6.3 mg/dl; 0.01% pravastatin, 88.5 ± 6.2 mg/dl; 0.1% pravastatin, 81.6 ± 2.6 mg/dl; n = 4–5 in each group). However, 0.1% but not 0.01% pravastatin significantly increased hepatic mRNA levels of HMG-CoA synthase 1 (Fig. 3A). Consistent with the in vitro findings of HepG2 cells, pravastatin treatment did not affect Abca1 transcript levels in the livers (Fig. 3B). Moreover, 0.1% pravastatin significantly increased LXRα mRNA levels (Fig. 3C). In the leukocytes, 2-week treatment with 0.1% pravastatin did not significantly affect either transcript levels of HMG-CoA synthase 1 (control, 1 ± 0.07; pravastatin, 1.06 ± 0.07; n = 4 in each group) or Abca1 (control, 1 ± 0.06; pravastatin, 1.05 ± 0.12), whereas the drug increased LXR mRNA levels (control, 1 ± 0.06; pravastatin, 1.38 ± 0.10; $P < 0.05$).

Because it remained possible that some of the effects of pravastatin observed in vitro were transient, we further investigated the effects of 24-h treatment with 0.1% pravastatin. Serum total cholesterol concentrations did not differ between the two groups (control, 73.5 ± 1.9 mg/dl; pravastatin, 72.3 ± 4.3 mg/dl; n = 4 in each group). As shown in Fig. 4A, the treatment still did not significantly increase hepatic HMG-CoA synthase 1 mRNA levels, but it had already increased LXRα transcript levels. Interestingly, pravastatin tended to increase hepatic Abca1 mRNA levels. In the leukocytes, 24-h pravastatin treatment also increased LXRα mRNA expression without affecting Abca1 mRNA levels (Fig. 4B).

Effects of Pravastatin on ABCA1 Protein Levels. We further investigated whether pravastatin affects protein levels of ABCA1. In RAW264.7 cells cultured without 22(*R*)-OHC, ABCA1 level was very low, and obvious effect of pravastatin was not detected (Fig. 5A). Moreover, in concordance with the effects on mRNA expression, pravastatin did not affect ABCA1 mass in RAW264.7 cells cultured with 22(*R*)-OHC (Fig. 5A) nor in HepG2 cells (Fig. 5B). Furthermore, oral dosing of pravastatin for 2 weeks to mice did not affect hepatic or leukocyte ABCA1 protein levels (Fig. 6).

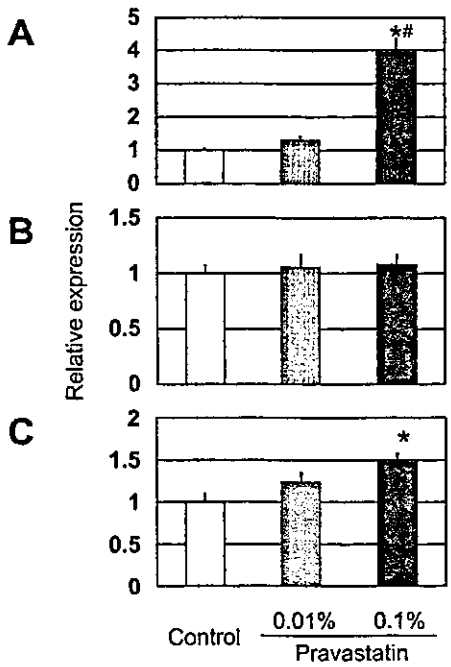

Fig. 3. Effects of 2-week pravastatin treatment on hepatic mRNA expression levels of HMG-CoA synthase 1 (A), Abca1 (B), and LXRα (C) in mice. Male C57BL/6 mice were divided into three groups at 8 weeks of age. These animals were given drinking water with or without pravastatin (0.01 or 0.1%) for 2 weeks, and thereafter mRNA expression levels of the target genes in the whole-liver were determined by the real-time quantitative reverse transcription-PCR. Data are means + S.E. of four to five mice in each group and expressed as relative value to control. *, $P < 0.05$ versus control. #, $P < 0.05$ versus 0.01% pravastatin.

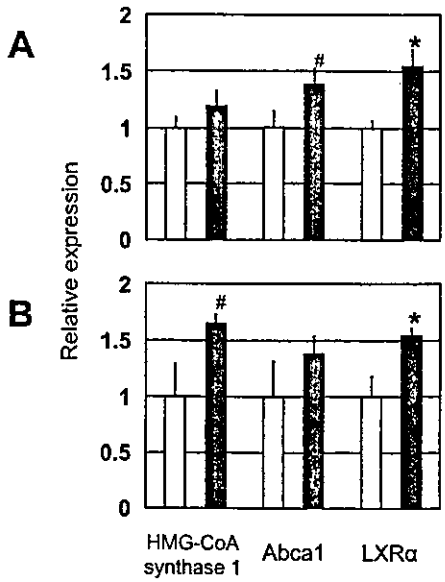

Fig. 4. Effects of 24-h pravastatin treatment on mRNA expression levels of HMG-CoA synthase 1, Abca1, and LXRα in the liver (A) and the leukocytes (B). Male C57BL/6 mice were divided into two groups at 8 weeks of age. These animals were given drinking water with (closed column) or without (open column) 0.1% pravastatin for 24 h, and thereafter hepatic and leukocyte transcript levels of the target genes were determined by the real-time quantitative reverse trnascription-PCR. Data are means + S.E. of three to four mice in each group and expressed as relative value to control. *, $P < 0.05$, #, $P < 0.1$ versus control.

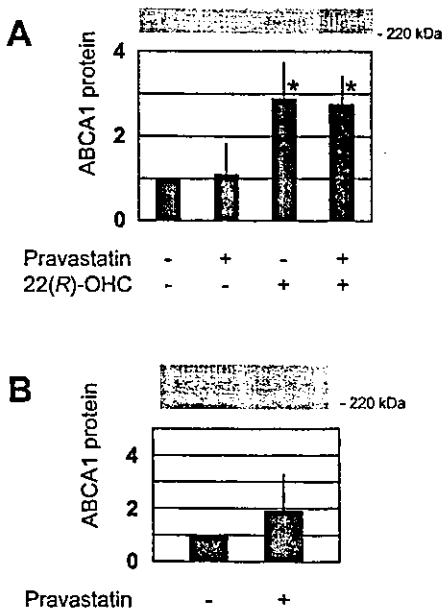

Fig. 5. Effects of pravastatin on ABCA1 protein levels in RAW264.7 (A) and HepG2 cells (B). Cells were cultured for 48 h in DMEM supplemented with 10% LPDS ± pravastatin (10 μM) in the presence or absence of 22(*R*)-OHC (25 μM). Western blot analysis of ABCA1 was performed as described under *Materials and Methods*. Equal quantities of protein (40 μg) were run in each lane. Samples from one representative experiment are shown. Data are means + S.E. of three independent experiments and expressed as relative value to control. *, $P < 0.05$ versus control.

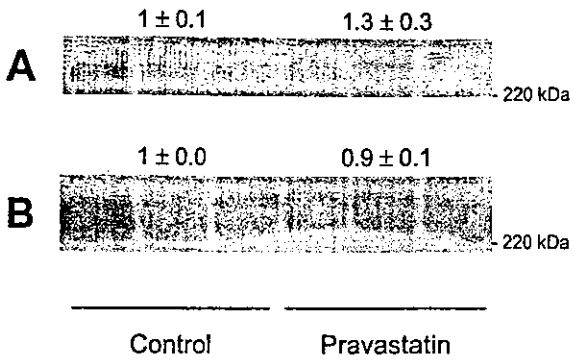

Fig. 6. Effects of pravastatin treatment on ABCA1 protein levels of the liver (A) and the leukocytes (B) in mice. Male C57BL/6 mice were divided into two groups at 8 weeks of age. These animals were given drinking water with or without 0.1% pravastatin for 2 weeks, and thereafter hepatic and leukocyte ABCA1 protein levels were determined by the Western blot analysis. Equal quantities of protein (40 μg) were run in each lane. Data are means ± S.E. of three mice in each group and expressed as relative value to control.

Discussion

HMG-CoA reductase inhibitors, which are widely used in the treatment of hypercholesterolemia, not only reduce LDL cholesterol but also increase HDL cholesterol (National Cholesterol Education Program, 2001). The increase in HDL is reported to be accompanied by the enhanced production of apolipoprotein A-I (Schaefer et al., 1999). Therefore, HMG-CoA reductase inhibitors seem to activate the reverse cholesterol transport pathway, especially at the first step. On the other hand, the previous studies clearly demonstrated that in vitro inhibition of HMG-CoA reductase causes the suppression of LXR activity (Forman et al., 1997). Because LXR regulates the expression of ABCA1, which is essential for the first step of the reverse cholesterol transport pathway, we examined the effects of HMG-CoA reductase inhibitors on ABCA1 expression.

Consistent with the findings of the effects on LXR activity, this study showed that pravastatin suppresses ABCA1 mRNA expression in RAW264.7 macrophages in the absence but not in the presence of an LXR agonist. Contrary to the results in RAW264.7 cells, pravastatin did not decrease ABCA1 mRNA levels in HepG2 hepatocytes. Moreover, in vivo treatment with pravastatin did not suppress either hepatic or leukocyte ABCA1 mRNA expression in mice. The precise mechanism that HMG-CoA reductase inhibition does not decrease ABCA1 mRNA levels in HepG2 cells or in vivo is not clear, but the present and previous results suggest the following mechanisms.

First, the increase in LXRα expression levels by pravastatin might compensate the suppression of LXR activity induced by the decrease of mevalonic acid. HMG-CoA reductase inhibitors have been shown to activate peroxisome proliferators-activated receptor α (Martin et al., 2001) and γ (Fajas et al., 1999). Ligand activation of either nuclear receptor can lead to primary induction of LXRα and subsequent induction of ABCA1 (Chawla et al., 2001; Chinetti et al., 2001). The results obtained in this study showed for the first time that in vivo treatment with pravastatin induces LXRα mRNA expression.

Second, the decrease of geranylgeranyl pyrophosphate, one of the major products of mevalonate pathway, by HMG-CoA reductase inhibition may be involved in the changes of ABCA1 mRNA expression. It has been reported that geranylgeranyl pyrophosphate can reduce ABCA1 expression by acting as an LXR antagonist (Forman et al., 1997; Gan et al., 2001) and through the activation of Rho proteins (Gan et al., 2001). It is possible that the depletion of geranylgeranyl pyrophosphate might obviously affect ABCA1 expression in presence but not in absence of LXR agonists.

Finally, there may be enough endogenous LXR agonists in HepG2 cells and in vivo, such as oxysterols, to compensate the decrease of mevalonic acid. In fact, ABCA1 mass in HepG2 cells or in vivo was more than that of RAW264.7 cells (cf. Figs. 5 and 6). In RAW264.7 cells, not only 22(*R*)-OHC but also cholesterol compensated the effects of HMG-CoA reductase inhibition on Abca1 and Abcg1 transcript levels (Fig. 1, B and C). Denis et al. (2003) also reported that cholesterol could increase ABCA1 expression levels probably through the formation of hydroxysterols in fibroblasts. Because both macrophages in atherosclerotic lesions and hepatocytes are relatively rich in cholesterol and oxysterols, it dose not seem likely that HMG-CoA reductase inhibitors suppress ABCA1 expression in those cells that are main components in the reverse cholesterol transport pathway. Moreover, ABCA1 mRNA levels in RAW264.7 cells were less than 1% of those in the murine leukocytes (data not shown), and its protein levels were very low without induction by LXR agonists (Fig. 5A). Accordingly, the effects of HMG-CoA reductase inhibitors in lowering ABCA1 expression from very low to even lower under these conditions might not be important.

Currently, LXR-activating therapy is expected to decrease atherosclerosis also in human (Lund et al., 2003). Administration of synthetic LXR agonists actually can inhibit the development of atherosclerosis in mice, but it also causes fatty liver and hypertriglyceridemia at least partly by the induction of sterol regulatory element binding protein-1c (Lund et al., 2003). Therefore, some strategies for dissociating antiatherosclerotic from triglyceride raising effects are needed to use LXR agonists for the treatment or prevention of cardiovascular disease. On the other hand, many previous data demonstrate both efficacy and safety of HMG-CoA reductase inhibitors and further suggest that they have not only lipid-lowering but various pleiotropic effects such as on macrophages (Ando et al., 2000, 2003; Ota et al., 2003). Because LXR agonists may also have various effects, it must be carefully evaluated whether addition of ABCA1-activating therapy to HMG-CoA reductase inhibitor treatment improves the outcome of patients with cardiovascular disease.

In conclusion, although the inhibition of HMG-CoA reductase suppressed ABCA1 mRNA expression in RAW264.7 macrophages in the absence of LXR agonists, pravastatin did not reduce its levels in the macrophages cultured with LXR agonists or HepG2 hepatocytes. Oral dosing of pravastatin to mice did not suppress either hepatic or leukocyte ABCA1 expression. It is unlikely that pravastatin has adverse effects on the action of the reverse cholesterol transport pathway by inhibiting ABCA1 expression.

References

Aiello RJ, Brees D, Bourassa PA, Royer L, Lindsey S, Coskran T, Haghpassand M, and Francone OL (2002) Increased atherosclerosis in hyperlipidemic mice with inactivation of ABCA1 in macrophages. *Arterioscler Thromb Vasc Biol* 22:630–637.

Ando H, Takamura T, Kobayashi K, Misu H, and Osawa K (2003) Does pravastatin affect circulating levels of soluble TNF receptor 2 in hypercholesterolemic patients? *Atherosclerosis* 166:413–414.

Ando H, Takamura T, Ota T, Nagai Y, and Kobayashi K (2000) Cerivastatin improves survival of mice with lipopolysaccharide-induced sepsis. *J Pharmacol Exp Ther* **294**:1043–1046.

Basso F, Freeman L, Knapper CL, Remaley A, Stonik J, Neufeld EB, Tansey T, Amar MJ, Fruchart-Najib J, Duverger N, et al. (2003) Role of the hepatic ABCA1 transporter in modulating intrahepatic cholesterol and plasma HDL cholesterol concentrations. *J Lipid Res* **44**:296–302.

Cao G, Beyer TP, Yang XP, Schmidt RJ, Zhang Y, Bensch WR, Kauffman RF, Gao H, Ryan TP, Liang Y, et al. (2002) Phospholipid transfer protein is regulated by liver X receptors in vivo. *J Biol Chem* **277**:39561–39565.

Chawla A, Boisvert WA, Lee CH, Laffitte BA, Barak Y, Joseph SB, Liao D, Nagy L, Edwards PA, Curtiss LK, et al. (2001) A PPAR gamma-LXR-ABCA1 pathway in macrophages is involved in cholesterol efflux and atherogenesis. *Mol Cell* **7**:161–171.

Chinetti G, Lestavel S, Bocher V, Remaley AT, Neve B, Torra IP, Teissier E, Minnich A, Jaye M, Duverger N, et al. (2001) PPAR-alpha and PPAR-gamma activators induce cholesterol removal from human macrophage foam cells through stimulation of the ABCA1 pathway. *Nat Med* **7**:53–58.

Christiansen-Weber TA, Voland JR, Wu Y, Ngo K, Roland BL, Nguyen S, Peterson PA, and Fung-Leung WP (2000) Functional loss of ABCA1 in mice causes severe placental malformation, aberrant lipid distribution and kidney glomerulonephritis as well as high-density lipoprotein cholesterol deficiency. *Am J Pathol* **157**:1017–1029.

Costet P, Luo Y, Wang N, and Tall AR (2000) Sterol-dependent transactivation of the ABC1 promoter by the liver X receptor/retinoid X receptor. *J Biol Chem* **275**: 28240–28245.

Denis M, Bissonnette R, Haidar B, Krimbou L, Bouvier M, and Genest J (2003) Expression, regulation and activity of ABCA1 in human cell lines. *Mol Genet Metab* **78**:265–274.

Fajas L, Schoonjans K, Gelman L, Kim JB, Najib J, Martin G, Fruchart JC, Briggs M, Spiegelman BM, and Auwerx J (1999) Regulation of peroxisome proliferator-activated receptor gamma expression by adipocyte differentiation and determination factor 1/sterol regulatory element binding protein 1: implications for adipocyte differentiation and metabolism. *Mol Cell Biol* **19**:5495–5503.

Forman BM, Ruan B, Chen J, Schroepfer GJ Jr, and Evans RM (1997) The orphan nuclear receptor LXRalpha is positively and negatively regulated by distinct products of mevalonate metabolism. *Proc Natl Acad Sci USA* **94**:10588–10593.

Francis GA, Knopp RH, and Oram JF (1995) Defective removal of cellular cholesterol and phospholipids by apolipoprotein A-I in Tangier disease. *J Clin Investig* **96**: 78–87.

Gan X, Kaplan R, Menke JG, MacNaul K, Chen Y, Sparrow CP, Zhou G, Wright SD, and Cai TQ (2001) Dual mechanisms of ABCA1 regulation by geranylgeranyl pyrophosphate. *J Biol Chem* **276**:48702–48708.

Glomset JA (1968) The plasma lecithins:cholesterol acyltransferase reaction. *J Lipid Res* **9**:155–167.

Goldstein JL and Brown MS (1990) Regulation of the mevalonate pathway. *Nature (Lond)* **343**:425–430.

Grefhorst A, Elzinga BM, Voshol PJ, Plosch T, Kok T, Bloks VW, van der Sluijs FH, Havekes LM, Romijn JA, Verkade HJ, et al. (2002) Stimulation of lipogenesis by pharmacological activation of the liver X receptor leads to production of large, triglyceride-rich very low density lipoprotein particles. *J Biol Chem* **277**:34182–34190.

Haghpassand M, Bourassa PA, Francone OL, and Aiello RJ (2001) Monocyte/macrophage expression of ABCA1 has minimal contribution to plasma HDL levels. *J Clin Investig* **108**:1315–1320.

Joseph SB, McKilligin E, Pei L, Watson MA, Collins AR, Laffitte BA, Chen M, Noh G, Goodman J, Hagger GN, et al. (2002) Synthetic LXR ligand inhibits the development of atherosclerosis in mice. *Proc Natl Acad Sci USA* **99**:7604–7609.

Kwak BR, Veillard N, Pelli G, Mulhaupt F, James RW, Chanson M, and Mach F (2003) Reduced connexin43 expression inhibits atherosclerotic lesion formation in low-density lipoprotein receptor-deficient mice. *Circulation* **107**:1033–1039.

Lund EG, Menke JG, and Sparrow CP (2003) Liver X receptor agonists as potential therapeutic agents for dyslipidemia and atherosclerosis. *Arterioscler Thromb Vasc Biol* **23**:1169–1177.

Martin G, Duez H, Blanquart C, Berezowski V, Poulain P, Fruchart JC, Najib-Fruchart J, Glineur C, and Staels B (2001) Statin-induced inhibition of the Rho-signaling pathway activates PPARalpha and induces HDL apoA-I. *J Clin Investig* **107**:1423–1432.

McNeish J, Aiello RJ, Guyot D, Turi T, Gabel C, Aldinger C, Hoppe KL, Roach ML, Royer LJ, de Wet J, et al. (2000) High density lipoprotein deficiency and foam cell accumulation in mice with targeted disruption of ATP-binding cassette transporter-1. *Proc Natl Acad Sci USA* **97**:4245–4250.

Narisawa T, Fukaura Y, Terada K, Umezawa A, Tanida N, Yazawa K, and Ishikawa C (1994) Prevention of 1,2-dimethylhydrazine-induced colon tumorigenesis by HMG-CoA reductase inhibitors, pravastatin and simvastatin, in ICR mice. *Carcinogenesis* **15**:2045–2048.

National Cholesterol Education Program (2001) Executive summary of the third report of the National Cholesterol Education Program (NCEP) expert panel on detection, evaluation, and treatment of high blood cholesterol in adults (adult treatment panel III). *J Am Med Assoc* **285**:2486–2497.

Ota T, Takamura T, Ando H, Nohara E, Yamashita H, and Kobayashi K (2003) Preventive effect of cerivastatin on diabetic nephropathy through suppression of glomerular macrophage recruitment in a rat model. *Diabetologia* **46**:843–851.

Remaley AT, Schumacher UK, Stonik JA, Farsi BD, Nazih H, and Brewer HB Jr (1997) Decreased reverse cholesterol transport from Tangier disease fibroblasts. Acceptor specificity and effect of brefeldin on lipid efflux. *Arterioscler Thromb Vasc Biol* **17**:1813–1821.

Repa JJ, Turley SD, Lobaccaro JA, Medina J, Li L, Lustig K, Shan B, Heyman RA, Dietschy JM, and Mangelsdorf DJ (2000) Regulation of absorption and ABC1-mediated efflux of cholesterol by RXR heterodimers. *Science (Wash DC)* **289**:1524–1529.

Rogler G, Trumbach B, Klima B, Lackner KJ, and Schmitz G (1995) HDL-mediated efflux of intracellular cholesterol is impaired in fibroblasts from Tangier disease patients. *Arterioscler Thromb Vasc Biol* **15**:683–690.

Sasaki M, Bharwani S, Jordan P, Joh T, Manas K, Warren A, Harada H, Carter P, Elrod JW, Wolcott M, et al. (2003) The 3-hydroxy-3-methylglutaryl-CoA reductase inhibitor pravastatin reduces disease activity and inflammation in dextran-sulfate induced colitis. *J Pharmacol Exp Ther* **305**:78–85.

Schaefer JR, Schweer H, Ikewaki K, Stracke H, Seyberth HJ, Kaffarnik H, Maisch B, and Steinmetz A (1999) Metabolic basis of high density lipoproteins and apolipoprotein A-I increase by HMG-CoA reductase inhibition in healthy subjects and a patient with coronary artery disease. *Atherosclerosis* **144**:177–184.

Schultz JR, Tu H, Luk A, Repa JJ, Medina JC, Li L, Schwendner S, Wang S, Thoolen M, Mangelsdorf DJ, et al. (2000) Role of LXRs in control of lipogenesis. *Genes Dev* **14**:2831–2838.

Schuster GU, Parini P, Wang L, Alberti S, Steffensen KR, Hansson GK, Angelin B, and Gustafsson JA (2002) Accumulation of foam cells in liver X receptor-deficient mice. *Circulation* **106**:1147–1153.

Singaraja RR, Brunham LR, Visscher H, Kastelein JJ, and Hayden MR (2003) Efflux and atherosclerosis: the clinical and biochemical impact of variations in the ABCA1 gene. *Arterioscler Thromb Vasc Biol* **23**:1322–1332.

Su YR, Linton MF, and Fazio S (2002) Rapid quantification of murine ABC mRNAs by real time reverse transcriptase-polymerase chain reaction. *J Lipid Res* **43**: 2180–2187.

Tangirala RK, Bischoff ED, Joseph SB, Wagner BL, Walczak R, Laffitte BA, Daige CL, Thomas D, Heyman RA, Mangelsdorf DJ, et al. (2002) Identification of macrophage liver X receptors as inhibitors of atherosclerosis. *Proc Natl Acad Sci USA* **99**:11896–11901.

van Eck M, Bos IS, Kaminski WE, Orso E, Rothe G, Twisk J, Bottcher A, Van Amersfoort ES, Christiansen-Weber TA, Fung-Leung WP, et al. (2002) Leukocyte ABCA1 controls susceptibility to atherosclerosis and macrophage recruitment into tissues. *Proc Natl Acad Sci USA* **99**:6298–6303.

Venkateswaran A, Laffitte BA, Joseph SB, Mak PA, Wilpitz DC, Edwards PA, and Tontonoz P (2000a) Control of cellular cholesterol efflux by the nuclear oxysterol receptor LXR alpha. *Proc Natl Acad Sci USA* **97**:12097–12102.

Venkateswaran A, Repa JJ, Lobaccaro JM, Bronson A, Mangelsdorf DJ, and Edwards PA (2000b) Human white/murine ABC8 mRNA levels are highly induced in lipid-loaded macrophages. A transcriptional role for specific oxysterols. *J Biol Chem* **275**:14700–14707.

Walter M, Gerdes U, Seedorf U, and Assmann G (1994) The high density lipoprotein- and apolipoprotein A-I-induced mobilization of cellular cholesterol is impaired in fibroblasts from Tangier disease subjects. *Biochem Biophys Res Commun* **205**: 850–856.

Address correspondence to: Dr. Akio Fujimura, Division of Clinical Pharmacology, Department of Pharmacology, Jichi Medical School, Tochigi 329-0498, Japan. E-mail: akiofuji@jichi.ac.jp

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Adsorption of oxacalcitriol by polysulphone haemodialyser in patients with secondary hyperparathyroidism

Shuichi Tsuruoka, Hisashi Yamamoto, Takashi Ioka, Hitoshi Ando, Tetsuo Saito[1] & Akio Fujimura

Department of Pharmacology, Division of Clinical Pharmacology, Jichi Medical School and [1]Haemodialysis Unit, Moka Hospital, Tochigi, Japan

Correspondence
Shuichi Tsuruoka MD, Department of Pharmacology Division of Clinical Pharmacology, Jichi Medical School, 3311 Yakushiji, Minamikawachi, Kawachi, Tochigi 329-0498, Japan.
Tel: + 81 284 58 7388
Fax: + 81 285 44 7562
E-mail: tsuru@jichi.ac.jp

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Aims
This study was undertaken to evaluate removal of 22-oxacalcitriol (OCT), an active and intravenously used vitamin D3 analogue with less calcaemic activity, by polysulphone haemodialyser *in vivo* and *in vitro*. We further compared the pharmacodynamic efficacy [suppression of intact parathyroid hormone (iPTH)] when given intravenously either during or at the end of the haemodialysis.

Methods
(i) Drug clearance by the polysulphone dialyser was measured during a single continuous infusion (5 μg) for 30 min into the arterial side of the dialyser in end-stage renal failure patients with secondary hyperparathyroidism ($n = 7$). (ii) The drug adsorption by the hollowfibre membrane during incubation for 30 min was measured *in vitro*. (iii) To evaluate efficacy, the drug was given (i.v. bolus) during or at the end of haemodialysis for 4 weeks in a cross-over fashion with a washout period of 8 weeks ($n = 9$). Serum Ca^{2+}, phosphate (P) and iPTH concentrations just before the initiation of the dialysis were monitored every week.

Results
(i) OCT was significantly cleared by the polysulphone haemodialyser, but the clearance declined in a time-dependent manner to approach zero at 30 min. Arterial (at the place between the drug infusion site and the haemodialyser column) drug concentrations did not change during the infusion (mean =2064 ± 233 pg ml^{-1}). Venous (just after the dialyser) drug concentrations at 10 min after the infusion were significantly lower than those of the arterial side (mean =784 ± 84 pg ml^{-1}); however, they increased with time and reached those of the arterial side at 30 min. (ii) *In vitro*, OCT was adsorbed by the membrane. The amount of adsorption was concentration-dependent and was lower in the presence of human serum (55 ± 4% without and 23 ± 4% with serum at 600 pg ml^{-1} of OCT). (iii) Although serum Ca^{2+} and P increased and iPTH decreased by both treatment regimens (i.e. OCT administered either during or at the end of haemodialysis), these changes did not significantly differ. Mean differences (and 95% confidence interval) of Ca^{2+}, P, and iPTH at the end of the trial were 0.03 (−0.04, 0.09) mM, 0.41 (−0.43, 1.26) mg dl^{-1} and 38 (−42, 88) pg ml^{-1}, respectively.

Conclusion
OCT is adsorbed by polysulphone dialyser *in vitro* and *in vivo*. However, the pharmacodynamic effectiveness was largely independent of the administration regimen of OCT given either during or at the end of haemodialysis.

Introduction

Hyperparathyroidism, with its related complications, is a serious threat to haemodialysis patients [1]. Treatment with vitamin D analogues, given either orally or intravenously, is the cornerstone of therapy [2]; however, their therapeutic use in suppressing intact parathyroid hormone (iPTH) may be limited by the development of hypercalcaemia [1]. 1,25 dihydroxy 22-oxacalcitriol (OCT), an analogue of calcitriol, is a newer active vitamin D analogue that is licensed in Japan for intravenous use in the treatment of secondary hyperparathyroidism. The substitution of an oxygen in place of carbon 22 in the side chain is the only difference between OCT and 1,25 dihydroxy-calcitriol. It is reported that OCT did not increase serum Ca concentration at doses which reduced plasma PTH concentrations while 1,25 calcitriol increased serum Ca concentrations at doses which affected PTH concentrations in normal [3], parathyroidectomized [4] and renal ablation [4, 5] rats. Although there are no definite clinical studies directly comparing OCT and conventional vitamin D analogues, it is believed that the chance of drug-related hypercalcaemia by OCT might be smaller than that associated with other vitamin D3 analogues in humans [6]. When OCT is given to patients on haemodialysis, the administration during a haemodialysis session offers advantages in terms of practicability and safety compared with administration immediately after the cessation of haemodialysis. However, if administered during dialysis, it is of great importance to know whether the chosen vitamin D analogue is cleared by haemodialysis.

Against this background, it was the aim of this study: (i) to determine the clearance of OCT by the polysulphone dialyser when given during haemodialysis into the dialysis system; (ii) to determine *in vitro* the amount of adsorption of OCT to the dialyser membrane; and (iii) to compare the pharmacodynamic efficacy of OCT (suppression of parathyroid hormone, PTH) when given intravenously either during or at the end of haemodialysis.

Methods

Three studies were performed: (i) a pharmacokinetic study after a single dosing of OCT during a haemodialysis session in end-stage renal failure patients; (ii) assessment of the adsorption of OCT *in vitro* by the hollowfibre membrane used for haemodialysis; and (iii) a pharmacodynamic study after repeated dosing of OCT during or at the end of the haemodialysis session in end-stage renal failure patients with secondary hyperparathyroidism.

Subjects

A total of nine patients (seven men and two women, mean 47 ± 4.5 years) on a long-term haemodialysis programme at Moka Hospital (Tochigi, Japan) participated in the study. Seven patients took part in the pharmacokinetic study and nine patients in the pharmacodynamic study. The protocol was approved by the Institutional Review Board and Ethics Committee of the hospital. All patients gave written informed consent before the initiation of the study. Mean predialysis serum concentrations of creatinine, inorganic calcium, phosphate, urea, Ca^{2+}, K, bicarbonate and intact PTH are shown in Table 1. We selected the secondary hyperparathyroidism patients with serum concentration of intact parathyroid hormone >400 pg ml^{-1} despite treatment with a small daily (0.25–0.5 μg) dose of oral calcitriol therapy. The diameter of the largest parathyroid gland measured by ultrasonography was >1.0 cm in each patient. Maintenance haemodialysis (4–4.5 h) was performed three times a week in each patient.

Although the prescription of haemodialysis was not changed until the end of the study, dry weight was changed as appropriate. Blood flow rate during haemodialysis (180–250 ml min^{-1}) was not altered throughout the study in any patient. Ca^{2+} concentration of the dialysate was 1.375 mM. All the patients used a hollowfibre-type haemodialyser made of polysulphone (APS-S, Asahi Medical, Tokyo, Japan). The surface area of their haemodialysers varied from 1.3 to 1.5 m^2, and size and type of the dialyser were not changed throughout the study in any patient. All patients received $CaCO_3$ as a phosphate binder (1.5–3.0 g day^{-1}) and seven received

Table 1
Patient profiles

Parameters	Value
Creatinine	9.2 ± 1.5 mg dl^{-1}
Urea	82.5 ± 1.9 mg dl^{-1}
Inorganic calcium	9.3 ± 0.3 mg dl^{-1}
Ca^{2+}	1.2 ± 0.08 mM
Phosphate	5.3 ± 0.3 mg dl^{-1}
Potassium	5.2 ± 0.3 mM
Bicarbonate	22.3 ± 0.8 mM
Haemoglobin	8.9 ± 0.5 g dl^{-1}
Intact PTH	552 ± 48 pg ml^{-1}
Body mass index	21.2 ± 1.3
KT/V	1.34 ± 0.09

Mean ± S.E, n = 9.

erythropoietin (epoietin alpha) before the initiation of the study. The dosage regimens were not changed until the end of the study. The patients did not adhere to a specific diet. Haematocrit of each patient was >25% during the study. Eight already received oral active vitamin D before the study. After obtaining informed consent from these patients, the D3 analogue was discontinued for 6 weeks just before the study.

Pharmacokinetic study

The pharmacokinetic study was performed 1 week before the initiation of the pharmacodynamic trial. The drug (Chugai Pharmaceutical Co., Ltd, Tokyo, Japan; 5 μg) was dissolved with 50 ml of saline and continuously infused from the arterial side of haemodialyser over 30 min. The syringe was covered with aluminium foil to avoid light degradation. During the infusion, blood samples were obtained from the arterial side (between the infusion site of the drug and haemodialyser) and venous site (after the haemodialyser) every 10 min to measure the haematocrit and plasma drug concentration. The dialyser clearance (CL) was calculated using the arterio–venous difference method according to the following equation [7–9]:

$$CL(ml\ min^{-1}\ m^{-2}) = Q_b \times (1 - HA) \times \{CA - CV \times [1 - (HV - HA)/HV]\}/CA/S$$

in which Q_b is blood flow rate (in ml min^{-1}), HA and HV are the haematocrits of the arterial and venous sides, CA and CV are plasma OCT concentrations of arterial and venous sides, and S is surface area of the dialyser. The elimination fraction (EF) was calculated as follows [7–9]:

$$EF\ (\%) = 100 - CV \times [1 - (HV - HA)/HV]/CA$$

We did not collect dialysate. Plasma was obtained and kept at −70 °C until the assay.

In vitro adsorption study

Hollowfibres of the haemodialyser used in this study (APS-S) were generously supplied by the manufacturer (Asahi Medical). The inner diameter of the fibre is 200 μm. One hundred fibres were bundled and cut to 1-cm lengths. The 100 bundled fibers were put in a small tube for incubation *in vitro*. OCT for clinical use (10 μg ampoule) was diluted with either 2 ml of saline or serum. The serum was obtained from three healthy male volunteers (age 28–42 years) after obtaining informed consent. Each volunteer was asked to donate 20 ml of blood and their serum was mixed before usage.

The diluted drug and bundle of fibres were added to the tube and incubated for either 10 or 30 min at 37 °C in the shaker [10]. The tubes were covered with aluminium foil during the study. After the end of the incubation, fibres were removed and fluid was kept at −80 °C until the assay. Adsorption rate was calculated as

$$(Cx - Cpre)/Cpre \times 100$$

where Cx and Cpre are OCT concentrations at the examined point and before experiments, respectively.

Pharmacodynamic study in secondary hyperparathyroidism patients

The study was performed by an open-labelled, randomized, cross-over design with a washout period of 8 weeks. OCT (5 μg, i.v. over 1 min) was injected into the venous side of haemodialysis at 2 h after the beginning or at the end of each haemodialysis session for 4 weeks. A blood sample was obtained from the A-V fistula every week, immediately before each haemodialysis session.

Assay

OCT concentrations were measured by liquid chromatography-mass spectrometry [11]. The detection limit was 10 pg ml^{-1} and the interassay coefficient of variation was 3.1% at 6000 pg ml^{-1}. All the measured plasma concentrations were within the range of standard solutions. Plasma intact PTH concentrations were measured by radioimmunoassay (Nichols Institute Diagnostics, San Clemente, CA,USA) [12, 13]. The detection limit was 2 pg ml^{-1} and coefficient of variation was 2.5%. Ionized Ca concentration (Ca^{2+}) was measured by a blood gas analyser (Model 288; Ciba-Corning East Walpole, MA, USA) [13]. Plasma urea nitrogen was measured by the urease-urtraviolet method with autoanalyser.

Statistical analysis

Data are presented as the mean ± SE. Statistical analysis was performed by one-way ANOVA and *t*-test with Bonferroni correction as appropriate, by personal computer software (StatView 5.0). A P-value <0.05 was regarded as significant.

Results

Pharmacokinetic study during the continuous infusion of OCT in haemodialysis patients

All the patients completed the protocol without any major adverse reactions. Figure 1A,B shows individual and mean plasma OCT concentrations taken from the arterial and venous sides of the haemodialyser during the continuous infusion. The mean arterial concentration at 10 min after the initiation of the infusion was 2064 ± 233 pg ml^{-1}, which was not different from those

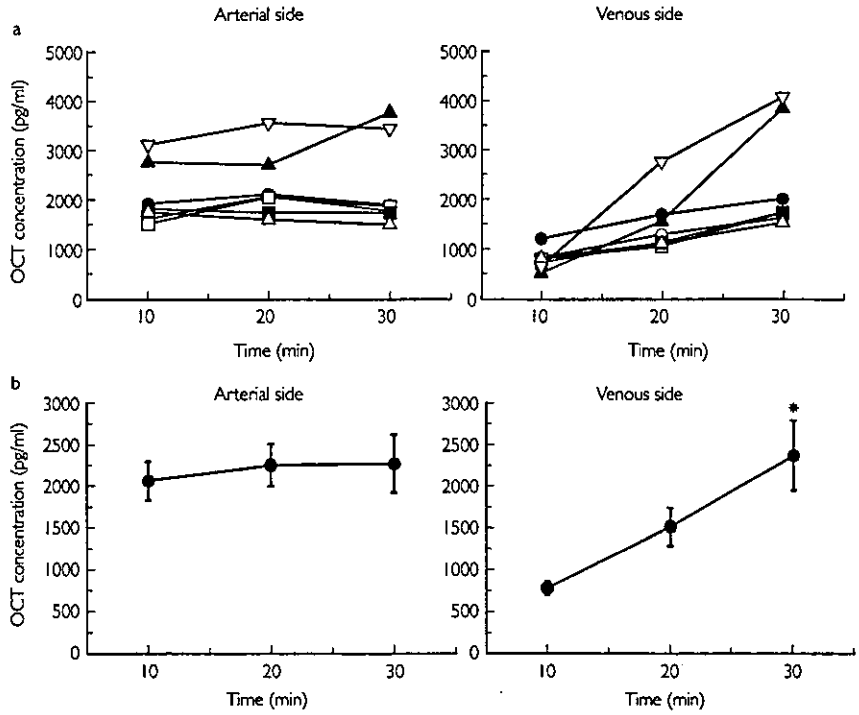

Figure 1
Individual (A) and mean (B) plasma concentration of OCT during continuous infusion of the drug for 30 min in haemodialysis patients. The drug was continuously infused into the arterial side of the haemodialyser for 30 min. Samples were taken at the arterial (between the drug infusion site and haemodialyser) and venous (just after the haemodialyser) sides during the infusion every 10 min. *$P < 0.05$ *vs.* arterial concentrations by ANOVA, mean ± SE, $n = 7$. Patient 1 (●), patient 2 (○), patient 3 (□), patient 4 (■), patient 5 (△), patient 6 (▲), patient 7 (▽)

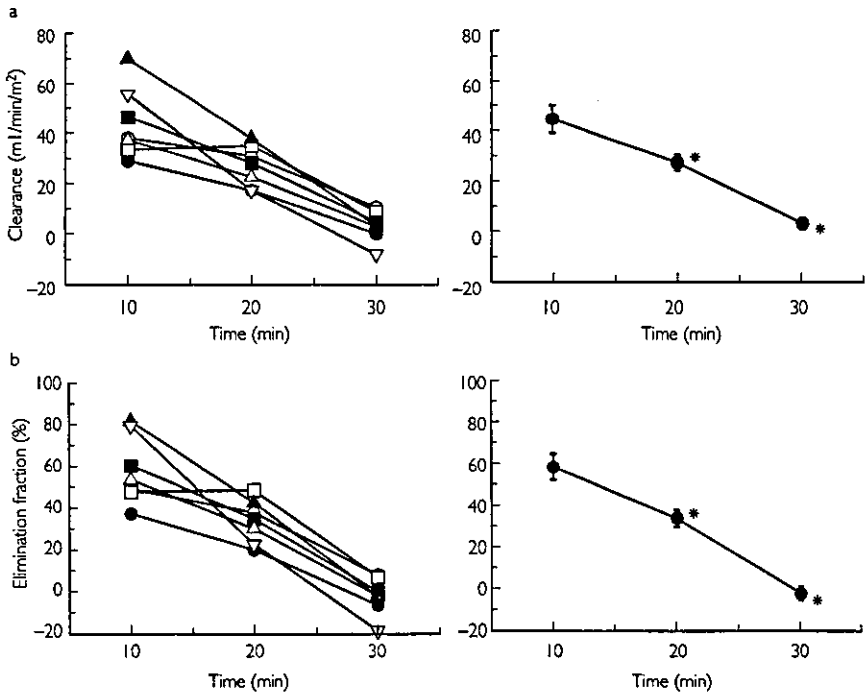

Figure 2
Calculated individual and mean clearance (A) and elimination fraction (B) of OCT during the continuous infusion of the drug for 30 min in haemodialysis patients. *$P < 0.05$ *vs.* 10 min by paired *t*-test. Mean ± SE, $n = 7$. Patient 1 (●), patient 2 (○), patient 3 (□), patient 4 (■), patient 5 (△), patient 6 (▲), patient 7 (▽)

at 20 and 30 min. On the other hand, the venous OCT concentration at 10 min was significantly ($P < 0.01$) lower than that of the arterial side (mean = 784 ± 84 pg ml^{-1}). However, the value went up to a level comparable to that of the arterial side at 30 min.

Individual and mean results of calculated dialyser clearance and elimination fraction are shown in Figure 2A,B. The mean clearance at 10 min was 44.2 ± 5.4 ml min^{-1} m^{-2}, which decreased thereafter and reached almost zero at 30 min. Elimination fraction showed a similar tendency (58.4 ± 6.3% at 10 min and −2.1 ± 3.4% at 30 min).

Plasma urea nitrogen concentration was measured using the same specimens. Its venous concentration was

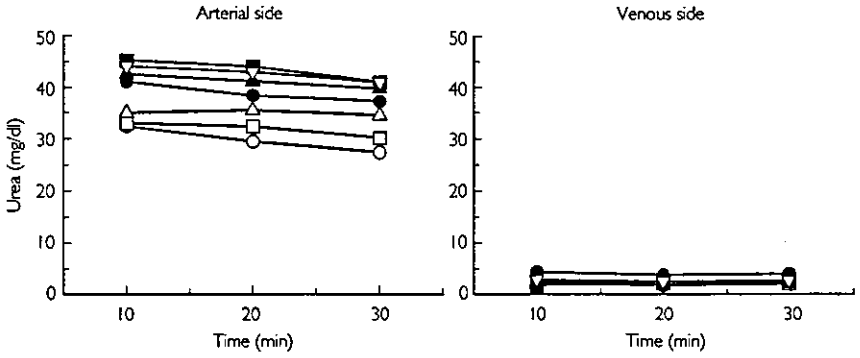

Figure 3
Individual plasma concentrations of urea during the continuous infusion of OCT for 30 min in the haemodialysis patients. Samples were taken at the arterial (between the drug infusion site and haemodialyser) and the venous (just after the haemodialyser) sides during the infusion every 10 min. Patient 1 (●), patient 2 (○), patient 3 (□), patient 4 (■), patient 5 (△), patient 6 (▲), patient 7 (▽)

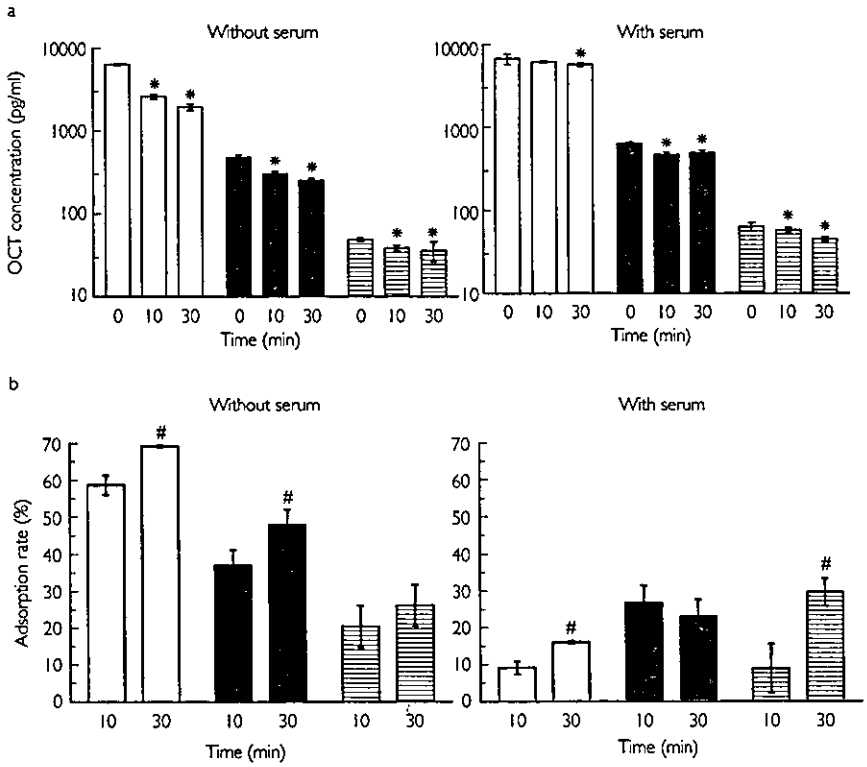

Figure 4
Change of OCT concentration (A) and its adsorption rate (B) during incubation with the haemodialyser membrane *in vitro*. The incubation was carried out with or without human serum obtained from healthy volunteers. *$P < 0.05$ *vs.* 0 min; #$P < 0.05$ *vs.* 10 min by ANOVA. Mean ± SE, $n = 5$. 6000 pg/ml (□), 600 pg/ml (■), 60 pg/ml (▤)

nearly zero in all the samples, indicating that the reduction of solute clearance was specific to OCT (Figure 3).

In vitro *adsorption of OCT*

Figure 4A shows OCT concentrations before and after the incubation with fibres used for haemodialysis *in vitro*. At all different concentrations, the OCT concentration significantly decreased with time after the incubation. The decrement with serum was significantly smaller than that without serum. The adsorption rate is shown in Figure 4B. It was nearly 58 ± 3% with 6000 pg ml^{-1} of the drug at 10 min after the initiation of the study and increased after 30 min to 78 ± 1%. Adsorption was also reduced by incubation with human serum (15 ± 1% at 30 min with the same concentration, the difference of 95% confidence interval 58, 67).

Effect of timing of OCT administration on Ca^{2+}, P and intact PTH concentrations after the repeated treatment

The time course of serum ionized Ca and P concentrations are shown in Figure 5A,B. The values of these parameters significantly increased 1 week after the treatment and persisted until the end of the study. On the other hand, serum intact PTH concentrations significantly decreased at 4 weeks after the treatment (Figure 5C). There were no significant differences in these values between the two different dosage regimens.

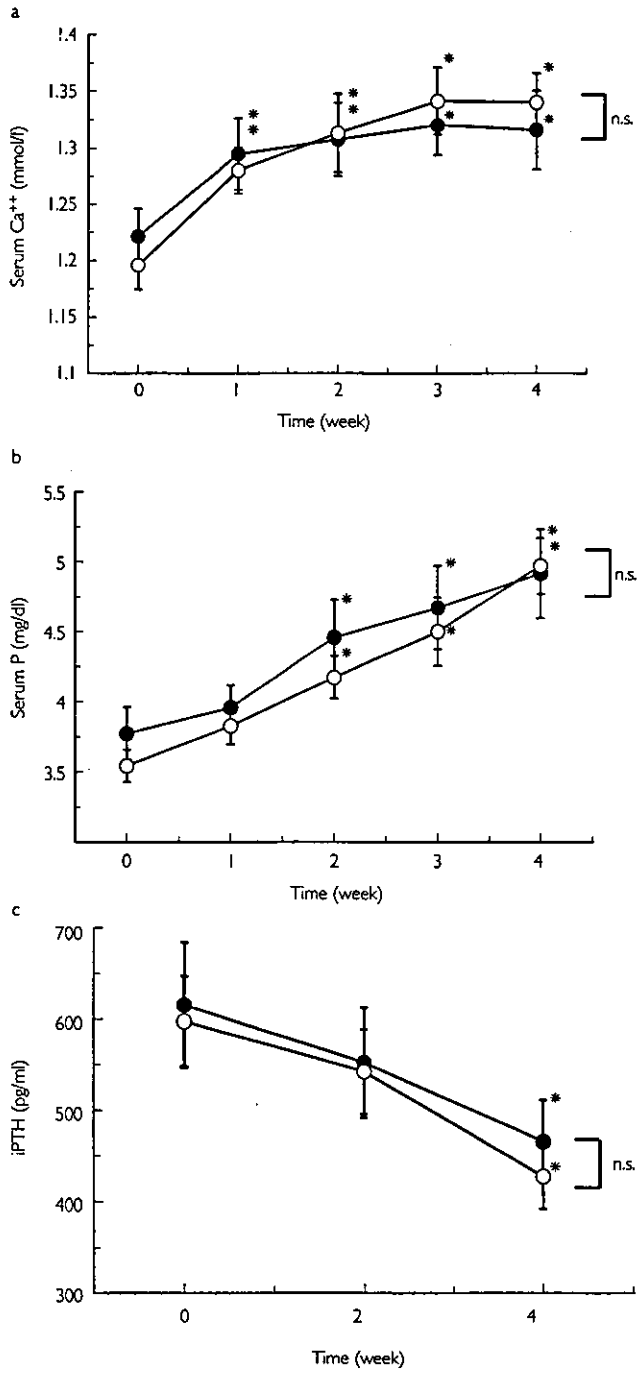

Figure 5

Serum concentrations of ionized Ca (A), phosphate (P) (B) and intact parathyroid hormone (iPTH, C) during the repeated infusion of OCT. Drug was injected during or just after the haemodialysis (HD) session for 4 weeks. Samples were taken just before starting haemodialysis every week. During HD (●); after HD (○). There was no difference between the two different dosing regimens but a significant time effect (ANOVA, $*P < 0.05$ *vs.* 0 week). Mean ± SE, $n = 9$

Discussion

In this study, we found that OCT was cleared by haemodialysis in the early period after the initiation of the infusion, but this gradually decreased to nearly zero at 30 min after the infusion. In the same samples, such a decrease in clearance was not detected for urea, indicating that the removal of OCT was specifically decreased. Although we did not measure drug concentration in dialysate, these results strongly indicate a possible adsorption of the drug by the membrane and its saturation during the infusion. The *in-vitro* study directly demonstrated adsorption of the drug in a concentration-dependent manner with human serum.

It is well-accepted that the haemodialyser membrane can adsorb some solutes, especially proteins. It is also reported that several drugs, such as erythropoietin, adsorb to dialyser membrane [14]. Previous studies on the adsorption of the drug were generally done *in vitro* without serum or protein. Thus, interpretation of these findings in clinical settings might be difficult. To our knowledge, this is the first study to show the adsorption of a vitamin D analogue by haemodialyser membrane in secondary hyperparathyroidsm patients *in vivo* as well as *in vitro*. To evaluate the removal of OCT by haemodialysis *in vivo*, the drug was continuously infused into the arterial side during a single dialysis session. Because the distribution volume of OCT is reported as 290 ml kg^{-1} [15], the maximum concentration after 5 μg of bolus infusion in patients of 50 kg body weight was estimated to be 344 pg ml^{-1}, which was within the range of concentrations that we selected for the *in-vitro* study.

Arterial concentrations of the drug did not change during the infusion in the pharmacokinetic study. This might be because either the amount of drug entered into the body is small or drug metabolism in the body is rapid. $T_{1/2\alpha}$ and $T_{1/2\beta}$ after a single injection were 0.71 ± 0.16 and 108.1 ± 45 min, respectively, in a Phase I study (unpublished manufacturer's observation), which may support the rapid drug clearance in the body. Low drug concentrations at the venous side from significant adsorption by the haemodialyser observed in this study may also support the first possibility. A future study is needed to resolve the question. In this study, venous drug concentrations at 30 min after the infusion were not different from those of the arterial side, which is compatible with the *in-vitro* study that OCT is not dialysed but adsorbed.

We used a polysulphone membrane, which is one of the high-flux membranes most used clinically. It was reported that nafamostat mesilate is the only substance reported to bind to polysulphone membranes [16]. The

mechanism of OCT adsorption by polysulphone is not clear at the present time. Direct adsorption of protein-unbound drug might be involved in the mechanism because the adsorption capacity was decreased by incubation with serum. It is reported that neutrally charged membranes have a small but significant adsorption capacity to hydrophobic proteins [17]. Because polysulphone membrane is neutrally charged [17], vitamin D, a hydrophobic drug, might be adsorbed to this membrane. We did not use serum secondary hyperparathyroidism patients in the *in-vitro* study because of the difficulty of obtaining a large volume of blood from these anaemic patients, and this is a limitation of the study. We did not reuse the dialyser in this study. The reused membrane is partly coated with intrinsic proteins and possesses better biocompatibility [18], which may affect adsorption. According to the present *in-vitro* data, reused dialyser may reduce the clearance of OCT *in vivo*.

As the final part of this study, we further tested the hypothesis that the repeated infusion of the drug at an intermediate period of the haemodialysis sessions may alter therapeutic efficacy due to the adsorption of the drug to the dialysis membrane observed in our *in-vitro* and *in-vivo* studies. We unexpectedly observed that the repeated injection of OCT at the middle point of the haemodialysis had similar efficacy (estimated by iPTH reduction) and safety (estimated by blood Ca and P elevations) to the injection of the drug just before the termination of the haemodialysis in the cross-over study. Drugs, including OCT, which are given by i.v. bolus infusion and removed by the haemodialyser membrane, are usually infused just before the termination of the haemodialysis session. The period just before the termination of the haemodialysis session is one of the critical times for intradialysis complications, mainly due to the reduction of blood pressure with ultrafiltration [19]. Thus, medical staff and nurses in the dialysis centre should watch the patients more carefully during the critical period, and we think that it would be safer if a drug was able to be given intravenously at a middle of the dialysis session if it were also effective. The pharmacokinetics of subcutaneously injected erythropoietin at the start of haemodialysis has been reported to be no different from that without haemodialysis [20]. These authors concluded that adsorption of erythropoietin to the dialyser is not significant in the clinical situation. Although OCT was developed to avoid the drug-related hypercalcaemia by conventional vitamin D analogues, there has been no definite comparative report and such a study is needed in the future.

In conclusion, this study showed that OCT was adsorbed by polysulphone haemodialyser both *in vivo* and *in vitro*. The amount of adsorption was lower in the presence of human serum. However, this adsorption does not appear to affect significantly the therapeutic efficacy of the drug since OCT given in the middle of the haemodialysis session exerts a similar efficacy in the suppression of iPTH compared with administration at the end of haemodialysis. Therefore if i.v. OCT is used for the treatment of secondary hyperparathyroidism, it can be administered during haemodialysis without loss of therapeutic efficacy.

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References

1 Llasch F, Bover J. Renal osteodystrophies. In Brenner and Rector's the Kidney, ed Brenner BB. Philadelphia: W.B. Saunders, 1996; 2187–273.

2 Slatopolsky E, Weerts C, Thielan J et al. Marked suppression of secondary hyperparathyroidism by intravenous administration of 1,25-dihydroxy- cholecalciferol in uremic patients. J Clin Invest 1984; 74: 2136–43.

3 Brown AJ, Ritter CR, Finch JL et al. The noncalcemic analogue of vitamin D, 22-oxacalcitriol, suppresses parathyroid hormone synthesis and secretion. J Clin Invest 1989; 84: 728–32.

4 Finch JL, Brown AJ, Mori T et al. Suppression of PTH and decreased action on bone are partially responsible for the low calcemic activity of 22-oxacalcitriol relative to 1,25-(OH) 2D3. J Bone Miner Res 1992; 7: 835–9.

5 Hirata M, Endo K, Katsumata K et al. A comparison between 1,25-dihydroxy-22-oxavitamin D(3) and 1,25-dihydroxyvitamin D(3) regarding suppression of parathyroid hormone secretion and calcaemic action. Nephrol Dial Transplant 2002; 17 (Suppl. 10): 41–5.

6 Tsukamoto Y, Hanaoka M, Matsuo T et al. Effect of 22-oxacalcitriol on bone histology of hemodialyzed patients with severe secondary hyperparathyroidism. Am J Kidney Dis 2000; 35: 458–64.

7 Gabutti L, Taminelli-Betraminelli L, Marone C. Clearance of ceftriaxone during hemodialysis using cuprophane, hemophane and polysulfone dialyzers. Eur J Clin Pharmacol 1997; 53: 123–6.

8 Gehr T, Sica D, Slugg P et al. The pharmacokinetics of pravastatin in patients on chronic hemodialysis. Eur J Clin Pharmacol 1997; 53: 117–21.

9 Tsuruoka S, Sugimoto KI, Hayasaka T et al. Ranitidine clearance during hemodialysis with high-flux membrane: comparison of polysulfone and cellulose acetate hemodialyzers. Eur J Clin Pharmacol 2000; 56: 581–3.

10 Tsuruoka S, Osono E, Nishiki K et al. Removal of digoxin by column for specific adsorption of beta(2)-microglobulin: a potential use for digoxin intoxication. Clin Pharmacol Ther 2001; 69: 422–30.

11 Ishigai M, Asoh Y, Kumaki K. Determination of 22-oxacalcitriol, a new analog of 1alpha,25-dihydroxyvitamin D3, in human serum by liquid chromatography-mass spectrometry. J Chromatogr B Biomed Sci Appl 1998; 706: 261–7.

12 Frolich M, Walma ST, Paulson C et al. Immunoradiometric assay for intact human parathyroid hormone: characteristics, clinical application and comparison with a radio-immunoassay. Ann Clin Biochem 1990; 27: 69–72.

13 Tsuruoka S, Sugimoto K, Ohmori M et al. Chronotherapy of high-dose 1,25-dihydroxyvitamin D3 in hemodialysis patients with secondary hyperparathyroidism: a single-dose study. Clin Pharmacol Ther 1999; 66: 609–16.

14 Mori H, Hiraoka K, Yorifuji R et al. Adsorption of human recombinant erythropoietin on dialysis membrane *in vitro*. Artif Organs 1994; 18: 725–8.

15 Kobayashi T, Tsugawa N, Okano T et al. The binding properties, with blood proteins, and tissue distribution of 22-oxa-1 alpha,25-dihydroxyvitamin D3, a noncalcemic analogue of 1 alpha, 25-dihydroxyvitamin D3, in rats. J Biochem (Tokyo) 1994; 115: 373–80.

16 Nakagawa K, Imagaki O, Iwaki R et al. Study of the binding capacity of FUT-175 on dialysis membrane. Jpn J Artif Organs 1991; 20: 27–31.

17 Moachon N, Boullange C, Fraud S et al. Influence of the charge of low molecular weight proteins on their efficacy of filtration and/or adsorption on dialysis membranes with different intrinsic properties. Biomaterials 2002; 23: 651–8.

18 Scott MK, Mueller BA, Sowinski KM. The effects of peracetic acid-hydrogen peroxide reprocessing on dialyzer solute and water permeability. Pharmacotherapy 1999; 19: 1042–9.

19 Amerling A, Cu G, Dubrow A et al. Complications during hemodialysis. In Clinical Dialysis, eds Nissenson AR, Fine RN, Gentile DE. London: Prentice Hall International Inc., 1995; 233–67.

20 Opatrny K Jr, Krouzecky A, Wirth J et al. The effects of a polyacrylonitrile membrane and a membrane made of regenerated cellulose on the plasma concentrations of erythropoietin during hemodialysis. Artif Organs 1998; 22: 816–20.

FULL PAPER *Immunology*

Effect of Lipopolysaccharide (LPS) Injection on the Immune Responses of LPS-Sensitive Mice

Yasuaki OGIKUBO[1], Mari NORIMATSU[2], Yoshimasa SASAKI[1], Akinori YASUDA[3], Junzo SAEGUSA[3] and Yutaka TAMURA[1,4]

[1]*National Veterinary Assay Laboratory, 1–15–1, Tokura, Kokubunji, Tokyo 185–8511, Japan,* [2]*Institute for Animal Health, Compton, Newbury, Berkshire, RG207NN, UK,* [3]*National Institute of Industrial Health, 6–21–1, Nagao, Tama, Kawasaki, Kanagawa 214–8585 and* [4]*Department of Veterinary Public Health, School of Veterinary Medicine, Rakuno Gakuen University, 582 Bunkyoudai-Midorimachi, Ebetsu, Hokkaido 069–8501, Japan*

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ABSTRACT. The effect of lipopolysaccharide (LPS) on humoral and cell-mediated immunity was assessed using LPS-sensitive C3H/HeN mice. A single injection of LPS significantly decreased the anti-sheep red blood cells (SRBC) antibody titers, but not the number of anti-SRBC antibody producing spleen cells. In contrast, double LPS injection did not significantly decrease the anti-SRBC titers and even increased the number of anti-SRBC antibody producing spleen cells. Similarly, single LPS injection significantly suppressed the swelling of the footpad, but double LPS injection caused milder suppression. These results suggest that a difference in the level and timing of exposure to LPS may influence the immune response to infection or vaccination.

KEY WORDS: C3H/HeN, immune response, lipopolysaccharide, tolerance.

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In an effort to improve the health of domestic animals, chronic infection is recently being paid more attention than acute epidemic infection, as most acute infections are under control. Since many chronic infections are caused by gram-negative bacteria, vaccines against these microbes have been increasingly produced, most of which are inactivated whole-cell vaccines. Lipopolysaccharide (LPS), which is a common component of the cell walls of gram-negative bacteria, is capable of eliciting a wide variety of pathophysiological effects such as endotoxin shock, tissue injury, and lethality in both man and animals [8, 10]. In addition, LPS, which is known to be a potent stimulant of the host immune system, stimulates proliferation and differentiation of B cells and initiates the activation of mononuclear phagocytes such as macrophages, thus enhancing immune responses [6, 9, 17, 18].

It has been reported that LPS can induce apoptosis of lymphocytes and the elevation of serum level of tumor necrosis factor (TNF)-α in both piglets and mice [4, 5, 13, 14]. Moreover, it has been recently hypothesized that Toll-like receptor 4 (TLR4) is the critical receptor for LPS signaling in host defense, and the innate immune system may be involved in the regulation of the adaptive immunity, which is mediated by B and T lymphocytes [16, 21]. However, little is known about the effects of LPS on the immune response of domestic animals as well as the mechanism behind.

In this report, we describe the effect of single and double LPS injections on the immune responses of an LPS-sensitive mouse strain as a model for domestic animals.

MATERIALS AND METHODS

LPS: LPS from *Escherichia coli* O55:B5 extracted by the hot phenol-water extraction method was purchased from Difco laboratories, U.S.A. and was suspended in pyrogen-free saline (Otsuka, Tokyo, Japan).

Quantification of antibody titers and antibody producing spleen cells: Eight-week-old female mice of the LPS-sensitive C3H/HeN strain (Japan SLC, Inc., Shizuoka, Japan) were intravenously immunized with 0.2 m*l* of 5% of sheep red blood cells (SRBC) and boosted 14 days later in an identical manner. The single LPS injection (single LPS) group was intravenously injected with 1 mg/kg of LPS 2 days before the booster immunization (n=5). Accordingly, the double LPS injection (double LPS) group was intravenously injected 2 days before both the priming and boosting immunizations (n=5). As a positive control, 5 mice were immunized with 5% of SRBC twice in the same way without LPS injection. Negative controls were injected with pyrogen-free saline. The anti-SRBC antibody titers and the number of anti-SRBC antibody producing spleen cells were measured 7 days after the second immunization by the SRBC hemagglutination test and by use of a chamber made up of two glass slides [2], respectively.

Delayed type hypersensitivity assay: LPS-sensitive C3H/HeN mice were intravenously immunized with 0.2 m*l* of 0.01% of SRBC. Three days after immunization, mice were challenged by injection of 0.03 m*l* of 20% SRBC intradermally into the left footpad. Delayed type hypersensitivity (DTH) responses were determined 3 days after challenge as the percentage increase in thickness of the left footpad compared with the right footpad, which was injected with 0.03 m*l* of pyrogen-free saline. The single LPS group was intravenously injected with 1 mg/kg of LPS 3 days before the immunization (n=10) and the double LPS group was injected both 3 days and 17 days before the immunization (n=6). Positive controls were treated in the same way with-

out LPS injection (n=6).

Flowcytometry analysis: C3H/HeN mice were injected with 1 mg/kg of LPS at day 0 (single LPS) or day 0 and 14 (double LPS). Either 3 or 6 animals were processed for flowcytometry at each time point: 0, 2, 5, 14 days post injection (dpi) and 2 days after the second LPS injection (14+2 dpi). For flowcytometry analysis, single cell suspensions containing approximately 10^6 cells were stained with monoclonal antibodies for 20 min at 4°C after incubation with anti-mouse Fcγ II/III receptor antibody (2.4G2) (PharMingen, San Diego, CA, U.S.A.) for 20 min at 4°C. Monoclonal antibodies used for staining included: fluorescent isothiocyanate-conjugated (FITC)-anti mouse CD3 epsilon (PharMingen), FITC-anti mouse CD8a (Ly-2) (PharMingen), Phycoerythrin-conjugated (PE)-anti mouse B220 (PharMingen), PE-anti mouse CD4 (L3T4) (Becton Dickinson, Franklin Lakes, NJ, U.S.A.). Analysis was conducted on a FACScan (Becton Dickinson) and the data were analyzed by software Consort 30 (Becton Dickinson). Dead cells and debris were gated out of the analysis on the basis of forward and side light scatter. The flowcytometry data were presented as the number of cells by calculating from the number of total cells of the organ and the percentage of each cell population.

Statistic analysis: Differences between groups were determined by analysis of variance using the StatView J-4.5 (Abacus Concepts, Inc., Berkeley, CA, U.S.A.).

RESULTS

The anti-SRBC antibody titers and the number of anti-SRBC antibody producing spleen cells: When LPS was injected 2 days before the second immunization (single LPS), the anti-SRBC antibody titers were significantly lower than those of the positive controls ($p< 0.01$). However, when mice were pretreated with the same dose of LPS 14 days before the first LPS injection (double LPS), the antibody titers were significantly higher than those of mice given a single LPS injection (Fig. 1).

When LPS was injected 2 days before the second immunization (single LPS), the number of anti-SRBC antibody producing spleen cells were similar to those of the positive control group. However, when mice were pretreated with the same dose of LPS 14 days before the first LPS injection (double LPS), the increase in the number of these cells was significantly higher than those of mice given a single injection with LPS and the positive control group ($p< 0.05$) (Fig. 2).

Influence of LPS injection on delayed type hypersensitivity responses: In the group given a single LPS injection, the footpad swelling was significantly lower than that of the positive control group ($p< 0.01$). However, swelling was significantly higher in the double LPS group than the single LPS group ($p< 0.05$) (Fig. 3).

Changes of thymus weight and leukocyte subpopulation in thymus and spleen by the LPS injection: In order to analyze the effect of LPS on immune system further, we also

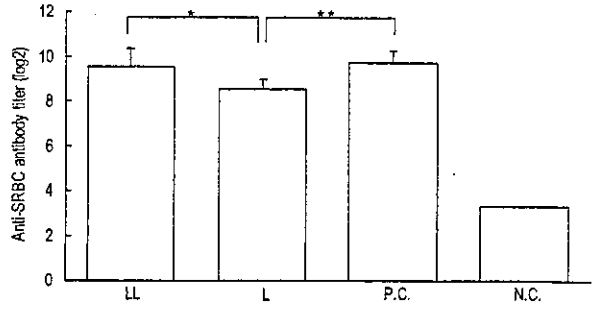

Fig. 1. Differences in the anti-SRBC antibody titers of C3H/HeN mice after the single and double LPS injection. L: single LPS injection, LL: double LPS injection, PC: positive controls, NC: negative controls. Data are expressed as mean ± standard deviation (n=5). Statistical analysis was conducted by the Fisher's protected least significant difference. Significant differences are expressed as *: $p<0.05$ and **: $p<0.01$.

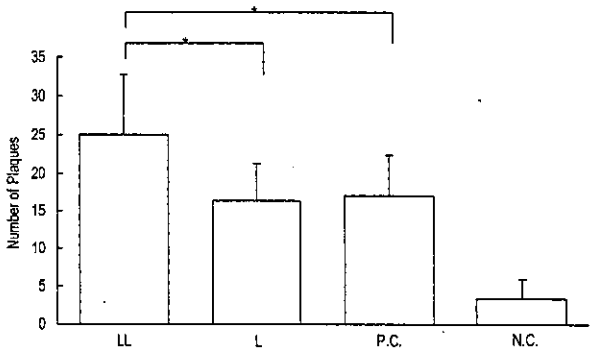

Fig. 2. Differences in the anti-SRBC antibody producing spleen cells of C3H/HeN mice after the single and double LPS injection. L: single LPS injection, LL: double LPS injection, PC: positive controls, NC: negative controls. Data are expressed as mean ± standard deviation (n=5). Statistical analysis was conducted by the Fisher's protected least significant difference. Significant differences are expressed as *: $p<0.05$.

examined the thymuses and spleens of the mice treated with either single or double LPS injection by flowcytometry and histology.

The weight of the thymus decreased at 2 dpi, accompanied by cortical atrophy by histological observation, and recovered to the negative control level by 14 dpi (Fig. 4a). Flowcytometry showed that the number of CD4+CD8+ cells significantly decreased at 2 dpi, almost disappeared at 5 dpi and recovered by 14 dpi (Fig. 4b), coinciding with the observation in the weight and histology. In contrast, 2 days after the second LPS injection, neither the weight nor the number of CD4+CD8+ cells in thymus decreased to the same level as 2 days after the fist LPS injection i.e. the suppressive effect of LPS was reduced after double LPS injection.

In the spleen, B cells represented by B220+ cells did not change significantly in number after the first LPS injection,

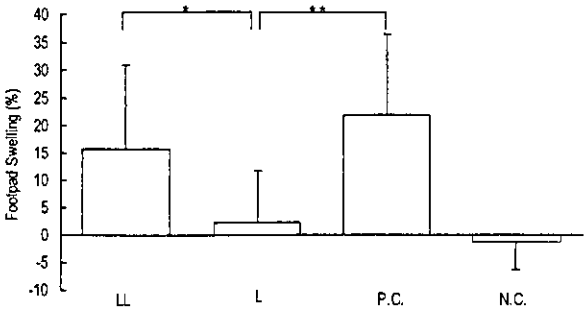

Fig. 3. Differences in footpad reaction in delayed type hypersensitivity (DTH) assay of C3H/HeN mice after the single and double LPS injection. L: single LPS injection, LL: double LPS injection, PC: positive controls, NC: negative controls. Data are expressed as mean ± standard deviation (n=5 to 10). Statistical analysis was conducted by the Fisher's protected least significant difference. Significant differences are expressed as *: $p< 0.05$ and **: $p<0.01$.

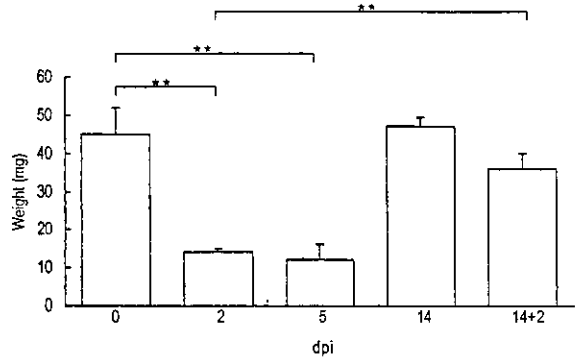

Fig. 4a. Changes of thymus weight of C3H/HeN mice after the LPS injection. Data are given as mean ± standard deviation. Statistical analysis was conducted by the Fisher's protected least significant difference. Significant differences are expressed as **: $p<0.01$.

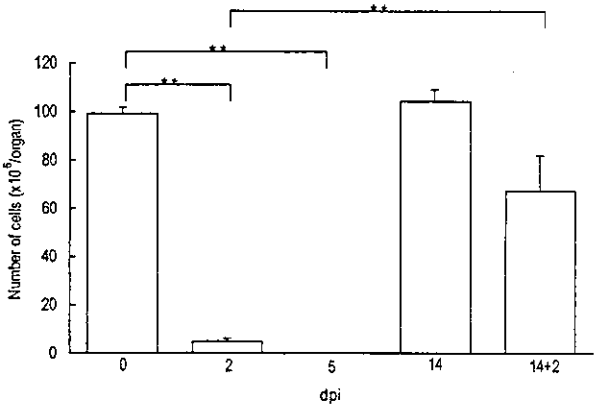

Fig. 4b. Changes of CD4+CD8+ phenocyte in thymus of C3H/HeN mice after the LPS injection. Data are given as mean ± standard deviation. Statistical analysis was conducted by the Fisher's protected least significant difference. Significant differences are expressed as **: $p<0.01$.

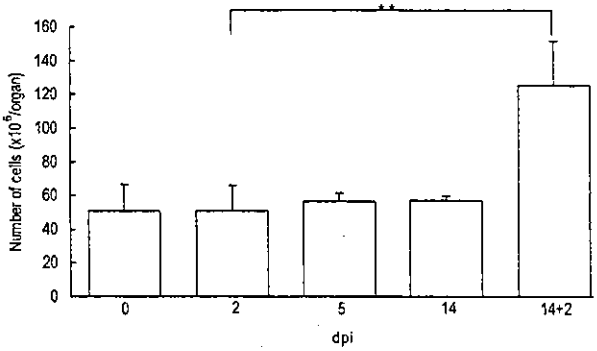

Fig. 4c. Changes of B lymphocyte in spleen of C3H/HeN mice after the LPS injection. Data are given as mean ± standard deviation. Statistical analysis was conducted by the Fisher's protected least significant difference. Significant differences are expressed as **: $p<0.01$.

but significantly increased after the second injection (Fig. 4c).

DISCUSSION

In this study we have shown that a single LPS injection into SRBC-immunized mice induces suppression of anti-SRBC antibody production and footpad swelling. Double LPS injection abrogated this immunosuppressive effect and enhanced the number of anti-SRBC antibody producing spleen cells.

T lymphocyte may be mainly classified into CD8+ cytotoxic T cell (CTL) which participates in the cell-mediated immune response and CD4+ helper T (Th) cell which may be divided into two functionally different Th1 and Th2 subset [11]. The Th1 cells are crucial for the activation of the CTL, whereas Th2 cells provide help to B cells leading to the production of antibodies [19, 20]. We revealed that the single LPS injection dramatically induced the depletion of CD4+CD8+ cells in the thymus at 2 dpi and completely recovered by 14 dpi (Fig. 4b). Moreover, the number of both CD4+ CD8- and CD4-CD8+ cells in the thymus decreased approximately one third of the control level at 5 dpi (data not shown). These results, therefore, suggest that the single LPS injection suppress the humoral and cell-mediated immune responses.

In our result, the change of B lymphocyte in the spleen corresponds to the change in number of anti-SRBC antibody producing spleen cells (Fig. 2 and Fig. 4c). T cells represented by CD3+ cells decreased after the first LPS injection in the spleen and almost recovered by 14 dpi, whilst they did not decrease after the second LPS injection (data not shown). Antibody production depends on B cell activation by antigen followed by the stimulation by antigen-specific Th cells [20]. Thus, the change in the number of B and T

cells may partly explain the observation that the anti-SRBC antibody titers were significantly lower following single LPS injection, but not in double LPS injection. It is also suggested that the function of T cells did not recover enough to activate antibody production by B cells in the double LPS injection, in which the anti-SRBC antibody titers did not increase compared with the positive control group.

It is well known that LPS can directly activate polyclonal B cells to differentiate into antibody producing cells [1]. However, this in turn may transiently exhaust the existing memory B cells that can interact with Th cells to stimulate proliferation, resulting in a suppressive effect on antibody production. This may partly explain the results presented herein that show a single LPS injection induces significant suppression of antibody titers, but does not affect on the number of antibody producing spleen cells. Simultaneous injection of SRBC and lipid A from *Salmonella Minnesota* R595 enhances the number of IgM-plaque-forming spleen cells in mice 3 and 4 days after injection and the serum IgM titer 4 and 5 days after injection [7]. Thus, the timing of LPS injection may complicate the consequent immune response to particular antigens.

It has been reported that LPS isolated from different bacterial strains exhibits a wide diversity in strength of adjuvant action in the induction of delayed type hypersensitivity to ovalbumin measured by footpad reaction in mice when antigen is simultaneously injected with each LPS [15]. However, it has also been reported that LPS can induce apoptosis mainly of CD4+CD8+ thymocytes and destruction of T cell area in the spleen in mice 1 to 3 days after LPS injection [4, 5, 14, 23]. Our results confirmed the latter observation, which may have caused the suppression of cell-mediated immunity measured by footpad reaction when mice were treated with single LPS injection 3 days before immunization.

Compared with the single LPS injection, the double LPS injection induced much less suppressive effects on both humoral and cell-mediated immune responses. In addition, we also observed that single LPS injection induced significant levels of mRNA expression of TNF-α and interferon-gamma in thymus 1 hr after injection, whereas in double LPS injection, the levels of these cytokines were dramatically reduced 1 hr after the second LPS injection (data not shown). This phenomenon is described as a late phase tolerance when a second LPS injection 5 days or more after the first induces little biological response. Late phase tolerance is thought to be associated with the development of specific antibodies against the polysaccharide side chain of LPS [3, 22]. This may partly explain our results, however, the precise mechanism of late phase tolerance is yet to be understood. It has recently been revealed that induction of early phase tolerance was regulated by TLR4 expressed on antigen-presenting cells, such as macrophages and dendritic cells, and production of pro-inflammatory cytokines [12, 21]. It is assumed that late phase tolerance is also regulated by Th cells and pro-inflammatory cytokines produced by antigen-presenting cells. The further study into the mechanism of late phase tolerance should also be conducted.

Our data suggest that LPS may induce suppression in both humoral and cell-mediated immunity on LPS-sensitive animals. The results also suggest that a difference in the level and timing of exposure to LPS, which may mimic the differences in the conditions where animals are reared (e.g. specific pathogen free environment or natural environment) and/or the history of infection with gram-negative bacteria, may influence the outcome of immune response. This may have profound consequences on the immune responses elicited during infection or vaccination.

REFERENCES

1. Anderson, J., Sjoberg, O. and Moller, G. 1972. Induction of immunoglobulin and antibody synthesis *in vitro* by Lipopolysaccharide. *Eur. J. Immunol.* **2**: 349–353.
2. Cunningham, A.J. 1965. A method of increased sensitivity for detecting single antibody-forming cells. *Nature (Lond.)* **207**: 1106–1107.
3. Greisman, S.E., Young, E. J. and Carozza F. A. Jr. 1969. Mechanisms of endotoxin tolerance. V. Specificity of the early and late phases of pyrogenic tolerance. *J. Immunol.* **103**: 1223–1236.
4. Kato, Y., Morikawa, A., Sugiyama, T., Koide, N., Jiang, G. Z., Takahashi, K. and Yokochi, T. 1995a. Role of tumor necrosis factor-alpha and glucocorticoid on lipopolysaccharide (LPS)-induced apoptosis of thymocytes. *FEMS Immunol. Med. Microbiol.* **12**: 195–204.
5. Kato, Y., Sugiyama, T., Takahashi, K., Kawai, M., Fukada, M. and Yokochi, T. 1995b. Mechanisms of LPS-induced apoptosis in thymus: cross talk among cytokines and glucocorticoids. *Jpn. J. Med. Sci. Biol.* **48**: 274.
6. Landy, M. and Baker, P. J. 1966. Cytodynamics of the distinctive immune response produced in regional lymph nodes by salmonella somatic polysaccharide. *J. Immunol.* **97**: 670–679.
7. Mashimo, J. and Mita, A. 1995. *In vivo* production of various cytokines in splenocytes of sheep erythrocyte-immunized mice after intravenous administration of bacterial lipid A. *Microbiol. Immunol.* **39**: 169–175.
8. Morrison, D.C. and Ulevitch, R. J. 1978. The effects of bacterial endotoxins on host mediation systems. A review. *Am. J. Pathol.* **93**: 527–617.
9. Morrison, D.C. and Ryan, J. L. 1979. Bacterial endotoxins and host immune responses. *Adv. Immunol.* **28**: 293–450.
10. Morrison, D.C. and Ryan, J. L. 1987. Endotoxins and disease mechanisms. *Annu. Rev. Med.* **38**: 417–432.
11. Mosmann, T.R. and Sad, S. 1996. The expanding universe of T-cell subsets: Th1, Th2 and more. *Immunol. Today* **17**: 138–146.
12. Nomura, F., Akashi, S., Sakao, Y., Sato, S., Kawai, T., Matsumoto, M., Nakanishi, K., Kimoto, M., Miyake, K., Takeda, K. and Akira, S. 2000. Cutting edge: endotoxin tolerance in mouse peritoneal macrophages correlates with down-regulation of surface Toll-like receptor 4 expression. *J. Immunol.* **164**: 3476–3479.
13. Norimatsu, M., Ono, T., Aoki, A., Ohishi, K., Takahashi, T., Watanabe, G., Taya, K., Sasamoto, S. and Tamura, Y. 1995a. Lipopolysaccharide-induced apoptosis in swine lymphocytes *in vivo*. *Infect. Immun.* **63**: 1122–1126.
14. Norimatsu, M., Ono, T., Aoki, A., Ohishi, K. and Tamura, Y. 1995b. *In-vivo* induction of apoptosis in murine lymphocytes

by bacterial lipopolysaccharide. *J. Med. Microbiol.* **43**: 251–257.

15. Ohta, M., Nakashima, I. and Kato, N. 1982. Adjuvant action of bacterial lipopolysaccharide in induction of delayed-type hypersensitivity to protein antigen. II. Relationships of intensity of the action to that of other immunological activities. *Immunobiology* **163**: 460–469.
16. Poltorak, A., He, X., Smirnova, I., Liu, M. Y., Huffel, C. V., Du, X., Birdwell, D., Alejos, E., Silva, M., Galanos, C., Freudenberg, M., Ricciardi-Castagnoli, P., Layton, B. and Beutler, B. 1998. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in *Tlr4* gene. *Science* **282**: 2085–2088.
17. Reed, N.D., Manning, J. K. and Rudbach, J. A. 1973. Immunologic responses of mice to Lipopolysaccharide from *Escherichia coli*. *J. Infect. Dis.* **128** (*Suppl.*): S70–S74.
18. Roeder, D.J., Lei, M-G. and Morrison, D. C. 1989. Endotoxic-lipopolysaccharide-specific binding proteins on lymphoid cells of various animal species: association with endotoxin susceptibility. *Infect. Immun.* **57**: 1054–1058.
19. Shaji, A.V., Kulkarni, M. Y. and Agrewala, J. N. 1998. Regulation of secretion of IL-4 and IgG1 isotype by melatonin-stimulated ovalbumin specific T cells. *Clin. Exp. Immunol.* **111**: 181–185.
20. Stevens, T.L., Bossie, A., Sanders, V. M., Fernandez, B. R., Coffman, R. L., Mosmann, T. R. and Vitetta, E. S. 1988. Regulation of antibody isotype secretion by subset of antigen specific helper T cells. *Nature (Lond.)* **334**: 2525–2528.
21. Werling, D. and Jungi, T. W. 2003. Toll-like receptors linking innate and adaptive immune response. *Vet. Immunol. Immunopathol.* **91**: 1–12.
22. West, M.A. and Heagy, W. 2002. Endotoxin tolerance: A review. *Crit. Care. Med.* **30**: S64–S73.
23. Zang, Y.H., Takahashi, K., Jiang, G. Z., Kawai, M., Fukada, M. and Yokochi, T. 1993. *In vitro* induction of apoptosis (programmed cell death) in mouse thymus by administration of lipopolysaccharide. *Infect. Immun.* **61**: 5044–5048.

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Original Article

Comparative Investigation of Several Sperm Analysis Methods for Evaluation of Spermatotoxicity of Industrial Chemical: 2-Bromopropane as an Example

Katsumi OHTANI[1]*, Shigeru YAMAZAKI[2], Hisayo KUBOTA[1], Muneyuki MIYAGAWA[1] and Junzo SAEGUSA[1]

[1]National Institute of Industrial Health, Independent Administrative Institution, 6–21–1, Nagao, Tama-ku, Kawasaki, Kanagawa 214-8585, Japan

[2]School of Medicine, Showa University, 1–5–8, Hatanodai, Shinagawa-ku, Tokyo 142-8555, Japan

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Abstract: Reproductive toxicity of 2-bromopropane (2BP), a substitute for ozone layer-depleting chloro-fluorocarbon, was found among the workers in an electronics factory in Korea in 1995. Furthermore the importance of testicular toxicity has been realized since the problem of endocrine disruptors arose all over the world, but manual methods must rely on subjective assessment. Recently, computer-assisted sperm analysis (CASA) was proposed but this system requires vast investment. We then investigated the applicability of the MTT method with a microplate and sperm quality analyzer (SQA) as simple, rapid, and economic instrumental methods for the examination of sperm quality in rats, comparing it with the manual microscopic method and CASA. Epididymal fluid derived from male F344/N Slc (Fischer) rats intraperitoneally injected with 2BP in the dose range of 125–1,000 mg/kg/d twice a week (total 8 times) were examined by these methods as a model experiment. Sperm count measured by the manual method and CASA in the epididymal fluid, absorbance by the MTT method and sperm motility index value by the SQA method were significantly lower in the 2BP 1,000 mg/kg administered group than in the control group. This result suggests that the MTT method can detect oligospermia. With the microplate and microplate reader, the efficiency of detection becomes much better. Sperm analyses by the MTT method with the microplate reader and the SQA method are available for reproductive toxicity study in rats.

Key words: 2-Bromopropane, Tetrazolium salt, MTT (3-(4,5-dimethylthioazol-2-yl)-2,5-diphenyl tetrazolium bromide), SQA (sperm quality analyzer), CASA (computer-assisted sperm analysis), Manual microscopic method, Reproductive toxicity, Rat

Introduction

Certain substances found in the environment can upset normal endocrine balance and become a health hazard. An example of growing concern is their effect on sperm[1]. Some workers in semiconductor factories in Korea were found to have affected in their reproductive functions after exposure to 2-bromopropane (2BP)[2,3]. Subsequently, the reproductive effects of 2BP were confirmed to the animal experimental studies[4–7]. These reports prompted close reappraisal of the efficacy and feasibility of mass screening for toxicity to male reproductive functions in industrial populations.

Surveying the method of investigating sperm activity, each method now in use has its own serious shortcomings. For instance, the conventional, manual method of sperm count and assessment of motility under the optical microscope is fraught with inevitable subjective variations which would make inter-institutional comparison of data practically

*To whom correspondence should be addressed.

impossible. The introduction of computer-assisted sperm analysis (CASA) eliminated variations due to subjective evaluation but this apparatus is expensive[8–13] and has not enjoyed wide acceptance. Meanwhile, the sperm quality analyzer (SQA), which measures sperm count and motility by the optical method, was introduced as a simple and inexpensive alternative[14, 15]. The third approach is the biochemical method (MTT method) which measures color changes in the tetrazolium reaction to mitochondrial reductase by absorption spectrometry reflecting the overall numerical and functional power of sperm activity[16, 17]. Although these methods of sperm testing have proliferated, their performance and efficacy have been evaluated individually and never collectively using the same test samples. In particular, comparative investigation of the MTT method with CASA has never been performed. Their performance in terms of mass handling of large numbers of samples has not been evaluated properly.

We attempted to develop a method for measuring toxicity to the sperm by combining an absorption spectrophotometer with a microplate reader so that a large number of specimen can be processed rapidly. The method is objective, simple, inexpensive and efficient and can be applied to mass screening of workers in suspicious environments. In the days when more and more clinical tests for male reproductive disability need to be performed on an everyday basis, the ability to processing a large number of samples will be an important prerequisite in the selection of test methods. Furthermore, the MTT method, with its speed and simplicity to deal with a large number of facilities, is a technique suitable for the animal studies of male reproductive disturbance induced by various chemicals. In this study, we induced reproductive toxicity with 2BP as a representative of bromopropanes, which are used widely in the industrial workplace.

We report the results of a study carried out on rats given repeated doses of 2BP using the MTT method with a microplate reader in comparison with other methods of sperm testing including CASA. The advantages and merits of various methods were compared and problems in performing the tests will be discussed.

Materials and Methods

Chemicals and supplements

2BP and MTT were purchased from Tokyo Kasei Kogyo (Tokyo, Japan) and Dojin (Kumamoto, Japan), respectively. Olive oil, HCl and isopropyl alcohol were from Wako Pure Chemical (Osaka, Japan). Bovine serum albumin Fraction V (BSA), Medium 199 and phosphate buffered saline (PBS) were from Seikagaku Kogyo (Tokyo, Japan), GIBCO (Grand Island, USA) and Nissui Pharmaceutical (Tokyo, Japan), respectively.

Instruments

The semen analyzer (HTM-IVOS Ver. 10.9i) was from Hamilton Thorne Research (Beverly, MA, USA). SQA was the product of Medical Electronic Systems (Migdal Haemek, Israel). The microplate reader (Immunoreader NJ2000) was purchased from Nalge Nunc (Tokyo, Japan). The optical microscope (Eclipse E600) was from Nikon (Tokyo, Japan).

Experimental protocol

F344/N Slc (Fischer) male rats (11 wk of age) from Japan SLC (Shizuoka, Japan) were kept in cages under standard conditions and received pellets (Oriental Yeast, Tokyo, Japan) and water *ad libitum*. The body weight was monitored just before each administration and sacrifice. Each of 4–5 rats (12 wk of age)/group received intraperitoneal instillations of 2BP dissolved in olive oil twice a week for 24 d in doses of 125, 250, 500 and 1,000 mg/kg. Control rats received an equal volume of olive oil. So each rat received a total of 8 injections. After a one week rest period following the last dose, the animals were sacrificed under ether anesthesia and the testes, epididymis and epididymal cauda were separated and weighed immediately. And then relative organ weights were calculated.

Preparation of epididymal cauda sample

Epididymal cauda was minced with scissors to release sperm in 2 ml of Medium 199 containing 0.5% BSA at 37°C. This sperm suspension sample served for the MTT and SQA methods. The aliquot of this sample was stored at −80°C. Before sperm count analysis, this aliquot was diluted 1:4 with PBS, further homogenized at room temperature, and served as a sample for the manual method and CASA.

Manual method

After staining with trypan blue the specimen was spread on a hemocytometer and the sperm heads were counted manually under the optical microscope. The data were expressed as the total number of sperm per one cauda epididymal tissue.

CASA

Each sample was stained with the attached staining kit (Supra Vital IDENT Stain Kit, Hamilton-Thorne Research, Bevely, MA, USA), dropped into a disposable counting chamber CELL-VU (Millennium Sciences Corp. NY, USA) and

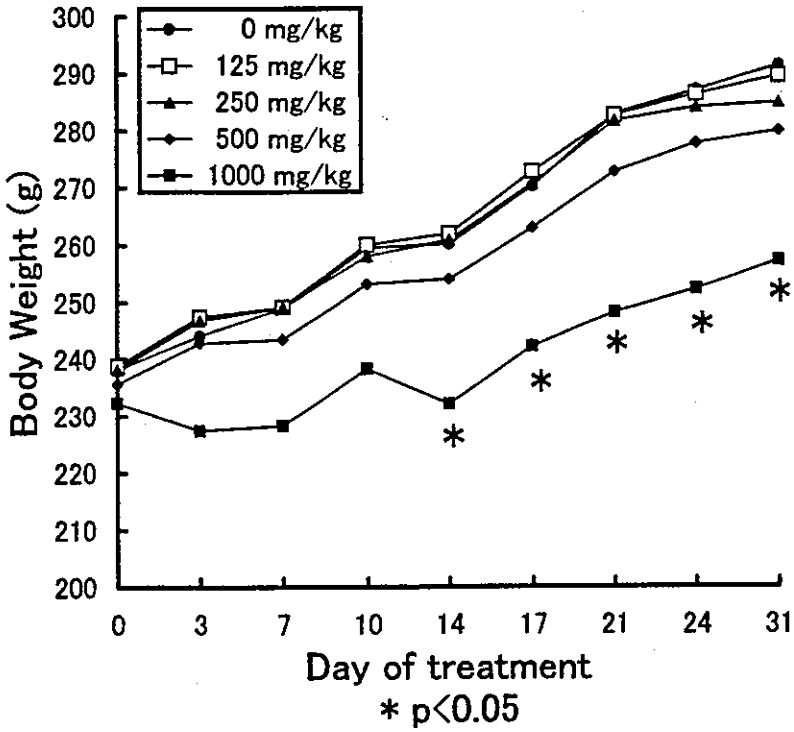

Fig. 1. Mean body weight of F344 rats exposed to 2BP ip.

mounted on the Semen Analyzer. Sperm heads fluorescence under an ultra-violet beam were counted in RAT-IDENT mode automatically. The data were expressed as the total number of sperm per cauda epididymal tissue sample.

SQA method

The disposable SQA capillary (Medical Electronic Systems, Migdal Haemek, Israel) containing the specimen was inserted into the slot in the SQA and the sperm motility index (SMI) was determined.

MTT method

Fifty microliters of the sperm suspension sample in the sterile 96-microplate well was incubated with MTT reagent (5 mg/ml in PBS, 25 micro liter) at 37°C for two hours. Then the reaction was stopped by rapid cooling. After the addition of 0.04 M HCl-isopropylalcohol and pipeting exhaustively to dissolve the formazan thus formed in the process, the absorbance in each well was estimated at 574 nm by the microplate reader.

Correlation among data from various methods

In an attempt at evaluation of various methods, we carried out the following experiments. Epididymis cauda obtained from an untreated rat (17 wk of age) was dissected with a pair of scissors in 2 ml of medium to suspend sperm. The sample consisted of this undiluted suspension and its dilutions with Medium 199 containing 0.5% BSA to 4 strength. Data on sperm count, SMI and absorbance obtained by pair of investigating methods were compared and correlation was sought.

Statistical analysis

The data were analyzed by one-way ANOVA. The statistical significance of difference between the control and 2BP-treated groups was determined with Fisher's PLSD test. In all cases, $P < 0.05$ was considered statistically significant.

Results

Body and organ weights

Body weight decreased in the groups with a dose of 1,000 mg/kg (Fig. 1) on and after day 14 as compared to the control group. The relative weights of testis (TE, right (R) and left (L)), epididymis (EP, right (R) and left (L)), and epididymis cauda (EPC, right (R) and left (L)) are shown in Table 1. The weight of both the right and left testis decreased in the 500 and 1,000 mg/kg dose groups. In the 250 mg/kg dose group, only the left testis weight decreased significantly, but no significant difference was found in the right testis.

Table 1. Relative weight (%) of reproductive organ in 2BP-treated (mg/kg) rats

2BP (mg/kg)	TER	TEL	EPR	EPL	EPCR	EPCL
0	0.503 ± 0.010	0.521 ± 0.018	0.172 ± 0.007	0.176 ± 0.011	0.080 ± 0.004	0.076 ± 0.005
125	0.505 ± 0.020	0.512 ± 0.020	0.186 ± 0.012	0.176 ± 0.017	0.089 ± 0.003*	0.082 ± 0.006
250	0.493 ± 0.023	0.480 ± 0.025*	0.183 ± 0.011	0.184 ± 0.014	0.087 ± 0.006	0.089 ± 0.011*
500	0.407 ± 0.021*	0.409 ± 0.038*	0.168 ± 0.012	0.179 ± 0.008	0.086 ± 0.006	0.076 ± 0.003
1000	0.193 ± 0.010*	0.199 ± 0.019*	0.131 ± 0.009*	0.130 ± 0.007*	0.056 ± 0.006*	0.055 ± 0.004*

TER: Right Testis, TEL: Left Testis, EPR: Right Epidydymis, EPL: Left Epididymis. EPCR: Right Epididymal Cauda, EPCL: Left Epididymal Cauda. Each value represents the mean ± SD. *: Significantly different at $p<0.05$.

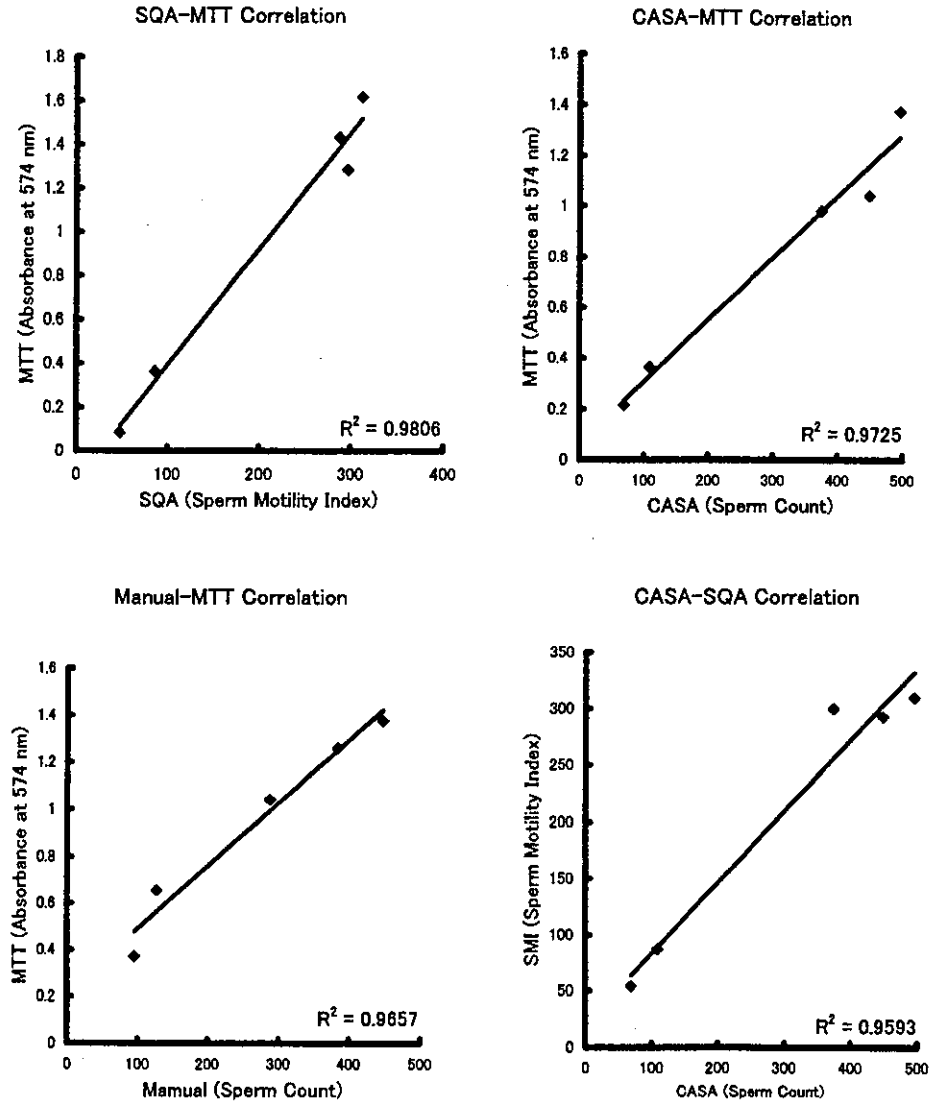

Fig. 2. Relationship between parameters of several methods.

And the weights of the epididymis and epididymal cauda decreased in the 1,000 mg/kg group, but some reverse results were found in lower dose groups (EPCR: 125 mg/kg, EPCL: 250 mg/kg), but they were not in both sides of the tissues.

Correlation between the sperm analysis methods

Figure 2 shows the correlation between the two methods. A high correlation was found between the SMI value found by the SQA method and absorption by the MTT method