white blood cells (WBC) were prepared by hypotonic shock (21). In some experiments, SC and thymocytes were used for chimeric assays. Staining with both donor-specific and T cell-specific mAbs was performed on each recipient and control mouse. Cells were incubated with a PE-conjugated anti-Lyt-1 (Lyt-1.1 and Lyt-1.2) (BD Pharmingen) mAb and a FITC-conjugated Lyt-1.1 (BD Pharmingen) mAb for 30 min at 4°C and then washed twice. To block nonspecific FcγR binding of labeled Abs, 10 μl of an undiluted culture supernatant of 2.4G2 (rat anti-mouse FcγR mAb) was used. All data were analyzed with a FACScan (BD Biosciences). Dead cells were excluded by gating out low forward scatter, high propidium iodide-retaining cells.

For the analysis of TCR expression on T cells of SC or WBC, two-color analysis was performed (21). WBC or SC were labeled with FITC-conjugated anti-Vβ6 or Vβ8.1/8.2 mAb (BD Pharmingen), and PE-conjugated anti-CD4 (BD Pharmingen) mAb. To determine the percentage of $CD4^+$ T cells that were $V\beta6^+$ or $V\beta8.1/8.2^+$, 10,000–20,000 gated $CD4^+$ cells were collected. For the analysis of TCR expression on thymocytes, three-color analysis was performed (21). Thymocytes were labeled with FITC-conjugated anti-Vβ6 or Vβ8.1/8.2 mAb (BD Pharmingen), PE-conjugated anti-CD4 (BD Pharmingen) mAb, and allophycocyanin-conjugated anti-CD8 (BD Pharmingen) mAb for 30 min at 4°C. To determine the percentage of CD4 single-positive cells that were $V\beta6^+$ or $V\beta8.1/8.2^+$, 5,000 to 10,000 gated $CD4^+$ and $CD8^-$ cells were collected. We investigated the effect of SC/CP on the ratio of $CD4^+V\beta6^+$ T cell or $CD4^+V\beta8^+$ T cell subsets to the total $CD4^+$ T cell number in the spleen or WBC and on the ratio of $CD4^+CD8^-V\beta6^+$ T cell or $CD4^+CD8^-V\beta8^+$ T cell subsets to the total $CD4^+CD8^-$ T cell number in the thymus. We also investigated the effect of SC/CP on the absolute number of $CD4^+V\beta6^+$ T cells or $CD4^+V\beta8^+$ T cells in the spleen and thymus.

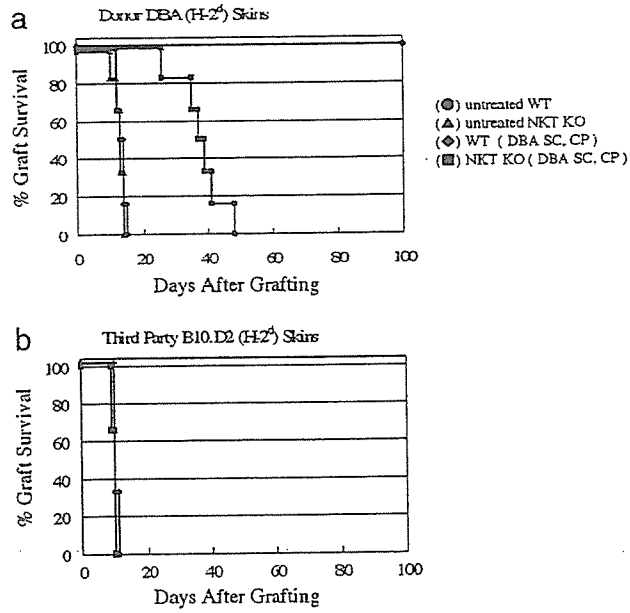

FIGURE 1. Skin allograft survival in the recipient BALB/c mice treated with DBA/2 SC. Recipient mice were grafted with skin from donor DBA/2 (DBA) (*a*) or third party B10.D2 (*b*) mice 4 wk after treatment. *a*, The groups and median skin graft survival times were as follows: ●, Untreated WT mice (n = 6; 13.5 days); ▲, untreated NKT KO mice (n = 6; 13 days); ◆, WT mice treated with DBA/2 SC and CP (n = 6; >100 days); and ■, NKT KO mice treated with DBA/2 SC and CP (n = 6; 38 days). *b*, B10.D2 skin grafts were rejected within 14 days after grafting in the following groups: ◆, WT mice treated with DBA/2 SC and CP (n = 3); ■, NKT KO mice treated with DBA/2 SC and CP (n = 3).

For the staining of NKT cells, SC or liver mononuclear cells (LMNC) were stained with PE-conjugated α-galactosyl ceramide (αGalCer)/CD1d tetramers and FITC-conjugated anti-CD3 mAb (BD Pharmingen). PE-conjugated αGalCer/CD1d tetramers were prepared as previously described (22). The liver was disrupted in RPMI 1640 medium (Invitrogen Life Technologies) supplemented with 10% FCS by pressing liver fragments between two glass slides and then washed, resuspended in a 40% isotonic Percoll solution (Amersham Biosciences) and underlaid with a 67.5% isotonic Percoll solution. Centrifugation for 30 min at 3 000 rpm at room temperature isolated the LMNC at the interface. Cells were washed two times with HBSS containing 2% FCS and resuspended in the same solution.

Adoptive transfer experiment

To elucidate the existence of regulatory cells in the tolerant recipients, adoptive transfer experiments were performed as described previously (14). Briefly, 1×10^8 or 4×10^7 SC from the recipient mice accepting DBA/2 skin allografts for over 100 days were transferred into WT mice that had been irradiated with 3 Gy on the same day. The SC were harvested from WT or NKT mice that had been thymectomized and treated with DBA/2 SC and CP. Skin grafting was performed 1 day following the adoptive transfer. In one experiment, $CD4^+CD8^+Thy1.2^+$ T cell depletion was performed using anti-CD4 mAb (L3/T4), anti-CD8 mAb (Ly2.2) (Cedarlane Laboratories), anti-Thy-1.2 mAb (Meiji), and complement (Low-Tox-M rabbit complement; Cedarlane Laboratories).

Table I. *Chimerism and clonal destruction in WBC of recipients treated with DBA/2 SC and CP*[a]

Group	Recipient	Treatment[a] SC (day 0)	Treatment[a] CP (day 2)	No. of Mice	Chimeric Analysis (percent positive cells ± SD) Lyt-1.1$^+$/Lyt-1$^+$ (%) .2 wk	Lyt-1.1$^+$/Lyt-1$^+$ (%) 8 wk	Analysis of TCR Expression (percent positive cells ± SD) CD4$^+$Vβ6$^+$/CD4$^+$ (%) 3 wk	CD4$^+$Vβ6$^+$/CD4$^+$ (%) 9 wk	CD4$^+$Vβ6$^+$/CD4$^+$ (%) 3 wk	CD4$^+$Vβ6$^+$/CD4$^+$ (%) 9 wk
1	BALB/c WT	(−)	(−)	6	0		10.7 ± 1.2		16.6 ± 1.6	
2	BALB/c NKT KO	(−)	(−)	6	0		11.3 ± 1.4		12.2 ± 1.7	
3	DBA/2	(−)	(−)	6	96.3 ± 2.4		0		13.0 ± 1.1	
4	BALB/c WT	DBA/2	200[b]	6	2.6 ± 0.8[c]	3.8 ± 1.0[c]	1.6 ± 0.5	1.1 ± 0.4	17.1 ± 1.9	16.6 ± 2.0
5	BALB/c NKT KO	DBA/2	200[b]	6	1.5 ± 0.1	0.9 ± 0.2	1.3 ± 0.3	0.8 ± 0.2	12.9 ± 1.2	2.6 ± 1.7

[a] The recipient mice were primed i.v. with 1 × 10^8 viable DBA/2 SC on day 0 and then given 200 mg/kg CP on day 2.
[b] Milligrams per kilogram (mg/kg).
[c] $p < 0.01$ compared with group 5.

Statistics

The statistical significance of the data was determined by a Mann-Whitney *U* test when the data were nonparametric or a Student's *t* test when the data were parametric. A value of $p < 0.05$ was considered to be statistically significant.

Results

Skin allograft prolongation in H-2-matched DBA/2 (H-2^d) → BALB/c WT (H-2^d) or BALB/c background Vα14 NKT KO (H-2^d) combination mice by using 1 × 10^8 DBA/2 SC followed by 200 mg/kg CP

When BALB/c WT (H-2^d) or BALB/c background NKT KO mice were grafted with H-2-matched DBA/2 skin allografts (H-2^d), the DBA/2 grafts were rejected within 14 days following grafting (Fig. 1*a*). Similarly, DBA/2 skin grafts were rejected within 14 days in BALB/c WT or NKT KO mice treated with DBA/2 SC alone or 200 mg/kg CP alone (data not shown). All of the DBA/2 skin allografts survived for >100 days in the recipient BALB/c WT mice treated with DBA/2 SC followed by CP (n = 6; MST, >100 days). When syngeneic (BALB/c) WT SC or PBS (0.5 ml) was used instead of DBA/2 SC or CP, respectively, the survival times of DBA/2 skin grafts were not prolonged (data not shown). In contrast, all DBA/2 skin grafts were rejected within 48 days in the recipient NKT KO mice treated with DBA/2 SC followed by CP (n = 6; MST, 38 days), although the survival of the grafts was moderately prolonged. The skin allograft prolongation in both BALB/c WT mice and NKT KO mice, which were treated with DBA/2 SC followed by CP, was tolerogen-specific, because the third party skin grafts of the B10.D2 strain (H-2^d) were rejected in a normal fashion (Fig. 1*b*).

Chimerism and reduction of Mls-1^a-reactive CD4$^+$Vβ6$^+$ T cells of WBC in the recipient mice treated with DBA/2 SC plus CP

As we previously reported (14), a minimal degree of mixed chimerism was detected in the BALB/c WT (Lyt-1.2) mice made tolerant of DBA/2 (Lyt-1.1) skin allografts. The mixed chimeric state induced with DBA/2 SC and CP was examined using

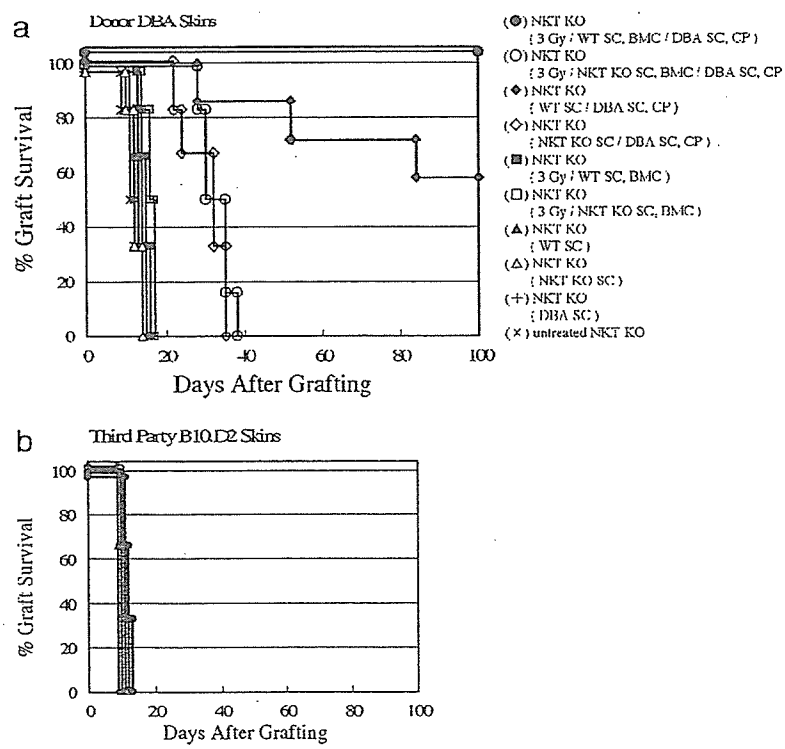

FIGURE 2. Skin allograft survival in recipient BALB/c NKT KO mice reconstituted with NKT cells and treated with DBA/2 SC and CP. Recipient mice were grafted with skin from donor DBA/2 (DBA) (*a*) or third party B10.D2 (*b*) mice 4 wk after treatment. *a* The groups and median skin graft survival times were as follows: ●, NKT KO mice irradiated with 3 Gy followed by reconstitution with WT SC and BMC and treatment with DBA/2 SC and CP (n = 6; >100 days); ○, NKT KO mice irradiated with 3 Gy followed by reconstitution with NKT KO SC and BMC and treatment with DBA/2 SC and CP (n = 6; 32.5 days); ◆, NKT KO mice reconstituted with WT SC and treated with DBA/2 SC and CP (n = 7; >100 days); ◇, NKT KO mice reconstituted with NKT KO SC and treated with DBA/2 SC and CP (n = 6; 32 days); ■, NKT KO mice irradiated with 3 Gy followed by reconstitution with WT SC and BMC (n = 6; 15 days); □, NKT KO mice irradiated with 3 Gy followed by reconstitution with NKT KO SC and BMC (n = 6; 16.5 days); ▲, NKT KO mice reconstituted with WT SC (n = 6; 13 days); △, NKT KO mice reconstituted with NKT KO SC (n = 6; 12 days); +, NKT KO mice treated with DBA/2 SC alone (n = 6; 14 days); and ×, untreated NKT KO mice (n = 6; 11.5 days). *b*, B10.BR skin grafts were rejected within 14 days after grafting in all of the groups described above in *a* (n = 3 in each group).

PE-conjugated anti-Lyt-1 (Lyt-1.1 and Lyt-1.2) mAb and FITC-conjugated Lyt-1.1 mAb. WBC were obtained from the recipient mice at 2 and 8 wk after tolerance induction (Table I).

In the T (Lyt-1$^+$) cells of BALB/c WT mice treated with DBA/2 SC and CP (Table I; group 4), 2–4% of Lyt-1.1 cells were clearly detected in the recipient WBC after tolerance induction. In contrast, a lower degree of chimerism was clearly detected at 2 wk (mean ± SD, 1.5 ± 0.1; $p < 0.01$ compared with group 4) and became <1% at 8 wk in the T (Lyt-1$^+$) cells of NKT KO mice treated with DBA/2 SC followed by CP (Table I; group 5). A higher degree of chimerism was always observed in recipient BALB/c WT mice treated with DBA/2 SC and CP. These results were reproducible in five independent experiments (data not shown).

We examined the expression of the Mls-1^a-reactive TCR Vβ6 in BALB/c WT or NKT KO (Mls-1^b) mice treated with DBA/2 (Mls-1^a) SC and CP. The WBC from the recipients were stained with FITC-conjugated anti-Vβ6 mAb and PE-conjugated anti-CD4 mAb (Table I).

In the WBC of untreated BALB/c WT or NKT KO mice, CD4$^+$Vβ6$^+$ T cells were detected (Table I; group 1 or 2, respectively), whereas they were hardly detected in the WBC of untreated DBA/2 mice (Table I; group 3). In all of the BALB/c WT mice treated with DBA/2 SC and CP (Table I; group 4), CD4$^+$Vβ6$^+$ T cells were significantly reduced by 3 wk. The same results were obtained in the WBC of NKT KO mice treated with DBA/2 SC and CP (Table I; group 5). There was no statistically significant difference in the results between groups 4 and 5. The disappearance of T cells from the WBC was specific for Vβ6$^+$ T cells, because the percentage of Vβ8.1/8.2$^+$ T cells was not significantly altered.

Induction of DBA/2 skin graft prolongation in NKT KO mice reconstituted with NKT cells from BALB/c WT mice

To clarify whether NKT cells were involved in the limitation of skin graft tolerance in CP-induced tolerance, NKT cells were reconstituted in NKT KO mice (Fig. 2). When SC and LMNC were stained with PE-conjugated αGalCer/CD1d tetramers and FITC-conjugated anti-CD3 mAb, αGalCer/CD1d tetramer$^+$CD3$^+$ cells accounted for ~1.0 ± 0.3 and 19.5 ± 5.4% of SC and LMNC in untreated BALB/c WT mice ($n = 3$), respectively, and 0.3 ± 0.1 and 1.2 ± 0.2% of SC and LMNC in untreated NKT KO mice ($n = 3$), respectively. A small percentage of αGalCer/CD1d tetramer$^+$CD3$^+$ cells were detected in NKT KO mice, because the NKT KO mice used in this study were generated by disruption of the *Jα281* gene (1). In contrast, αGalCer/CD1d tetramer$^+$CD3$^+$ cells accounted for ~0.4 ± 0.1 and 4.3 ± 0.5% in SC and LMNC of NKT KO mice ($n = 3$) injected with BALB/c WT SC 7 days earlier, respectively. Therefore, we planned an additional experiment to further reconstitute NKT cells in NKT KO mice. For this purpose, recipient NKT KO mice were irradiated with 3 Gy on day −28 and then injected with 1×10^7 SC and 5×10^6 untreated BMC from WT mice on the same day. In NKT KO mice ($n = 5$) irradiated and injected with BALB/c WT SC and BMC 28 days earlier, αGalCer/CD1d tetramer$^+$CD3$^+$ cells accounted for ~0.7 ± 0.1 and 9.5 ± 2.6% of SC and LMNC, respectively. When NKT KO mice were injected with 1×10^8 SC from BALB/c WT mice on day −7 and treated with SC on day 0 and CP on day 2, the survival of DBA/2 skin grafts was significantly prolonged ($n = 7$; MST, >100 days), and four of seven recipients accepted donor DBA/2 skin grafts for >100 days (Fig. 2*a*). DBA/2 skin grafts were accepted for >100 days in all of the NKT KO mice irradiated with 3 Gy on day −28, reconstituted with 1×10^7 SC and 5×10^6 BMC from BALB/c WT mice on day −28, and then treated with DBA/2 SC on day 0 and CP on day 2 (Fig. 2*a*). Survival of donor skin grafts was not significantly prolonged in NKT KO mice reconstituted with SC and/or BMC from NKT KO mice and treated with DBA/2 SC and CP as compared with that for NKT KO mice treated with DBA/2 SC and CP. In contrast, no skin graft prolongation was observed in NKT KO mice reconstituted with BALB/c WT SC or BMC, irradiated NKT KO mice reconstituted with BALB/c WT SC and BMC, NKT KO mice reconstituted with NKT KO SC or BMC, or irradiated NKT KO mice reconstituted with NKT SC and BMC if the recipient mice were not treated with donor SC and CP (Fig. 2*a*). This skin allograft prolongation was tolerogen-specific, because the third party skin of the B10.D2 strain (H-2^d) was rejected in a normal fashion (Fig. 2*b*).

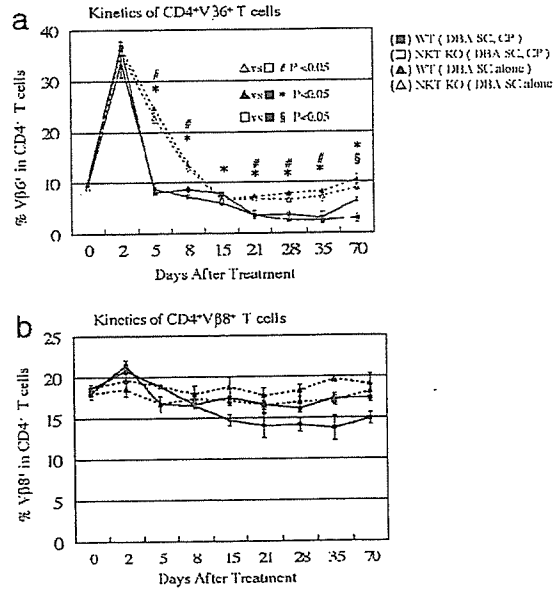

FIGURE 3. Clonal destruction in the periphery of recipient mice. The kinetics of CD4$^+$Vβ6$^+$ (*a*) or CD4$^+$Vβ8.1/8.2$^+$ (*b*) T cells in spleen cells harvested from the recipient mice are shown. SC were labeled with FITC-conjugated anti-Vβ6 or Vβ8.1/8.2 mAb and PE-conjugated anti-CD4 mAb. To determine the percentage of CD4$^+$ T cells that were Vβ6$^+$ or Vβ8.1/8.2$^+$, 10,000–20,000 gated CD4$^+$ cells were collected. SC cells were obtained from WT (H-2^d; Mls-1^b) mice treated with DBA/2 (DBA) (H-2^d; Mls-1^a) SC and CP (■; $n = 4$), NKT KO mice treated with DBA/2 SC and CP (□; $n = 4$), WT mice treated with DBA/2 SC alone (▲; $n = 4$), and NKT KO mice treated with DBA/2 SC alone (△; $n = 4$). Vertical bars represent the SD. The statistical significance of the differences among groups was analyzed and the results are given in *a*.

Analysis of splenic clonal destruction and intrathymic clonal deletion and mixed chimerism in BALB/c WT or NKT KO mice treated with DBA/2 SC and CP

As reported previously (12, 13), the induction mechanism of CP-induced tolerance is the clonal destruction of Ag-stimulated and proliferating T cells by the antimitotic drug CP. To further analyze the role of NKT cells in the tolerance induction, we examined the kinetics of Mls-1^a-reactive CD4$^+$Vβ6$^+$ T cells in the CD4$^+$ T cells of SC in recipient BALB/c WT or NKT KO mice. When DBA/2 SC were injected into untreated BALB/c WT mice on day 0, CD4$^+$Vβ6$^+$ T cells significantly increased to ~35% on day 2 and then eventually declined to the normal range by days 15–21 (Fig. 3*a*). The same result was observed in NKT KO mice. In BALB/c WT mice treated with DBA/2 SC on day 0 and CP on day 2, CD4$^+$Vβ6$^+$ T cells significantly increased to ~35% on day 2, rapidly decreased to the normal range on day 5, and then gradually

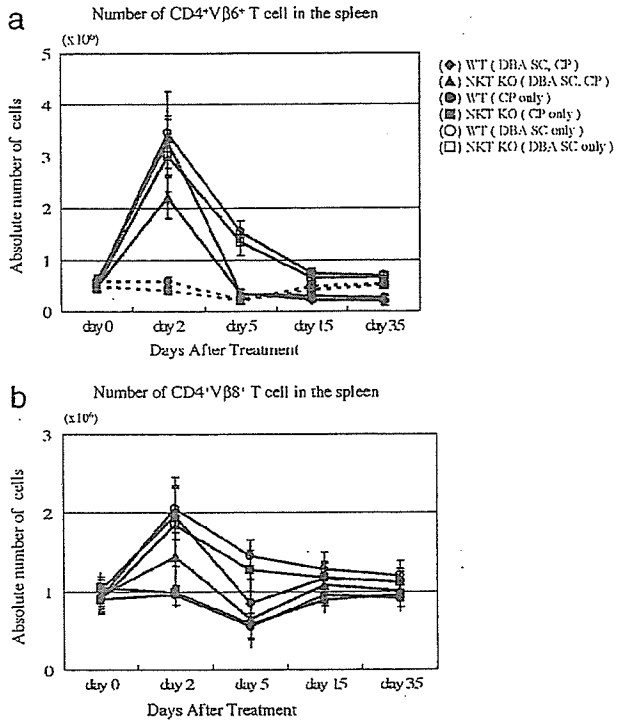

FIGURE 4. Absolute number of cells in the spleen of recipients treated with DBA/2 SC and CP. The kinetics of $CD4^+$ $V\beta6^+$ (*a*) and $CD4^+$ $V\beta8.1/8.2^+$ (*b*) T cells in spleen cells harvested from the recipient BALB/c mice are shown. *a*, The numbers of $CD4^+$ $V\beta6^+$ cells in the spleens from WT mice treated with DBA/2 (DBA) SC and CP (◆; $n = 4$), NKT KO mice treated with DBA/2 SC and CP (▲; $n = 4$), WT mice treated with CP (●; $n = 4$), NKT KO mice treated with CP (■; $n = 4$), WT mice treated with DBA/2 SC (○; $n = 4$), and NKT KO mice treated with DBA/2 SC (□; $n = 4$). *b*, The numbers of $CD4^+V\beta8^+$ cells in the spleens from WT mice treated with DBA/2 SC and CP (◆; $n = 4$), NKT KO mice treated with DBA/2 SC and CP (▲; $n = 4$), WT mice treated with CP (●; $n = 4$), NKT KO mice treated with CP (■; $n = 4$), WT mice treated with DBA/2 SC (○; $n = 4$), and NKT KO mice treated with DBA/2 SC (□; $n = 4$).

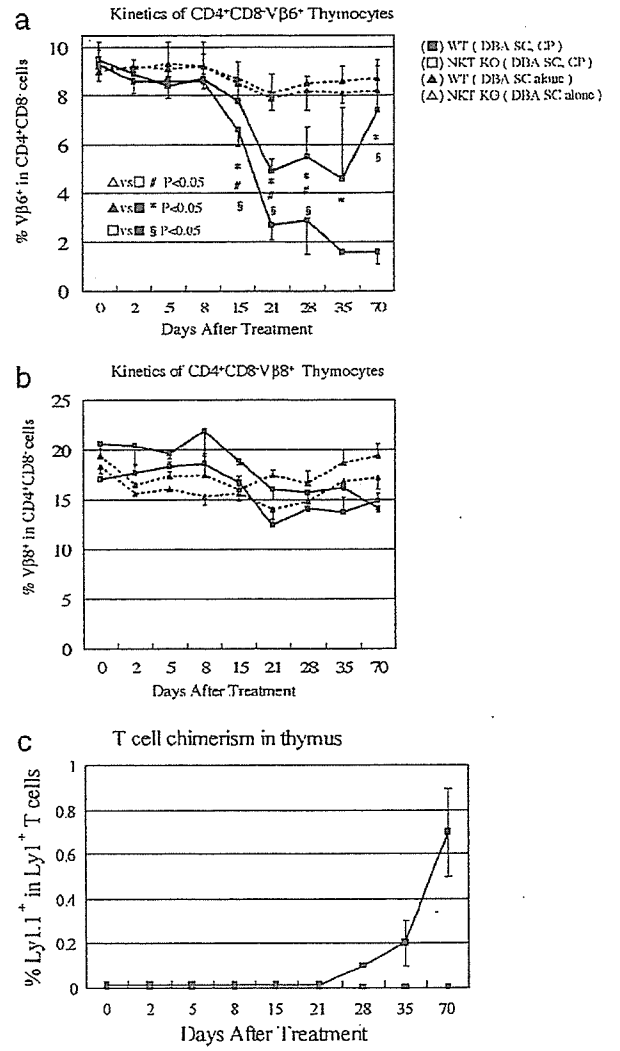

FIGURE 5. Intrathymic clonal deletion in the recipient mice. *a* and *b*, The kinetics of $CD4^+CD8^-V\beta6^+$ (*a*) or $CD4^+CD8^-$ $V\beta8.1/8.2^+$ (*b*) T cells in thymocytes harvested from the recipient BALB/c mice are shown. Thymocytes were labeled with FITC-conjugated anti-Vβ6 or Vβ8.1/8.2 mAb, PE-conjugated anti-CD4 mAb, and allophycocyanin-conjugated anti-CD8 mAb. To determine the percentage of $CD4^+$ T cells that were $V\beta6^+$ or $V\beta8.1/8.2^+$, 10,000–20,000 gated $CD4^+CD8^-$ cells were collected. Thymocytes were obtained from WT mice treated with DBA/2 (DBA) SC and CP (■; $n = 4$), NKT KO mice treated with DBA/2 SC and CP (□; $n = 4$), WT mice treated with DBA/2 SC alone (▲; $n = 4$), and NKT KO mice treated with DBA/2 SC alone (△; $n = 4$). Vertical bars represent SD. The statistical significance of the differences among groups was analyzed and the results are given in *a*. *c*, Intrathymic chimerism in the recipient mice. Thymocytes were labeled with FITC-conjugated anti-Lyt 1.1mAb and PE-conjugated anti-Lyt 1(1.1 + 1.2) mAb. To determine the percentages of T cell chimerism that were $Lyt\ 1.1^+$, 10,000–20,000 gated $Lyt\ 1^+$ cells were collected. Thymocytes were obtained from WT (Lyt-1.2) mice treated with DBA/2 (Lyt-1.1) SC and CP (■; $n = 4$) and NKT KO mice treated with DBA/2 SC and CP (□; $n = 4$). Chimerism was undetectable in WT or NKT KO mice treated with DBA/2 SC alone (data not shown). Vertical bars represent SD.

decreased to ~3%. The percentage of $CD4^+V\beta6^+$ T cells was significantly reduced in BALB/c WT mice treated with DBA/2 SC and CP as compared with that for BALB/c WT mice treated with DBA/2 SC alone. The disappearance of T cells in WBC was specific for $V\beta6^+$ T cells, because the percentage of $V\beta8.1/8.2^+$ T cells was not significantly altered (Fig. 3*b*). Furthermore, the absolute number of $CD4^+V\beta6^+$ T cells in the spleen was analyzed, and similar results were obtained (Fig. 4). We have already reported this phenomenon, which we termed clonal destruction (12, 13), and similar results were obtained in NKT KO mice treated with DBA/2 SC on day 0 and CP on day 2 (Fig. 4). In contrast, when BALB/c WT or NKT KO mice were treated with CP alone on day 2, a transient reduction of both the $CD4^+V\beta6^+$ and $CD4^+V\beta8^+$ T cell subsets was observed.

To further investigate the cellular events in the thymuses of BALB/c mice made tolerant of DBA/2 mice, the association of the clonal deletion with the mixed chimerism was examined (Fig. 5). Whole thymocytes were stained with FITC-conjugated anti-Vβ6 mAb, PE-conjugated anti-CD4 mAb, and allophycocyanin-conjugated anti-CD8 mAb. We previously reported that intrathymic clonal deletion occurs by 6 wk after SC and CP treatment (12, 13), but we did not investigate whether intrathymic CD4 single-positive T cells are depleted by clonal destruction or when intrathymic clonal deletion begins. The present analysis was performed by gating $CD4^+CD8^-$ single-positive thymocytes.

Among the $CD4^+CD8^-$ thymocytes of the BALB/c WT or NKT KO mice, $CD4^+V\beta6^+$ T cells represented ~9% (Fig. 5*a*),

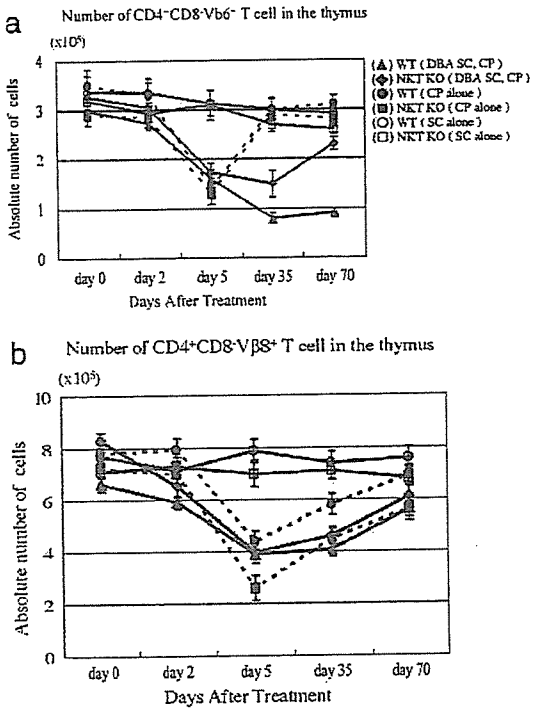

FIGURE 6. Absolute number of cells in the thymuses of recipients treated with DBA/2 (DBA) SC and CP. The kinetics of $CD4^+CD8^-V\beta6^+$ (*a*) and $CD4^+CD8^-$ $V\beta8.1/8.2^+$ (*b*) T cells in thymocytes harvested from the recipient mice are shown. *a*, The numbers of $CD4^+CD8^-V\beta6^+$ cells in the thymuses from WT mice treated with DBA/2 SC and CP (▲; $n = 4$), NKT KO mice treated with DBA/2 SC and CP (◆; $n = 4$), WT mice treated with CP (●; $n = 4$), NKT KO mice treated with and CP (■; $n = 4$), WT mice treated with SC (O; $n = 4$), and NKT KO mice treated with SC (□; $n = 4$). *b*, The numbers of $CD4^+CD8^-V\beta8^+$ cells in the thymuses from WT mice treated with DBA/2 SC and CP (▲; $n = 4$), NKT KO mice treated with DBA/2 SC and CP (◆; $n = 4$), WT mice treated with CP (●; $n = 4$), NKT KO mice treated with CP (■; $n = 4$), WT mice treated with SC (O; $n = 4$), and NKT KO mice treated with SC (□; $n = 4$).

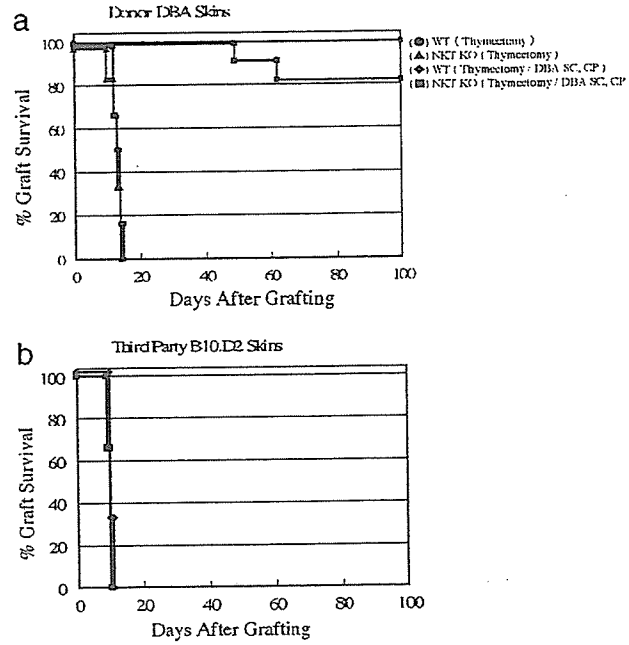

FIGURE 7. Permanent DBA/2 (DBA) skin graft acceptance in the thymectomized BALB/c NKT KO mice treated with DBA/2 SC and CP. Recipient mice were grafted with skin from donor DBA/2 (*a*) or third party B10.D2 (*b*) mice 4 wk after treatment. *a*, The groups and median skin graft survival times were as follows: ●, thymectomized WT mice ($n = 6$; 10 days); ▲, thymectomized NKT KO mice ($n = 6$; 10 days); ◆, thymectomized WT mice treated with DBA/2 SC and CP ($n = 6$; >100 days); ■, thymectomized NKT KO mice treated with DBA/2 SC and CP ($n = 11$; >100 days). *b*, B10.D2 skin grafts were rejected within 14 days after grafting in the following groups: ◆, thymectomized WT mice treated with DBA/2 SC and CP ($n = 3$); and ■, thymectomized NKT KO mice treated with DBA/2 SC and CP ($n = 3$).

and the injection of DBA/2 SC did not significantly alter the percentage of $CD4^+V\beta6^+$ T cells during our observation. In the thymuses of BALB/c WT mice treated with DBA/2 SC and CP, the percentage of $CD4^+V\beta6^+$ T cells was not significantly changed by day 8 but then declined to ~3% by day 21 and reached <2% on day 35. The reduction in $CD4^+V\beta6^+$ T cells was strongly associated with the intrathymic mixed chimerism (Fig. 5*c*). After 28 days, mixed chimerism was detected in the thymuses of BALB/c WT mice treated with DBA/2 SC and CP. In contrast, in the thymuses of NKT KO mice treated with DBA/2 SC and CP, the percentage of $CD4^+V\beta6^+$ T cells was not significantly changed by day 8, then declined to ~5% on day 21, and returned to the normal range by day 70 (Fig. 5*a*). Mixed chimerism was not detected in the thymuses of BALB/c NKT KO mice treated with DBA/2 SC and CP during our observation (Fig. 5*c*). The intrathymic clonal deletion in the tolerant BALB/c mice was specific for Mls-1^a-reactive T cells expressing TCR Vβ6, because $V\beta8.1/8.2^+$ thymocytes were not deleted (Fig. 5*b*). Furthermore, the absolute number of $CD4^+CD8^-V\beta6^+$ thymocytes was analyzed and similar results were obtained (Fig. 6). When BALB/c WT or NKT KO mice were treated with CP alone on day 2, a transient reduction of both $CD4^+V\beta6^+$ and $CD4^+V\beta8^+$ T cell subsets in the thymus was observed.

Induction of skin allograft prolongation in thymectomized NKT KO mice

The previous results indicated that the effector T cells ($CD4^+CD8^-$ $V\beta6^+$) in the thymuses of WT mice were not depleted until intrathymic clonal deletion occurred and that intrathymic clonal deletion was associated with the establishment of mixed chimerism. Thus, we supposed that the effector T cells generated in the thymus at the early phase of tolerance induction were regulated by NKT cells. To confirm this hypothesis, recipients were thymectomized on day −14. As shown in Fig. 7*a*, DBA/2 skin graft survival was permanently prolonged in 9 of 11 recipient NKT KO mice thymectomized on day −14 and treated with SC on day 0 and CP on day 2 (MST, >100 days). Similar results were obtained in thymectomized WT mice ($n = 6$; MST, >100 days). This skin graft prolongation was tolerogen-specific, because third party B10.D2 ($H\text{-}2^d$) allografts were rejected in a normal fashion (Fig. 7*b*).

Generation of tolerogen-specific regulatory T cells in both WT and NKT KO recipients at the late stage of tolerance

Previous studies have demonstrated that the third mechanism of cyclophosphamide-induced tolerance is a regulatory mechanism at the late stage of tolerance (11, 14). To examine whether NKT cells were involved in the generation of regulatory T cells, adoptive transfer experiments were conducted (Fig. 8). BALB/c WT mice were irradiated with 3 Gy and then received an i.v. transfer of 1×10^8 SC from thymectomized WT or NKT KO recipients that had accepted DBA/2 skin grafts for >100 days. With respect to the T

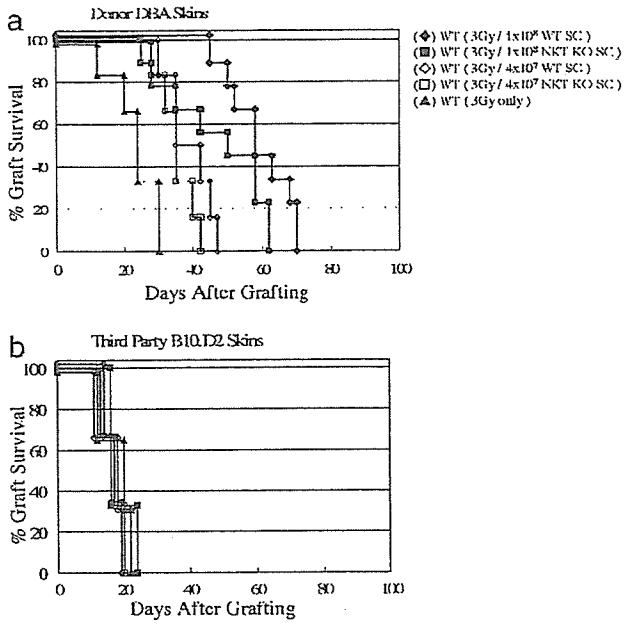

FIGURE 8. Generation of regulatory cells in the recipient mice accepting donor DBA/2 (DBA) skins. BALB/c WT mice were irradiated with 3 Gy and injected i.v. with 1×10^8 or 4×10^7 SC from the thymectomized WT or NKT KO recipients accepting DBA/2 skin grafts >100 days. Recipient mice were grafted with skin from donor DBA/2 (*a*) or third party B10.D2 (*b*) mice 1 day following the transfer of tolerant SC. The groups and median skin graft survival times were as follows: ◆, irradiated WT mice treated with 1×10^8 WT SC ($n = 9$; 58 days); ■, irradiated WT mice treated with 1×10^8 NKT KO SC ($n = 9$; 50 days); ◇, irradiated WT mice treated with 4×10^7 WT SC ($n = 6$; 38.5 days); □, irradiated WT mice treated with 4×10^7 NKT KO SC ($n = 6$; 35 days); and ▲, irradiated WT mice ($n = 6$; 24 days). *b*, B10.BR skin grafts were rejected within 24 days after grafting in all groups.

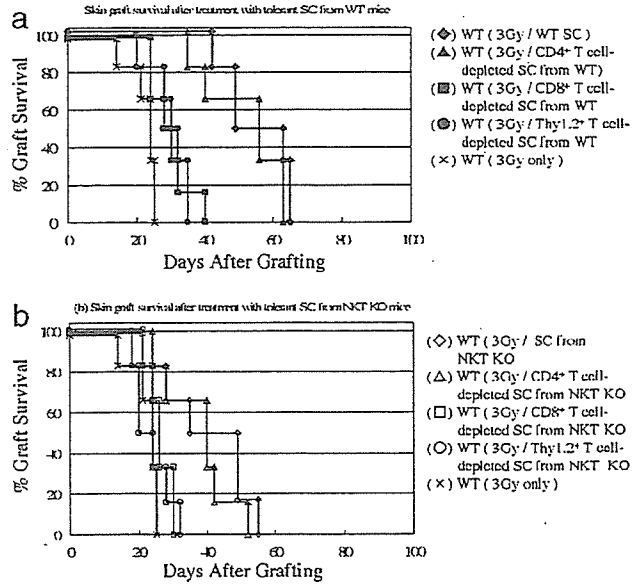

FIGURE 9. Generation of regulatory cells in the recipient mice accepting donor DBA/2 skins. BALB/c WT mice were irradiated with 3 Gy and injected i.v. with 1×10^8 SC from the thymectomized WT or NKT KO recipients accepting DBA/2 skin grafts over 100 days. Recipient mice were grafted with skin from donor DBA/2 mice 1 day following the transfer of tolerant SC. Skin grafting was performed on the same day in all groups. *a*, The groups and median skin graft survival times after treatment with tolerant SC from WT mice were as follows: ◆, irradiated WT mice treated with SC from WT recipients ($n = 6$; 63 days); ▲, irradiated WT mice treated with $CD4^+$ T cell-depleted SC from WT recipients ($n = 6$; 56 days); ■, irradiated WT mice treated with $CD8^+$ T cell-depleted SC from WT recipients ($n = 6$; 29 days); ●, irradiated WT mice treated with $Thy1.2^+$ T cell-depleted SC from WT recipients ($n = 6$; 32.5 days); and ×, irradiated WT mice ($n = 6$; 24 days). *b*, The groups and median skin graft survival times after treatment with tolerant SC from NKT KO mice were as follows: ◇, irradiated WT mice treated with SC from NKT KO recipients ($n = 6$; 42 days); △, irradiated WT mice treated with $CD4^+$ T cell-depleted SC from NKT KO recipients ($n = 6$; 35 days); □, irradiated WT mice treated with $CD8^+$ T cell-depleted SC from NKT KO recipients ($n = 6$; 26 days); ○, irradiated WT mice treated with $Thy1.2^+$ T cell-depleted SC from NKT KO recipients ($n = 6$; 22 days). ×, irradiated WT mice ($n = 6$; 24 days).

cell percentage of the SC, no significant difference was observed between thymectomized NKT KO mice and BALB/c WT donors (20–25%). Skin grafting was performed 1 day following the transfer of the SC. DBA/2 skin grafts were rejected within 30 days after grafting in the BALB/c WT mice treated with irradiation alone (Fig. 8*a*; $n = 6$; MST ± SD = 23.3 ± 6.8 days; median = 24 days). The survival of the DBA/2 skin grafts was further prolonged in the irradiated BALB/c WT mice by transferring the SC from thymectomized WT mice that had accepted DBA/2 skin grafts ($n = 9$; MST ± SD = 59.3 ± 9.1 days; median = 58 days). Similarly, in the irradiated BALB/c WT mice which received the SC transferred from thymectomized NKT KO mice that had accepted DBA/2 skin grafts, the survival of DBA/2 skin grafts was moderately prolonged ($n = 9$; MST ± SD = 46.7 ± 14.6 days; median = 50 days). There was a statistically significant difference between the graft survivals in irradiated BALB/c WT mice receiving SC transfers from thymectomized WT and NKT KO mice that had accepted DBA/2 skin grafts ($p < 0.05$). In addition, we investigated whether a lower dose of tolerant SC (4×10^7) could induce prolongation of graft survival. Skin graft survival was mildly prolonged in the irradiated BALB/c WT mice by transferring 4×10^7 SC from thymectomized NKT KO mice that had accepted DBA/2 skin grafts ($n = 6$; MST ± SD = 35.3 ± 5.1 days; median = 35 days). The survival time of the DBA/2 skin grafts was also prolonged in the irradiated BALB/c WT mice by transferring 4×10^7 SC from thymectomized WT mice that had accepted DBA/2 skin grafts ($n = 6$; MST ± SD = 39.0 ± 6.7 days; median = 38.5 days). In the case of the transfer experiment using low-dose SC, there was no statistically significant difference in survival between the groups treated with 4×10^7 SC from DBA/2 skin graft-accepting thymectomized WT mice and those treated with an equivalent number of SC from DBA/2 skin graft-accepting thymectomized NKT KO mice. The graft survival times in the irradiated BALB/c WT mice treated with a low dose (4×10^7) of SC from DBA/2 skin graft-accepting thymectomized BALB/c WT or NKT KO mice were shorter than those in the irradiated BALB/c WT mice treated with a high dose (1×10^8) of SC. These skin allograft prolongations were tolerogen-specific, because third party skin B10.D2 ($H\text{-}2^d$) allografts were rejected within 24 days after grafting (Fig. 8*b*).

Furthermore, we investigated which T cell subset was dominant in the regulatory function. SC from tolerant BALB/c WT mice were treated with anti-CD4, -CD8, or -Thy-1.2 mAb and complement ex vivo, and 1×10^8 mAb-treated SC were transferred to the irradiated WT mice. Recipient mice were grafted 1 day following the transfer of tolerant SC (Fig. 9*a*). The graft survival time of the recipient treated with $CD4^+$ T cell-depleted SC from tolerant BALB/c WT mice was moderately prolonged ($n = 6$; MST ±

SD = 52.2 ± 11.9 days; median = 56 days). There was no statistically significant difference compared with the graft survival of the recipient treated with non-T cell-depleted tolerant SC (n = 6; MST ± SD = 55.2 ± 9.7 days; median = 56 days). In contrast, the graft survival of the recipients treated with $CD8^+$ or $Thy1.2^+$ T cell-depleted tolerant SC was significantly shorter than that of the recipients treated with non-T cell-depleted tolerant SC (n = 6; MST ± SD = 29.7 ± 6.0 days; median = 29 days; and n = 6; MST ± SD = 30.0 ± 5.6 days; median = 31 days; respectively). These data indicated that the regulatory cells induced in CP-induced tolerance are mainly $CD8^+$ T cells rather than $CD4^+$ T cells. When SC from tolerant NKT KO mice were used, similar results were obtained (Fig. 9*b*).

Discussion

By using the H-2-matched murine combination of DBA/2 into BALB/c WT and mAbs against T cell markers (Lyt-1.1 and Lyt-1.2) and TCR Vβ6, we have demonstrated the sequential mechanisms of CP-induced tolerance (11, 14). These mechanisms are as follows: 1) clonal destruction of Ag-stimulated and then proliferating T cells by CP at the early stage; 2) intrathymic clonal deletion at the intermediate stage; and 3) regulatory mechanisms at the late stage of tolerance. These three conditions are achieved by SC and 200 mg/kg CP alone without any other supportive treatment in most H-2-matched mouse combinations. In the present study, we have elucidated the roles of NKT cells in the induction of skin allograft tolerance in CP-induced tolerance.

The first mechanism essential to CP-induced tolerance is the selective destruction of Ag-stimulated and then proliferating T cells by CP treatment. This mechanism is considered to be responsible for destroying mature T cells but not immature T cells. As shown in Fig. 3, the $CD4^+V\beta6^+$ T cells that are responsible for the MLR against $Mls\text{-}1^a$-encoded Ag (14) and probably the effector T cells that are responsible for the rejection of DBA/2 skin selectively proliferated on day 2 and were depleted by day 5 in the periphery of the WT mice given DBA/2 SC and CP, leaving most of the nonproliferative $CD4^+V\beta8^+$ T cells. The same results were observed in NKT KO mice given DBA/2 SC and CP, suggesting that NKT-mediated immunoregulation was not required for the induction of clonal destruction in the periphery.

The second mechanism is the intrathymic clonal deletion, which is essential for maintaining the central tolerance in CP-induced tolerance and other chimerism-based tolerance systems (12, 13). By days 28–35 after the treatments with DBA/2 SC and CP, intrathymic chimerism was established due to regeneration of the stem cells of donor origin contained in the tolerogenic SC, and then clonal deletion of $V\beta6^+$ T cells began in the thymuses of WT recipients (Fig. 4). In fact, intrathymic clonal deletion was well correlated with intrathymic mixed chimerism. Notably, in the thymuses of NKT KO recipients given DBA/2 SC and CP, the percentage of $CD4^+V\beta6^+$ T cells decreased only transiently from day 21 through day 35 and returned to the normal level by day 70. Consistently, intrathymic chimerism was not established in NKT KO recipients given DBA/2 SC and CP. Because donor Ag-reactive effector T cells can break mixed chimerism in the periphery, it can be speculated that the effector T cells generated in the thymuses of recipient WT mice by DBA/2 SC administration must be suppressed or regulated by an unsolved mechanism to establish the intrathymic mixed chimerism, which is essential for clonal deletion of donor Ag-specific T cells in the thymus. We hypothesized that this unsolved mechanism could be mediated by the NKT cells. To confirm this hypothesis, we performed a thymectomy and then conditioned the mice with DBA/2 SC and CP (Fig. 7). The results showed that skin graft tolerance was induced in 9 of 11 of the thymectomized NKT KO mice given DBA/2 SC and CP (Fig. 7).

It is important to consider why chimerism or clonal deletion was poorly observed in NKT recipients (group 5; Table I and Fig. 5*a*). Regarding the reduced level of chimerism, we conjectured that chimerism was established by the clonal destruction but was gradually rejected by effector T cells from the thymus. In fact, the level of chimerism was reduced from 2 to 8 wk (group 5; Table I). In BALB/c WT mice, as described above, effector T cells from the thymus were suggested as being regulated by NKT cells, chimerism was stably maintained, and donor skins were permanently accepted. By performing thymectomies in NKT KO mice, a higher level of chimerism could be induced compared with that in non-thymectomized NKT KO mice (group 6 vs 7; Table II). As a result, skin allograft tolerance could be induced in thymectomized NKT KO mice treated with DBA/2 SC and CP. However, the level of chimerism in thymectomized NKT KO mice treated with DBA/2 SC and CP tended to be lower than that in thymectomized BALB/c WT mice treated with DBA/2 SC and CP (group 6 vs group 4; Table II), although this difference did not reach the level of statistical significance. These results may be explained in the following ways. First, we detected T cell chimerism, which may not correlate with bone marrow chimerism. Second, NKT-mediated immunity may contribute to the homeostatic proliferation or self-renewal of T cells. Regarding the poor level of deletion of $CD4^+CD8^-V\beta6^+$ thymocytes in NKT mice (Fig. 5*a*), we can hypothesize that NKT cells may regulate negative selection in the

Table II. *Chimerism and clonal destruction in recipients treated with thymectomy, DBA/2 SC and CP*[a]

Group	Recipient	Treatment[a] Thymectomy (day −14)	SC (day 0)	CP (day 2)	No. of Mice	Chimeric Analysis (percent positive cells ± SD) Lyt-1.1+/Lyt-1.+ (%) 2 wk	8 wk	Analysis of TCR Expression (percent positive cells ± SD) $CD4^+V\beta6^+/CD4^+$ (%) 3 wk	9 wk	$CD4^+V\beta6^+/CD4^+$ (%) 3 wk	9 wk
1	BALB/c WT	(+)	(−)	(−)	6	0		11.7 ± 0.9		18.7 ± 2.1	
2	BALB/c NKT KO	(+)	(−)	(−)	6	0		10.1 ± 1.2		18.9 ± 1.5	
3	DBA/2	(+)	(−)	(−)	6	98.0 ± 2.2		0		13.2 ± 2.0	
4	BALB/c WT	(+)	DBA/2	200[b]	6	3.0 ± 1.0[c]	3.5 ± 1.2[c]	1.1 ± 0.2	0.9 ± 0.7	18.7 ± 0.7	16.9 ± 3.3
5	BALB/c WT	Sham	DBA/2	200[b]	6	2.4 ± 0.9	3.2 ± 1.2	1.7 ± 0.4	1.3 ± 0.5	19.2 ± 1.5	17.4 ± 1.1
6	BALB/c NKT KO	(+)	DBA/2	200[b]	6	2.6 ± 0.5[d]	2.0 ± 0.7[d]	1.3 ± 0.3	1.3 ± 0.2	16.2 ± 0.8	14.7 ± 2.4
7	BALB/c NKT KO	Sham	DBA/2	200[b]	6	1.4 ± 0.3	0.8 ± 0.1	1.5 ± 0.5	1.0 ± 0.4	15.9 ± 1.0	16.7 ± 1.3

[a] The recipient mice were primed i.v. with 1×10^8 viable DBA/2 SC on day 0 and then given 200 mg/kg CP on day 2. Thymectomies were performed on some groups on day −14.
[b] Milligrams per kilogram (mg/kg).
[c] No statistical significance as compared with group 6.
[d] $p < 0.01$ compared with group 7.

thymus. We intend to elucidate these unsolved mechanisms in a future study.

The third mechanism is the generation of regulatory cells in the late stage of tolerance (11, 14). Any significant contribution of suppressor factors, such as enhancing Abs or anti-idiotypic Abs, was excluded from the transfer experiments by using the serum from long-term tolerant mice (11). Recent reports have clarified that the regulatory mechanism is mediated by both $CD25^+CD4^+$ and $CD25^-CD4^+$ T cells via CTLA-4 molecules and Th2 cytokines in mAb-induced tolerance systems (23–25). Furthermore, another study has reported that CP depleted $CD25^+CD4^+$ T cells (26). We have reported that $CD8^+$ T cells are generally involved in the suppressor activity in CP-induced tolerance, whereas $CD4^+$ T cells are not (11, 14). The present study confirmed that $CD8^+$ T cells exhibit the main suppressor activity, indicating that $CD25^+CD4^+$ T cells are not involved in the regulatory mechanisms. One of the aims in the present study was to examine the role of NKT cells in the generation of regulatory cells. The results showed that regulatory cells could be generated without the contribution of NKT cells. However, regarding the suppressor activity, NKT may have some effects on the suppression of the alloreactivity in the recipients, because the survival of DBA/2 skin grafts was significantly longer in irradiated recipients receiving a high dose (1×10^8) of SC from tolerant WT mice than in those receiving the same amount of SC from tolerant NKT KO mice.

Two reports have described the critical role of NKT cells in inducing transplantation tolerance (5, 6). However, the precise mechanisms at the cellular and molecular levels have remained unclear. It has been well documented that NKT cells produce large amounts of both IL-4 and IFN-γ upon activation (27–29). Given that IL-4 and IFN-γ have opposite effects on the development of Th1 and Th2 cells, extensive analyses have been performed with various experimental systems, and conflicting results have been reported (30–32). By using IL-4 KO and IFN-γ KO mice, two groups analyzed the mechanisms of the NKT-mediated role in transplantation tolerance induction and produced conflicting results (5, 6). Ikehara et al. (6) suggested that there was little involvement of these two cytokines in C57BL/6 mice injected with anti-CD4 mAb and grafted with rat islets. In contrast, Seino et al. (5) suggested that IFN-γ partially contributes to tolerance induction in C57BL/6 mice injected with anti-LFA-1 and ICAM-1 mAbs and grafted with heart grafts from BALB/c ($H\text{-}2^d$) mice. However, these results did not seem to be definitive, because they could not show clearly whether the IFN-γ produced by NKT cells was involved in one or more of the steps that induce and maintain transplantation tolerance, i.e., activation of effector T cells, apoptosis of effector T cells, reprogramming of effector T cells (anergy induction), and the generation of regulatory T cells. In the present study, we can strongly suggest two roles for NKT cells in CP-induced tolerance. One is to regulate the effector T cells generated in the thymuses of recipient WT mice by DBA/2 SC administration through the establishment of intrathymic clonal deletion. The other is to allow generation of regulatory cells without NKT cell-mediated immunoregulation.

As for the NKT reconstitution assay (Fig. 2), unfortunately we could not show how many NKT cells are needed to completely reconstitute NKT-mediated immunoregulation. In our laboratory, the Vα14 transgenic mice (RAG-1 KO background) needed for reconstituting NKT cells in NKT (Vα14) KO mice are unavailable. However, even in the experiments using the Vα14 transgenic mice, a previous attempt to perform adoptive transfer of $V\alpha14^+$ cells from Vα14 transgenic mice in an allogeneic tolerance system was not successful, probably because the dose of $V\alpha14^+$ cells was not sufficient to restore these cells to the normal level (Y. Yasunami, unpublished observation). We initially transferred 1×10^8 SC from WT mice to NKT KO mice but could not induce permanent acceptance donor skin grafts in three of seven recipients. NKT (αGalCer/CD1d tetramer$^+CD3^+$) cells were restored to 0.4 and 4.3% in SC and LMNC of these mice, respectively, suggesting that the level of NKT reconstitution was not enough. In contrast, Seino et al. had reconstituted WT BMC (including NKT cells and progenitors) in irradiated NKT KO mice (5). To further reconstitute NKT cells, recipient NKT KO mice were irradiated with 3 Gy and reconstituted with SC and BMC from WT mice. Although NKT cells were not fully restored (0.7 and 9.5% in SC and LMNC, respectively), permanent skin graft acceptance was induced in all of the irradiated and reconstituted NKT KO mice.

Acknowledgments

We thank Dr. Hisanori Mayumi, General Manager, Watanabe Hospital (Kagoshima, Japan) for reviewing this manuscript and giving helpful comments. We also thank the Edanz Editing Co. Ltd. (Fukuoka, Japan) and KN International, Inc. (Iowa City, IA) for the English editing of this manuscript.

Disclosures

The authors have no financial conflict of interest.

References

1. Cui, J., T. Shin, T. Kawano, H. Sato, E. Kondo, I. Toura, Y. Kaneko, H. Koseki, M. Kanno, and M. Taniguchi. 1997. Requirement for Vα14 NKT cells in IL-12-mediated rejection of tumors. *Science* 278: 1623–1626.
2. Mieza, M. A., T. Itoh, J. Q. Cui, Y. Makino, T. Kawano, K. Tsuchida, T. Koike, T. Shirai, H. Yagita, A. Matsuzawa, et al. 1996. Selective reduction of $V\alpha14^+$ NK T cells associated with disease development in autoimmune-prone mice. *J. Immunol.* 156: 4035–4040.
3. Slifka, M. K., R. R. Pagarigan, and J. L. Whitton. 2000. NK markers are expressed on a high percentage of virus-specific $CD8^+$ and CD4+ T cells. *J. Immunol.* 164: 2009–2015.
4. Naiki, Y., H. Nishimura, T. Kawano, Y. Tanaka, S. Itohara, M. Taniguchi, and Y. Yoshikai. 1999. Regulatory role of peritoneal $NK1.1^+$ αβ T cells in IL-12 production during *Salmonella* infection. *J. Immunol.* 163: 2057–2063.
5. Seino, K. I., K. Fukao, K. Muramoto, K. Yanagisawa, Y. Takada, S. Kakuta, Y. Iwakura, L. Van Kaer, K. Takeda, T. Nakayama, et al. 2001. Requirement for natural killer T (NKT) cells in the induction of allograft tolerance. *Proc. Natl. Acad. Sci. USA* 98: 2577–2581.
6. Ikehara, Y., Y. Yasunami, S. Kodama, T. Maki, M. Nakano, T. Nakayama, M. Taniguchi, and S. Ikeda. 2000. $CD4^+$ Vα14 natural killer T cells are essential for acceptance of rat islet xenografts in mice. *J. Clin. Invest.* 105: 1761–1767.
7. Shin, T., H. Mayumi, K. Himeno, H. Sanui, and K. Nomoto. 1984. Drug-induced tolerance to allografts in mice. I. Difference between tumor and skin grafts. *Transplantation* 37: 580–584.
8. Mayumi, H., T. Shin, K. Himeno, and K. Nomoto. 1985. Drug-induced tolerance to allografts in mice II. Tolerance to tumor allografts of large doses associated with rejection of skin allografts and tumor allografts of small doses. *Immunobiology* 169: 147–161.
9. Mayumi, H., K. Himeno, T. Shin, and K. Nomoto. 1985. Drug-induced tolerance to allografts in mice. IV. Mechanisms and kinetics of cyclophosphamide-induced tolerance. *Transplantation* 39: 209–215.
10. Mayumi, H., K. Himeno, K. Tanaka, N. Tokuda, J. L. Fan, and K. Nomoto. 1986. Drug-induced tolerance to allografts in mice. IX. Establishment of complete chimerism by allogeneic spleen cell transplantation from donors made tolerant to H-2-identical recipients. *Transplantation* 42: 417–422.
11. Tomita, Y., H. Mayumi, M. Eto, and K. Nomoto. 1990. Importance of suppressor T cells in cyclophosphamide-induced tolerance to the non-H-2-encoded alloantigens. Is mixed chimerism really required in maintaining a skin allograft tolerance? *J. Immunol.* 144: 463–473.
12. Eto, M., H. Mayumi, Y. Tomita, Y. Yoshikai, and K. Nomoto. 1990. Intrathymic clonal deletion of $V\beta6^+$ T cells in cyclophosphamide-induced tolerance to H-2-compatible, Mls-disparate antigens. *J. Exp. Med.* 171: 97–113.
13. Tomita, Y., Y. Nishimura, N. Harada, M. Eto, K. Ayukawa, Y. Yoshikai, and K. Nomoto. 1990. Evidence for involvement of clonal anergy in MHC class I and class II disparate skin allograft tolerance after the termination of intrathymic clonal deletion. *J. Immunol.* 145: 4026–4036.
14. Eto, M., H. Mayumi, Y. Tomita, Y. Yoshikai, Y. Nishimura, and K. Nomoto. 1990. Sequential mechanisms of cyclophosphamide-induced skin allograft tolerance including the intrathymic clonal deletion followed by late breakdown of the clonal deletion. *J. Immunol.* 145: 1303–1310.
15. Zhang, Q. W., H. Mayumi, M. Umesue, Y. Tomita, K. Nomoto, and H. Yasui. 1997. Fractionated dosing of cyclophosphamide for establishing long-lasting skin allograft survival, stable mixed chimerism, and intrathymic clonal deletion in mice primed with allogeneic spleen cells. *Transplantation.* 63: 1667–1673.
16. Maeda, T., M. Eto, Y. Nishimura, K. Nomoto, and Y. Y. Kong. 1993. Role of peripheral hemopoietic chimerism in achieving donor-specific tolerance in adult mice. *J. Immunol.* 150: 753–762.

17. Yoshikawa, M., Y. Tomita, T. Uchida, Q. W. Zhang, and K. Nomoto. 1999. Lack of pluripotent stem cell engraftment in cyclophosphamide-induced tolerance. *Transplant. Proc.* 31: 898–899.
18. Yoshikawa, M., Y. Tomita, T. Uchida, Q. W. Zhang, I. Shimizu, and K. Nomoto. 1999. Cyclophosphamide 200 mg/kg lacks ability to induce pluripotent stem cell engraftment in mice. *Transplant. Proc.* 31: 1939.
19. Tomita, Y., M. Yoshikawa, Q. W. Zhang, I. Shimizu, S. Okano, T. Iwai, H. Yasui, and K. Nomoto. 2000. Induction of permanent mixed chimerism and skin allograft tolerance across fully MHC-mismatched barriers by the additional myelosuppressive treatments in mice primed with allogeneic spleen cells followed by cyclophosphamide. *J. Immunol.* 165: 34–41.
20. Mayumi, H., K. Nomoto, and R. A. Good. 1988. A surgical technique for experimental free skin grafting in mice. *Jpn. J. Surg.* 18: 548–557.
21. Tomita, Y., A. Khan, and M. Sykes. 1994. Role of intrathymic clonal deletion and peripheral anergy in transplantation tolerance induced by bone marrow transplantation in mice conditioned with a nonmyeloablative regimen. *J. Immunol.* 153: 1087–1098.
22. Matsuda, J. L., O. V. Naidenko, L. Gapin, T. Nakayama, M. Taniguchi, C. R. Wang, Y. Koezuka, and M. Kronenberg. 2000. Tracking the response of natural killer T cells to a glycolipid antigen using CD1d tetramers. *J. Exp. Med.* 192: 741–754.
23. Graca, L., S. Thompson, C. Y. Lin, E. Adams, S. P. Cobbold, and H. Waldmann. 2002. Both $CD4^+CD25^+$ and $CD4^+CD25^-$ regulatory cells mediate dominant transplantation tolerance. *J. Immunol.* 168: 5558–5565.
24. Taylor, P. A., R. J. Noelle, and B. R. Blazar. 2001. $CD4^+CD25^+$ immune regulatory cells are required for induction of tolerance to alloantigen via costimulatory blockade. *J. Exp. Med.* 193: 1311–1318.
25. Kingsley, C. I., M. Karim, A. R. Bushell, and K. J. Wood. 2002. $CD25^+CD4^+$ regulatory T cells prevent graft rejection: CTLA-4- and IL-10-dependent immunoregulation of alloresponses. *J. Immunol.* 168: 1080–1086.
26. Ghiringhelli, F., N. Larmonier, E. Schmitt, A. Parcellier, D. Cathelin, C. Garrido, B. Chauffert, E. Solary, B. Bonnotte, and F. Martin. 2004. $CD4^+CD25^+$ regulatory T cells suppress tumor immunity but are sensitive to cyclophosphamide which allows immunotherapy of established tumors to be curative. *Eur. J. Immunol.* 34: 336–344.
27. Arase, H., N. Arase, K. Ogasawara, R. A. Good, and K. Onoe. 1992. An $NK1.1^+$ $CD4^+8^-$ single-positive thymocyte subpopulation that expresses a highly skewed T-cell antigen receptor $V\beta$ family. *Proc. Natl. Acad. Sci. USA* 89: 6506–6510.
28. Kawano, T., J. Cui, Y. Koezuka, I. Toura, Y. Kaneko, K. Motoki, H. Ueno, R. Nakagawa, H. Sato, E. Kondo, et al. 1997. CD1d-restricted and TCR-mediated activation of $V\alpha14$ NKT cells by glycosylceramides. *Science* 278: 1626–1629.
29. Yoshimoto, T., and W. E. Paul. 1994. CD4pos, NK1.1pos T cells promptly produce interleukin 4 in response to in vivo challenge with anti-CD3. *J. Exp. Med.* 179: 1285–1295.
30. Cui, J., N. Watanabe, T. Kawano, M. Yamashita, T. Kamata, C. Shimizu, M. Kimura, E. Shimizu, J. Koike, H. Koseki, et al. 1999. Inhibition of T helper cell type 2 cell differentiation and immunoglobulin E response by ligand-activated $V\alpha14$ natural killer T cells. *J. Exp. Med.* 190: 783–792.
31. Burdin, N., L. Brossay, and M. Kronenberg. 1999. Immunization with α-galactosylceramide polarizes CD1-reactive NK T cells towards Th2 cytokine synthesis. *Eur. J. Immunol.* 29: 2014–2025.
32. Singh, N., S. Hong, D. C. Scherer, I. Serizawa, N. Burdin, M. Kronenberg, Y. Koezuka, and L. Van Kaer. 1999. Cutting edge: activation of NK T cells by CD1d and α-galactosylceramide directs conventional T cells to the acquisition of a Th2 phenotype. *J. Immunol.* 163: 2373–2377.

Physical activity and colorectal cancer: The Fukuoka Colorectal Cancer Study

Kayoko Isomura,[1,12] Suminori Kono,[1] Malcolm A. Moore,[1] Kengo Toyomura,[1] June Nagano,[1] Tetsuya Mizoue,[1] Ryuichi Mibu,[2] Masao Tanaka,[2] Yoshihiro Kakeji,[3] Yoshihiko Maehara,[3] Takeshi Okamura,[4] Koji Ikejiri,[5] Kitaroh Futami,[6] Yohichi Yasunami,[7] Takafumi Maekawa,[8] Kenji Takenaka,[9] Hitoshi Ichimiya[10] and Nobutoshi Imaizumi[11]

Departments of [1]Preventive Medicine, [2]Surgery and Oncology, and [3]Surgery and Science, Graduate School of Medical Sciences, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582; [4]Department of Gastroenterological Surgery, National Kyushu Cancer Center, 3-1-1 Notame, Minami-ku, Fukuoka 811-1395; [5]Division of Surgery, National Kyushu Medical Center, 1-8-1 Jigyohama, Chuo-ku, Fukuoka 810-8563; [6]Department of Surgery, Fukuoka University Chikushi Hospital, 377-1 Oaza-zokumyoin, Chikushino-shi 818-0067; [7]the First and [8]Second Departments of Surgery, Fukuoka University School of Medicine, 4-45-1 Nanakuma, Jonan-ku, Fukuoka 814-0180; [9]Division of Surgery, Fukuoka City Hospital, 13-1 Yoshizuka-honmachi, Hakata-ku, Fukuoka 812-0046; [10]Division of Surgery, Hamanomachi General Hospital, 3-5-27 Maizuru, Chuo-ku, Fukuoka 810-8539; [11]Division of Surgery, Fukuoka Red Cross Hospital, 3-1-1 Ogusu, Minami-ku, Fukuoka 815-8555, Japan

(Received March 22, 2006/Revised June 15, 2006/Accepted June 18, 2006/Online publication August 11, 2006)

The number of cases of colorectal cancer in Japan has increased over the past few decades, and incidence rates are now among the highest in the world. The present investigation within the Fukuoka Colorectal Cancer Study, including 778 cases and 767 controls aged 20–74 years, examined the association between physical activity and colorectal cancer risk by subsite. Employment-associated and leisure time physical activity was assessed by a questionnaire and interview. Division of sites into the proximal and distal colon, as well as the rectum, revealed clear site-dependent protective effects, with adjustment for smoking, alcohol consumption, BMI and age. In males, greater job-related physical activity was associated with significant reduction of risk in the distal colon and rectum ($P = 0.047$ and 0.02, respectively), whereas total and moderate or hard non-job physical activity exerted effects limited to the rectum ($P = 0.01$ and 0.004, respectively). In females, job-related physical activity and moderate or hard non-job physical activity was also protective, but only in the distal colon. Separate assessment of the influence of BMI 10 years previous to the study showed increase in risk with obesity in males but not in females, limited to distal colon and rectum. The results of the present study indicate that physical activity associated with work and leisure-time exerts beneficial effects in Japanese, but not on the proximal colon. (*Cancer Sci* 2006; 97: 1099–1104)

Colorectal cancer is one of the most common cancers world-wide, accounting for approximately 10% of incident cases.[1] Both incidence and mortality have markedly increased over the past decades in Japan,[2] now among the countries with the highest rates of colorectal cancer in the world.[3] It has been argued that the change from low to high incidence is due to the adoption of a Westernized lifestyle, characterized by high intake of animal foods and low levels of physical exercise.[4] Many cohort and case-control studies have consistently shown that physical activity, as assessed by different methods, is associated with decreased risk of colorectal or colon cancer.[5,6] It is notable that a decreased risk associated with physical activity is more evident for colon cancer than colon and rectal cancer combined. In the case of rectal cancer, a protective association with physical activity has rarely been observed. According to a review published in 2002, two cohort studies and 10 case-control studies addressed the relationship between physical activity and rectal cancer, and only four case-control studies suggested a protective association.[5] An unsolved question is whether total energy expenditure or physical activity is more relevant to protection against colon cancer, and it is unsolved whether protective effects of physical activity might differ by the site of colon cancer. Several investigations have found a stronger protective association between physical activity and distal colon cancer than proximal colon lesions,[7–10] but others have noted a stronger risk reduction in the proximal segment,[11,12] or no difference.[13–16]

We therefore examined the relationship between physical activity at work, and during leisure time, commuting, housework, and shopping, and colorectal cancer risk in the Fukuoka Colorectal Cancer Study in Japan, with particular attention to the impact of physical activity on occurrence of cancer in the proximal colon, distal colon, and rectum. Because obesity has been reported to be associated with increased risk of colorectal cancer,[5] and is also closely associated with the level of physical activity, we also investigated the relationship between BMI and colorectal cancer development.

Materials and Methods

Details of methodological issues have been described elsewhere.[17] In brief, both cases and controls were residents of Fukuoka City and three adjacent areas. Cases were patients undergoing surgery for a first diagnosis of colorectal cancer at two university hospitals or six affiliated hospitals. Other eligibility criteria included the following characteristics: age of 20–74 years at the time of diagnosis; no prior history of partial or total removal of the colorectum, familial adenomatous polyposis, or inflammatory bowel disease; and mental and physical competence to give informed consent and to complete the interview. Of 1053 eligible cases, a total of 840 cases (80%) participated in the interview. Eligibility criteria for controls were the same as described for cases except for two items, that is, having no diagnosis of colorectal cancer and age of 20–74 years at the time of selection. A total of 1500 persons were selected as control candidates by two-stage random sampling. Fifteen small areas out of 178 in total were randomly selected, and approximately 100 persons were randomly selected in each small area. Numbers of control candidates by sex and 10-year age class were determined *a priori* in accordance with sex and age-specific numbers of incident cases of colorectal cancer in the Osaka Cancer Registry during the period 1988–1992.[18] A letter of invitation was sent to each candidate, and a telephone call was made if the candidate was listed in the telephone directory. At most three additional letters of invitation were mailed to non-respondents. A total of 833 persons (60%) participated in the survey.

[12]To whom correspondence should be addressed.
E-mail:kisomura@phealth.med.kyushu-u.ac.jp
Abbreviations: BMI, body mass index; CI, confidence interval; MET, metabolic equivalent; OR, odds ratio.

doi: 10.1111/j.1349-7006.2006.0282.x

The cases were interviewed at each hospital during the period 2000–2003, and controls were surveyed during the period 2001–2002. Excluded from this analysis were 60 cases and 65 controls who had conditions that might have influenced their physical activities (prior history of angina pectoris, myocardial infarction, or cerebral infarction), and who were not perfectly independent in the activities of daily living. One control subject whose height was not measured and two cases whose cancers were both in colon and rectum were also excluded. Thus, 778 cases (456 men and 322 women) and 767 controls (470 men and 297 women) remained in the analysis.

Physical activity assessment. Questions on physical activities elicited information regarding the respondents' type of job, activities in commuting, housework, and shopping, and leisure-time activities at the time of 5 years prior to the interview. Five options were prepared to describe the type of job: sedentary or standing work (e.g., clerical work, taxi driving, housework); work with walking (e.g., delivery by walking, patrolling on foot), labor work (e.g., construction work, agricultural work, loads transport), hard labor work (e.g., digging or chopping with heavy tools, carrying heavy loads), and no job. Weekly minutes spent in walking, bicycling, and jogging were each ascertained regarding commuting, housework, and shopping on average in the year. Regular leisure-time activities were ascertained, on average over 1 year, with regularity defined as at least once per week. Information was obtained for up to three activities, in terms of the type of activity, numbers of months and of days per week that individuals participated in each activity, and minutes of participation per occasion.

Intensity of each non-job physical activity, including physical activity at leisure time, commuting, housework, and shopping, was classified into light, moderate, hard and very hard in terms of the MET on the basis of a published compilation for physical activity.[19] The time spent in non-job physical activity was multiplied by the corresponding MET value (light 2, moderate 4, hard 6 and very hard 8) to yield MET-hours per week of non-job activities. Twenty-eight subjects completed the questionnaire again after an interval of 1 year. The Spearman correlations for job-related and non-job physical activities were 0.67 and 0.57, respectively.

Job-related physical activities were categorized into three levels of sedentary (not employed, or sedentary or standing work), moderate (walking with work), and hard (labor work or hard labor work) for men. Because of the small number of female subjects engaged in jobs of moderate or hard activity, these two categories were combined into one. Non-job physical activity was categorized into three levels of 0, 0.1–15.9, or 16 + MET-hours per week, based on the distribution of physical activity among controls.

Anthropometric parameters. In this study, referent dates were the date of the onset of symptoms or screening for cases, and the date of interview for controls.

Participants reported height (cm) and body weight (kg) at the referent date and also body weight 10 years before the referent date. BMI, weight in kilograms divided by squared height in meters, was calculated as a measure of obesity at the referent date and 10 years before. Information on body weight 10 years before was missing with five cases and 11 controls, and their BMI 10 years before was based on the recent body weight. BMI was categorized into three groups of <23, 23–24.9, and 25+ kg/m^2, with reference to the results from a prospective study that examined the association between BMI and mortality in Japan.[20]

Other lifestyle parameters. To assess smoking habits, individuals were first asked whether they had ever smoked cigarettes every day for 1 year or longer. Then they were asked about the current status (before the symptoms or screening in the cases). For past smokers, their age when they started smoking, and that of quitting smoking, were ascertained. Years of smoking and numbers of cigarettes smoked per day were ascertained for each decade of age from the second to eighth decade.

Alcohol consumption at the time of 5 years prior to the referent dates was elicited, with alcohol use defined as drinking alcoholic beverages at least once per week over the period of 1 year or longer. Then individuals answered open-ended questions regarding the frequency of consumption (number of days per week) and the amount of alcohol consumed on the day of alcohol drinking, on average over a year at the time 5 years prior to the referent date. The amount of alcohol was expressed in conventional units: one go (180 mL) of sake, one large bottle (633 mL) of beer, and one half go (90 mL) of shochu were each expressed as one unit; and one drink (30 mL) of whisky or brandy and one glass (100 mL) of wine were each converted to a half unit.

Prior histories of medical conditions and surgeries were elicited, and activities of daily living were ascertained with classification into four categories (perfect independence, need of help for going outside, support needed at home, and bedridden).

Statistical analysis. In examining the association between physical activity and confounding factors, the Kruskal–Wallis test was used for continuous confounding variables, and the χ^2-test for dichotomous confounding variables. Association of physical activity and obesity with the risk of colorectal cancer was examined for men and women separately by use of multiple logistic regression analysis in terms of OR and 95% CI. Adjustment was made for age (<50, 50–54, 55–59, 60–64, 65–69, and 70+ years), cigarette smoking (0, 1–399, 400–799 and 800+ cigarette-years), alcohol use (0, 0.1–0.9, 1.0–1.9, or 2+ units per day), residential area (Fukuoka City or suburban area) and BMI 10 years before. Job-related physical activity and non-job physical activity were adjusted for each other. Statistical significance was concluded if the two-sided P-value was less than 0.05 or if 95% CI did not include unity. Those aged below 30 years were few (two cases and eight controls). Repeated analysis excluding these subjects did not change the results, and we presented the results based on the whole subjects. All statistical analyses were performed using SAS version 8.2 (SAS Institute, Cary, NC).

Results

Table 1 shows the association between several confounding factors and job-related physical activity among controls. In men, non-job physical activity was less among subjects whose job was physically hard than those who were sedentary at work. Sedentary men were older than those whose job was physically active. Such associations were not observed in women. In terms of non-job physical activity, the mean age was higher in both men and women with greater activity. None of the other confounding factors was related to level of non-job physical activity (Table 2).

Table 3 shows associations of physical activity with colon and rectal cancer risk in men. Hard work in terms of physical activity was associated with lower risks of both colon and rectal cancer as compared with sedentary work. A decrease in risk associated with job-related physical activity was noted for both distal and proximal colon cancer. Non-job physical activity was associated with a significantly decreased risk of only rectal cancer, although a similar tendency was noted for distal colon cancer. We also examined associations between moderate or hard non-job physical activity and colorectal cancer, and the results were similar to those for total non-job physical activity.

In women (Table 4), job-related physical activity was associated with a slightly decreased risk of colon cancer, and a significantly decreased risk of distal colon cancer, but not of rectal cancer. Similarly, total non-job physical activity was associated with a lower risk of colon cancer overall, and of distal colon cancer specifically, but not of either proximal colon or rectal cancer.

The current BMI was unrelated to either colon or rectal cancer in men and women (data not shown). BMI 10 years before was

doi: 10.1111/j.1349-7006.2006.00282.x

Table 1. Characteristics of controls by job-related physical activity

Variable	Men				Women		
	Sedentary	Moderate	Hard	P*	Sedentary	Hard	P*
Number	290	75	105	NA	245	52	NA
Mean age (years)	59.2	56.4	57.8	0.03	58.1	58.1	0.57
Mean BMI (kg/m^2)	23.0	23.3	23.2	0.37	22.4	22.4	0.78
Mean MET-hours/week†	16.5	10.0	8.8	<0.0001	15.9	14.0	0.36
Alcohol user (%)‡	76.2	88.0	76.2	0.08	28.6	36.5	0.25
Heavy alcohol user (%)§	47.9	58.7	60.0	0.05	NS	NS	NS
Ever-smoker (%)¶	82.1	78.7	81.0	0.75	20.8	25.0	0.51
Heavy smoker (%)††	37.6	26.7	28.6	0.09	NS	NS	NS
Residents in Fukuoka City (%)	63.5	68.0	52.4	0.06	71.0	59.6	0.11

*P-values (two-sided) were based on the Kruskal–Wallis test for continuous variables and χ^2-test for proportions. †Physical activity during leisure time, commuting, housework, and shopping. ‡Drinking alcohol at least once per week 5 years before. §Drinking alcohol at least 1.0 unit per day. Because of a small number of heavy drinkers in women ($n = 19$), only results for men are presented. ¶Smoking cigarettes daily for at least 1 year. ††Smoking at least 800 cigarette-years. Because of a small number of heavy smokers in women ($n = 6$), only results for men are presented. NA, not applicable; NS, result not significant.

Table 2. Characteristics of controls according to level of non-job physical activity† (MET-hours/week)

Variable	Men				Women			
	0	0.1–15.9	16.0+	P*	0	0.1–15.9	16.0+	P*
Number	161	151	158	NA	60	124	113	NA
Mean age (years)	57.5	57.7	60.2	0.03	55.2	57.7	60.2	0.0005
Mean BMI (kg/m^2)	23.2	23.0	23.2	0.30	22.5	22.2	22.6	0.52
Alcohol use (%)‡	75.2	82.8	76.6	0.23	23.3	30.7	32.7	0.43
Heavy alcohol use (%)§	52.2	57.0	48.1	0.30	NS	NS	NS	NS
Ever-smoker (%)¶	82.0	81.5	81.0	0.98	28.3	17.7	22.1	0.26
Heavy smoker (%)††	36.0	33.8	31.7	0.71	NS	NS	NS	NS
Residents in Fukuoka City (%)	57.1	64.2	63.9	0.34	61.7	69.4	72.6	0.33

*P-values (two-sided) were based on the Kruskal–Wallis test for continuous variables and χ^2-test for proportions. †Physical activity during leisure time, commuting, housework, and shopping. ‡Drinking alcohol at least once per week 5 years before. §Drinking alcohol at least 1.0 unit per day. Because of a small number of heavy drinkers in women ($n = 19$), only results for men are presented. ¶Smoking cigarettes daily for at least 1 year. ††Smoking at least 800 cigarette-years. Because of a small number of heavy smokers in women ($n = 6$), only results for men are presented. NA, not applicable; NS, result not significant.

Table 3. Adjusted OR and 95% CI of colon and rectal cancer in relation to physical activity in men

	No. of controls	Colon		Proximal colon		Distal colon		Rectum	
		No.	OR (95% CI)†	No.	OR (95% CI)†	No.	OR (95% CI)†	No.	OR (95% CI)†
Job-related physical activity									
Sedentary	290	167	1.0 (referent)	56	1.0 (referent)	110	1.0 (referent)	138	1.0 (referent)
Moderate	75	37	0.9 (0.6–1.4)	16	1.2 (0.6–2.2)	21	0.8 (0.4–1.4)	33	0.9 (0.5–1.4)
Hard	105	44	0.7 (0.4–1.0)	16	0.7 (0.4–1.4)	28	0.6 (0.4–1.0)	37	0.6 (0.4–0.9)
Trend	NA	NA	$P = 0.06$	NA	$P = 0.45$	NA	$P = 0.047$	NA	$P = 0.02$
Total non-job physical activity‡									
0.0	161	87	1.0 (referent)	27	1.0 (referent)	60	1.0 (referent)	91	1.0 (referent)
0.1–15.9	151	83	0.9 (0.6–1.4)	33	1.2 (0.6–2.1)	49	0.8 (0.5–1.3)	61	0.6 (0.4–0.9)
16.0+	158	78	0.8 (0.5–1.2)	28	0.9 (0.5–1.7)	50	0.7 (0.4–1.1)	56	0.5 (0.3–0.8)
Trend	NA	NA	$P = 0.22$	NA	$P = 0.69$	NA	$P = 0.19$	NA	$P = 0.01$
Moderate or hard non-job physical activity‡									
0.0	184	105	1.0 (referent)	31	1.0 (referent)	74	1.0 (referent)	104	1.0 (referent)
0.1–14.9	137	68	0.8 (0.6–1.2)	29	1.1 (0.6–2.1)	38	0.7 (0.4–1.1)	53	0.6 (0.4–0.9)
15.0+	149	75	0.8 (0.5–1.1)	28	1.0 (0.6–1.8)	47	0.7 (0.4–1.0)	51	0.5 (0.3–0.8)
Trend	NA	NA	$P = 0.24$	NA	$P = 0.99$	NA	$P = 0.12$	NA	$P = 0.004$

†Adjustment was made for age, cigarette smoking, alcohol use, residential area, BMI of 10 years before, and non-job physical activities or job-related activities. ‡MET-hours/week. Physical activities during leisure time, commuting, housework, and shopping. NA, not applicable.

Table 4. Adjusted OR and 95% CI of colon and rectal cancer in relation to physical activity in women

	No. of controls	Colon		Proximal colon		Distal colon		Rectum	
		No.	OR (95% CI)†	No.	OR (95% CI)†	No.	OR (95% CI)†	No.	OR (95% CI)†
Job-related physical activity									
Sedentary	245	166	1.0 (referent)	72	1.0 (referent)	94	1.0 (referent)	107	1.0 (referent)
Active	52	24	0.7 (0.4–1.2)	15	1.2 (0.6–2.3)	9	0.4 (0.2–0.8)	25	1.1 (0.6–1.9)
Trend	NA	NA	$P = 0.18$	NA	$P = 0.65$	NA	$P = 0.02$	NA	$P = 0.81$
Total non-job physical activity‡									
0.0	60	41	1.0 (referent)	11	1.0 (referent)	30	1.0 (referent)	26	1.0 (referent)
0.1–15.9	124	78	0.9 (0.5–1.5)	37	1.5 (0.7–3.3)	41	0.7 (0.4–1.3)	61	1.2 (0.7–2.3)
16.0+	113	71	0.8 (0.5–1.4)	39	1.6 (0.7–3.6)	32	0.6 (0.3–1.1)	45	0.9 (0.5–1.8)
Trend	NA	NA	$P = 0.45$	NA	$P = 0.41$	NA	$P = 0.12$	NA	$P = 0.47$
Moderate or hard non-job physical activity‡									
0	69	47	1.0 (referent)	15	1.0 (referent)	32	1.0 (referent)	30	1.0 (referent)
0.1–14.9	121	81	1.0 (0.6–1.6)	37	1.3 (0.6–2.5)	44	0.8 (0.5–1.5)	62	1.3 (0.7–2.2)
15.0+	107	62	0.8 (0.5–1.4)	35	1.3 (0.6–2.7)	27	0.5 (0.3–1.1)	40	0.9 (0.5–1.7)
Trend	NA	NA	$P = 0.35$	NA	$P = 0.59$	NA	$P = 0.09$	NA	$P = 0.41$

†Adjustment was made for age, cigarette smoking, alcohol use, residential area, BMI of 10 years before, and non-job physical activities or job-related activities. ‡MET-hours/week. Physical activities at leisure time, commuting, housework, and shopping. NA, not applicable.

Table 5. Adjusted OR and 95% CI of colon and rectal cancer in relation to BMI of 10 years before in men and women

	No. of controls	Colon		Proximal colon		Distal colon		Rectum	
		No.	OR (95% CI)†	No.	OR (95% CI)†	No.	OR (95% CI)†	No.	OR (95% CI)†
BMI‡ of 10 years before									
Men									
<23.0	237	96	1.0 (referent)	39	1.0 (referent)	56	1.0 (referent)	91	1.0 (referent)
23.0–24.9	118	68	1.3 (0.9–2.0)	26	1.3 (0.8–2.3)	42	1.4 (0.9–2.3)	51	1.1 (0.7–1.7)
25.0+	115	84	1.7 (1.2–2.5)	23	1.2 (0.7–2.1)	61	2.1 (1.4–3.3)	66	1.5 (1.0–2.3)
Trend	NA	NA	$P = 0.0063$	NA	$P = 0.49$	NA	$P = 0.0009$	NA	$P = 0.049$
Women									
<23.0	182	120	1.0 (referent)	52	1.0 (referent)	68	1.0 (referent)	75	1.0 (referent)
23.0–24.9	56	32	0.8 (0.5–1.3)	17	0.9 (0.5–1.8)	15	0.7 (0.3–1.3)	29	1.5 (0.8–2.7)
25.0+	59	38	0.9 (0.5–1.4)	18	0.8 (0.4–1.6)	20	0.9 (0.5–1.6)	28	1.2 (0.7–2.1)
Trend	NA	NA	$P = 0.45$	NA	$P = 0.51$	NA	$P = 0.58$	NA	$P = 0.53$

†Adjustment was made for age, cigarette smoking, alcohol use, residential area, and physical activity. ‡kg/m². NA, not applicable.

associated with a significant increase in the risk of colon and rectal cancer in men. Increased risk associated with obesity was noted almost exclusively for distal colon cancer. However, there was no such association in women (Table 5).

Discussion

The present study adds to evidence that physical activity confers decreased risk of colon cancer, especially of distal colon cancer in both men and women. A notable finding in the present study was that physical activity was also protective against rectal cancer in men exclusively. Obesity was also related to increased risks of distal colon and rectal cancer in men only.

Most previous epidemiological studies have suggested physical activity reduces colon cancer risk independent of race, society, and sex, whereas associations of physical activity with rectal cancer risk have been inconsistent. At least 12 case-control studies[10,11,13–16,21–26] and three cohort studies[12,27,28] have examined the relationship between physical activity and rectal cancer risk. Some case-control studies found a decreased risk associated with occupational physical activity in men and women combined[11] and in men,[23] with leisure-time physical activity in men not in women,[13] and with total physical activity in both men and women.[22,25] Other case-control studies failed to find a protective association with physical activity assessed differently in men and women,[15,26] in men and women combined[21] and in men.[10,14,16,24] Colbert *et al.* observed a substantial decrease in the risk of rectal cancer associated with physically moderate to heavy work in a cohort study of male smokers,[28] but no association was observed between occupational activity and rectal cancer in either men or women,[12] and in men[27] in two other cohort studies. We have no clear explanation to our finding that physical activity was protective against rectal cancer only in men. The observed gender difference might have been due to chance. In the present study, both occupational activity and non-job physical activity were associated with reduced risk of rectal cancer in men, adding to evidence that physical activity is protective against rectal cancer.

As reviewed elsewhere, the suggested mechanisms of risk reduction of colon cancer by physical activity include shortening the bowel intestinal transit time, enhancing immune function, increasing prostaglandin F and decreasing prostaglandin E2, maintenance of insulin sensitivity, lowering activity of insulin-like growth factor, sex hormones and bile acid secretion, and decreasing adiposity.[5,29–31] However, it is unclear which mechanisms are important for colorectal cancer prevention. Taking the available information on site-specific influence into account,[32] our present results might provide some clarification, as the

doi: 10.1111/j.1349-7006.2006.00282.x

effects were limited to the distal colon and rectum. Hyperinsulinemia and type 2 diabetes mellitus have been shown to be associated with increased risk of proximal colon cancer rather than distal segment,[33,34] and the possibility of physical activity impacting on insulin levels does not appear to be a likely mechanism to explain our findings. However, our results for BMI are of great interest and difficult to explain.

Given the fact that the distal colon mainly functions in fecal storage, it could be that the characteristics of feces and their passage is most important. Constipation is a possible risk factor of colon cancer,[35–38] and this can be relieved by exercise.[39–42] High physical activity has been reported to be related to low prevalence of constipation in some,[43,44] but not all, studies.[45] One possibility that deserves further consideration is that there could be an association between exercise and sunlight, and therefore vitamin D levels, thought to be protective against neoplasia in the colon and rectum.[46–48]

Several previous studies suggested that more vigorous exercise had a stronger association with risk reduction than light or moderate activity.[9,25,49] The most frequent activities among participants in the present study were walking, gardening, and golf, these being light or moderate in intensity. In addition, more than half of participants reported that they never engaged in moderate or hard activities at leisure time. The observed decrease in the risk with moderate or hard activity was not greater than that with total activity.

Obesity has been shown to be associated with increased risk of colon cancer, but the association seems to be weaker and less consistent in women than in men.[5] Although reasons for the gender difference in risk have not been clarified, estrogen might be an explanation. Several observational studies reported a reduced risk of colon cancer associated with hormone replacement therapy.[50–52] It has also been observed that obesity is not associated with increased risk of colon cancer in postmenopausal women in several observational studies.[53–55] Increased estrogen levels related to obesity, particularly in postmenopausal women, probably confer reduction in the risk linked with obesity. Again, the present study showed the gender difference in the association between obesity and rectal cancer. This finding is in agreement with the previous observations in case-control studies in Sweden[56] and in Hawaii.[13]

The present case-control study has advantages of being a population-based study with an adequate number of participants, who were all interviewed about all types of physical activity, with attention to the job setting and calculation of energy expenditure. However, there were several limitations that should be noted. The first is that we did not directly validate the method of assessing physical activities, although the measurement was highly reproducible. Another limitation concerned the referent period as to physical activity. In the present study, physical activity only in the recent past was ascertained. Long-term, habitual physical exercise at work or leisure has been suggested to be more protective.[57,58] Dietary factors such as vegetables and red meat, which seem to modify the risk of colorectal cancer, were not taken into account in the present study. We actually carried out a dietary survey in the Fukuoka Colorectal Cancer Study, but validation of the dietary assessment was not completed. It is unlikely that the observed associations with physical activity and obesity were due to the uncontrolled confounding effect of dietary factors. Reduced risk of colon or colorectal cancer associated with physical activity has been observed regardless of adjustment for dietary factors in many studies.[9,11,13,15,21,24,25]

Despite some inconsistencies with earlier studies, the present investigation suggests that physical activity and weight control are important for prevention of cancer in the distal colon and rectum, especially in males.

Acknowledgments

This work was supported by a Grant-in-Aid for Scientific Research on Priority Areas (12218226) from the Ministry of Education, Culture, Sports, Science and Technology, Japan. The authors acknowledge support from Emeritus Professor Keizo Sugimachi; Professors Seiyo Ikeda, Takayuki Shirakusa, and Sumitaka Arima; and Drs Motonori Saku, Yoichi Ikeda, Soichiro Maekawa, Kazuo Tanoue, Kinjiro Sumiyoshi, and Shoichiro Saito in conducting the survey of cases. The following physicians kindly supervised the survey of controls at their clinics: Drs Hideaki Baba, Tomonori Endo, Hiroshi Hara, Yoichiro Hirokata, Motohisa Ikeda, Masayoshi Ishibashi, Fumiaki Itoh, Yasuhiro Iwanaga, Hideki Kaku, Shoshi Kaku, Minoru Kanazawa, Akira Kobayashi, Ryunosuke Kumashiro, Shinichi Matsumoto, Soukei Mioka, Umeji Miyakoda, Osamu Nakagaki, Nobuyoshi Nogawa, Nobuyuki Ogami, Toyoaki Okabayashi, Hironao Okabe, Nishiki Saku, Masafumi Tanaka, Masahiro Ueda, Bunichi Ushio, and Koheisho Yasunaga. The authors are grateful to research nurses: Ms. Nobuko Taguchi, Yuriko Moroe, Yuko Noda, Ryoko Tanaka, Hisako Nakagawa, and Yoko Mikasa, and research clerk Ms. Hiroko Mizuta, for their self-sacrificing work.

References

1 Parkin DM, Bray F, Ferlay J, Pisani P. Estimating the world cancer burden: Globocan 2000. *Int J Cancer* 2001; **94**: 153–6.
2 Kono S. Dietary factors for gastrointestinal cancers: a worldwide overview. *GANN Monogr Cancer Res* 1996; **44**: 29–39.
3 Parkin DM. *Cancer Incidence in Five Continents*, Vol. VIII. Lyon: IARC Press, 2002.
4 Kono S. Secular trend of colon cancer incidence and mortality in relation to fat and meat intake in Japan. *Eur J Cancer Prev* 2004; **13**: 127–32.
5 International Agency for Research on Cancer. *IARC Handbooks of Cancer Prevention: Vol. 6 Weight Control and Physical Activity*. Lyon: IARC Press, 2002.
6 World Cancer Research Fund and American Institute for Cancer Research. *Food, Nutrition and the Prevention of Cancer: A Global Perspective*. Washington DC: American Institute for Cancer Research, 1997.
7 Martinez ME, Giovannucci E, Spiegelman D, Hunter DJ, Willett WC, Colditz GA. Leisure-time physical activity, body size, and colon cancer in women. Nurses' Health Study Research Group. *J Natl Cancer Inst* 1997; **89**: 948–55.
8 Fredriksson M, Bengtsson NO, Hardell L, Axelson O. Colon cancer, physical activity, and occupational exposures. A case-control study. *Cancer* 1989; **63**: 1838–42.
9 Giovannucci E, Ascherio A, Rimm EB, Colditz GA, Stampfer MJ, Willett WC. Physical activity, obesity, and risk for colon cancer and adenoma in men. *Ann Intern Med* 1995; **122**: 327–34.
10 Garabrant DH, Peters JM, Mack TM, Bernstein L. Job activity and colon cancer risk. *Am J Epidemiol* 1984; **119**: 1005–14.
11 Levi F, Pasche C, Lucchini F, Tavani A, La Vecchia C. Occupational and leisure-time physical activity and the risk of colorectal cancer. *Eur J Cancer Prev* 1999; **8**: 487–93.
12 Thune I, Lund E. Physical activity and risk of colorectal cancer in men and women. *Br J Cancer* 1996; **73**: 1134–40.
13 Le Marchand L, Wilkens LR, Kolonel LN, Hankin JH, Lyu LC. Associations of sedentary lifestyle, obesity, smoking, alcohol use, and diabetes with the risk of colorectal cancer. *Cancer Res* 1997; **57**: 4787–94.
14 Kato I, Tominaga S, Ikari A. A case-control study of male colorectal cancer in Aichi Prefecture, Japan: with special reference to occupational activity level, drinking habits and family history. *Jpn J Cancer Res* 1990; **81**: 115–21.
15 Tavani A, Braga C, La Vecchia C *et al.* Physical activity and risk of cancers of the colon and rectum: an Italian case-control study. *Br J Cancer* 1999; **79**: 1912–6.
16 Fraser G, Pearce N. Occupational physical activity and risk of cancer of the colon and rectum in New Zealand males. *Cancer Causes Control* 1993; **4**: 45–50.
17 Kono S, Toyomura K, Yin G, Nagano J, Mizoue T. A case-control study of colorectal cancer in relation to lifestyle factors and genetic polymorphisms: design and conduct of the Fukuoka colorectal cancer study. *Asian Pac J Cancer Prev* 2004; **5**: 393–400.
18 Parkin DM, Whelan SL, Ferlay J, Raymond L, JY. *Cancer Incidence in Five Continents*, Vol. VII. Lyon: IARC Press, 1997.

19 Ainsworth BE, Haskell WL, Whitt MC *et al.* Compendium of physical activities: an update of activity codes and MET intensities. *Med Sci Sports Exerc* 2000; **32**: S498–504.
20 Tsugane S, Sasaki S, Tsubono Y. Under- and overweight impact on mortality among middle-aged Japanese men and women: a 10-y follow-up of JPHC study cohort I. *Int J Obes Relat Metab Disord* 2002; **26**: 529–37.
21 Gerhardsson de Verdier M, Steineck G, Hagman U, Rieger A, Norell SE. Physical activity and colon cancer: a case-referent study in Stockholm. *Int J Cancer* 1990; **46**: 985–9.
22 Whittemore AS, Wu-Williams AH, Lee M *et al.* Diet, physical activity, and colorectal cancer among Chinese in North America and China. *J Natl Cancer Inst* 1990; **82**: 915–26.
23 Markowitz S, Morabia A, Garibaldi K, Wynder E. Effect of occupational and recreational activity on the risk of colorectal cancer among males: a case-control study. *Int J Epidemiol* 1992; **21**: 1057–62.
24 Longnecker MP, Gerhardsson le Verdier M, Frumkin H, Carpenter C. A case-control study of physical activity in relation to risk of cancer of the right colon and rectum in men. *Int J Epidemiol* 1995; **24**: 42–50.
25 Slattery ML, Edwards S, Curtin K *et al.* Physical activity and colorectal cancer. *Am J Epidemiol* 2003; **158**: 214–24.
26 Mao Y, Pan S, Wen SW, Johnson KC. Physical inactivity, energy intake, obesity and the risk of rectal cancer in Canada. *Int J Cancer* 2003; **105**: 831–7.
27 Gerhardsson M, Norell SE, Kiviranta H, Pedersen NL, Ahlbom A. Sedentary jobs and colon cancer. *Am J Epidemiol* 1986; **123**: 775–80.
28 Colbert LH, Hartman TJ, Malila N *et al.* Physical activity in relation to cancer of the colon and rectum in a cohort of male smokers. *Cancer Epidemiol Biomarkers Prev* 2001; **10**: 265–8.
29 Quadrilatero J, Hoffman-Goetz L. Physical activity and colon cancer. A systematic review of potential mechanisms. *J Sports Med Phys Fitness* 2003; **43**: 121–38.
30 Friedenreich CM, Orenstein MR. Physical activity and cancer prevention: etiologic evidence and biological mechanisms. *J Nutr* 2002; **132**: 3456S–64S.
31 Bianchini F, Kaaks R, Vainio H. Weight control and physical activity in cancer prevention. *Obes Rev* 2002; **3**: 5–8.
32 Moore MA, Sobue T, Kuriki K, Tajima K, Tokudome S, Kono S. Comparison of Japanese, American-Whites and African-Americans – pointers to risk factors to underlying distribution of tumours in the colorectum. *Asian Pac J Cancer Prev* 2005; **6**: 412–9.
33 Hu FB, Manson JE, Liu S *et al.* Prospective study of adult onset diabetes mellitus (type 2) and risk of colorectal cancer in women. *J Natl Cancer Inst* 1999; **91**: 542–7.
34 Limburg PJ, Anderson KE, Johnson TW *et al.* Diabetes mellitus and subsite-specific colorectal cancer risks in the Iowa Women's Health Study. *Cancer Epidemiol Biomarkers Prev* 2005; **14**: 133–7.
35 Ghadirian P, Maisonneuve P, Perret C, Lacroix A, Boyle P. Epidemiology of sociodemographic characteristics, lifestyle, medical history, and colon cancer: a case-control study among French Canadians in Montreal. *Cancer Detect Prev* 1998; **22**: 396–404.
36 Jacobs EJ, White E. Constipation, laxative use, and colon cancer among middle-aged adults. *Epidemiology* 1998; **9**: 385–91.
37 Watanabe T, Nakaya N, Kurashima K, Kuriyama S, Tsubono Y, Tsuji I. Constipation, laxative use and risk of colorectal cancer: The Miyagi Cohort Study. *Eur J Cancer* 2004; **40**: 2109–15.
38 Roberts MC, Millikan RC, Galanko JA, Martin C, Sandler RS. Constipation, laxative use, and colon cancer in a North Carolina population. *Am J Gastroenterol* 2003; **98**: 857–64.
39 De Schryver AM, Keulemans YC, Peters HP *et al.* Effects of regular physical activity on defecation pattern in middle-aged patients complaining of chronic constipation. *Scand J Gastroenterol* 2005; **40**: 422–9.
40 Koffler KH, Menkes A, Redmond RA, Whitehead WE, Pratley RE, Hurley BF. Strength training accelerates gastrointestinal transit in middle-aged and older men. *Med Sci Sports Exerc* 1992; **24**: 415–9.
41 Oettle GJ. Effect of moderate exercise on bowel habit. *Gut* 1991; **32**: 941–4.
42 Cordain L, Latin RW, Behnke JJ. The effects of an aerobic running program on bowel transit time. *J Sports Med Phys Fitness* 1986; **26**: 101–4.
43 Dukas L, Willett WC, Giovannucci EL. Association between physical activity, fiber intake, and other lifestyle variables and constipation in a study of women. *Am J Gastroenterol* 2003; **98**: 1790–6.
44 Nakaji S, Tokunaga S, Sakamoto J, Todate M, Shimoyama T, Umeda T *et al.* Relationship between lifestyle factors and defecation in a Japanese population. *Eur J Nutr* 2002; **41**: 244–8.
45 Klauser AG, Peryrl C, Schindlbeck NE, Muller-Lissner SA. Nutrition and physical activity in chronic constipation. *European J Gastroenterol Hepatol* 1992; **4**: 227–33.
46 Cho E, Smith-Warner SA, Spiegelman D *et al.* Dairy foods, calcium, and colorectal cancer: a pooled analysis of 10 cohort studies. *J Natl Cancer Inst* 2004; **96**: 1015–22.
47 Martinez ME, Willett WC. Calcium, vitamin D, and colorectal cancer: a review of the epidemiologic evidence. *Cancer Epidemiol Biomarkers Prev* 1998; **7**: 163–8.
48 Garland CF, Garland FC. Do sunlight and vitamin D reduce the likelihood of colon cancer? *Int J Epidemiol* 1980; **9**: 227–31.
49 Lee IM, Manson JE, Ajani U, Paffenbarger RS Jr, Hennekens CH, Buring JE. Physical activity and risk of colon cancer: the Physicians' Health Study (United States). *Cancer Causes Control* 1997; **8**: 568–74.
50 Calle EE, Miracle-McMahill HL, Thun MJ, Heath CW Jr. Estrogen replacement therapy and risk of fatal colon cancer in a prospective cohort of postmenopausal women. *J Natl Cancer Inst* 1995; **87**: 517–23.
51 Persson I, Yuen J, Bergkvist L, Schairer C. Cancer incidence and mortality in women receiving estrogen and estrogen-progestin replacement therapy – long-term follow-up of a Swedish cohort. *Int J Cancer* 1996; **67**: 327–32.
52 Kampman E, Potter JD, Slattery ML, Caan BJ, Edwards S. Hormone replacement therapy, reproductive history, and colon cancer: a multicenter, case-control study in the United States. *Cancer Causes Control* 1997; **8**: 146–58.
53 Terry P, Giovannucci E, Bergkvist L, Holmberg L, Wolk A. Body weight and colorectal cancer risk in a cohort of Swedish women: relation varies by age and cancer site. *Br J Cancer* 2001; **85**: 346–9.
54 Terry PD, Miller AB, Rohan TE. Obesity and colorectal cancer risk in women. *Gut* 2002; **51**: 191–4.
55 Slattery ML, Ballard-Barbash R, Edwards S, Caan BJ, Potter JD. Body mass index and colon cancer: an evaluation of the modifying effects of estrogen (United States). *Cancer Causes Control* 2003; **14**: 75–84.
56 Gerhardsson de Verdier M, Hagman U, Steineck G, Rieger A, Norell SE. Diet, body mass and colorectal cancer: a case-referent study in Stockholm. *Int J Cancer* 1990; **46**: 832–8.
57 Lee IM, Paffenbarger RS Jr, Hsieh C. Physical activity and risk of developing colorectal cancer among college alumni. *J Natl Cancer Inst* 1991; **83**: 1324–9.
58 Slattery ML, Edwards SL, Ma KN, Friedman GD, Potter JD. Physical activity and colon cancer: a public health perspective. *Ann Epidemiol* 1997; **7**: 137–45.

doi: 10.1111/j.1349-7006.2006.00282.x