doi:10.1006/cyto.2001.0940, available online at http://www.idealibrary.com on IDEAL®

# EFFECT OF MIDAZOLAM ON INTERLEUKIN-6 mRNA EXPRESSION IN HUMAN PERIPHERAL BLOOD MONONUCLEAR CELLS IN THE ABSENCE OF LIPOPOLYSACCHARIDE

Takuya Miyawaki,[1] Norio Sogawa,[2] Sigeru Maeda,[1] Atsushi Kohjitani,[1] Masahiko Shimada[1]

**Midazolam, a benzodiazepine, has an hypnotic effect via benzodiazepine receptors and is widely used as an anaesthetic. Recently, it has been suggested that benzodiazepines modulate cytokine responses. The purpose of the present study was to evaluate the effect of midazolam on interleukin-6 (IL-6) response by observing mRNA expression levels in human peripheral blood mononuclear cells (PBMCs) in the absence of lipopolysaccharide (LPS). PBMCs were isolated from healthy volunteers in endotoxin-free 0.9% sodium chloride solution. The cells were incubated for 2 h at 37°C immediately after isolation. IL-6 mRNA expression levels in the cells were quantified using reverse transcription and competitive polymerase chain reaction. It was found that midazolam time-dependently inhibited the IL-6 mRNA expression in PBMCs in the absence of LPS, and significantly inhibited the IL-6 mRNA expression at 1 μg/ml ($P<0.05$) or 10 μg/ml ($P<0.01$) in the absence of LPS. However, neither a specific agonist of peripheral-type benzodiazepine receptors, Ro5-4864, nor a specific agonist of central-type benzodiazepine receptors, clonazepam, inhibited IL-6 mRNA expression. These findings indicated a suppression of the IL-6 response in human PBMCs by midazolam in the absence of LPS, and suggests that midazolam has its effect not via benzodiazepine receptors, but by another mechanism.**



Interleukin-6 (IL-6), a proinflammatory cytokine, is consistently released in the systemic circulation following surgical injury and plays an important role in post-operative states, including the immune response, inflammatory response, metabolism, and healing.[1] Furthermore, it has been suggested to be associated with post-operative complications in patients undergoing surgery.[2] Thus, IL-6 is thought to be a key mediator of immune and inflammatory cascades following surgical injury. Recently, attention has focused on anaesthetic agents that modulate the cytokine response to surgical injury.[3–5] Larsen *et al.*[5] reported on the effects of various type of intravenous anaesthetics, including ketamine, thiopentone, etomidate, propofol, and midazolam, on cytokine responses in cultured whole blood cells, and suggested a complicated modulation of the cytokine responses by these anaesthetics.

Midazolam, a benzodiazepine, has an hypnotic effect via benzodiazepine receptors coupled to type A γ-aminobutyric acid ($GABA_A$) receptor in the brain and is widely used as an anaesthetic.[6] Benzodiazepines have recently been suggested to modulate cytokine responses, including IL-6 response.[7–9] Benzodiazepine receptors consist of two types of receptors, central-type benzodiazepine receptors (CBRs) and peripheral-type benzodiazepine receptors (PBRs) which are not coupled to $GABA_A$ receptors. Benzodiazepines have been suggested to have some effects on peripheral tissue,[10] and PBRs identified in blood cells have been suggested to be associated with immune response.[11,12] However, the effect of benzodiazepine on immune response has not been fully clarified. Midazolam is known to bind to both receptor types and was also reported to have the effect on tumour necrosis factor activity in mouse macrophages.[13] With regard to the

From the [1]Department of Dental Anesthesiology, Okayama University Hospital of Dentistry, and [2]Department of Dental Pharmacology, Okayama University Dental School, 2-5-1 Shikata-cho, Okayama 700-8525, Japan

Correspondence to: Takuya Miyawaki, Department of Dental Anesthesiology, Okayama University Hospital of Dentistry, 2-5-1 Shikata-cho, Okayama 700-8525, Japan. Fax: +81-86-235-6721, E-mail: miyawaki@md.okayama-u.ac.jp

Received 1 April 2001; received in revised form 30 June 2001; accepted for publication 16 July 2001



1043–4666/01/180320+08 $35.00/0

KEY WORDS: benzodiazepine receptors/human peripheral blood mononuclear cells/interleukin-6/midazolam/mRNA

effect on IL-6 response, the agonists of PBRs, including midazolam, were reported to modulate the lipopolysaccharide (LPS)–induced IL-6 response,[7–9] whereas in the absence of LPS they had no effect.[9] However, few studies have investigated this in the absence of LPS and the finding has not been fully demonstrated. Furthermore, differing from sepsis, where LPS stimulation is a major inducer of IL-6 production, the IL-6 response following surgical injury is thought to be relatively independent of LPS stimulation because surgical wounds are disinfected.

Thus, the purpose of the present study was to evaluate the effect of midazolam on the IL-6 response in human peripheral blood mononuclear cells (PBMCs) in the absence of LPS. Furthermore, in order to clarify the mechanism, we evaluated whether the effect of midazolam on the IL-6 response was mediated by benzodiazepine receptors, using a specific ligand of CBRs or PBRs.

Since the samples obtained in the present study were limited and never stimulated by LPS, we determined the IL-6 activity by observing messenger RNA (mRNA) expression levels in PBMCs using the competitive polymerase chain reaction (PCR) technique preceded by reverse transcription of mRNA to cDNA, a sensitive and useful molecular biology technique.[14–17]

## RESULTS

### *Viability of PBMCs*

The viability of PBMCs in the presence of midazolam (10 μg/ml) was similar to that in endotoxin-free 0.9% sodium chloride (NaCl) solution without midazolam (control) until 8 h (Fig. 1). It decreased time-dependently, but did not greatly differ from the baseline values during 2 h in both the midazolam-treated and the control PBMCs. The 2 h viability of PBMCs in the absence and presence of midazolam (10 μg/ml) were $98.3 \pm 1.78\%$ and $98.8 \pm 1.24\%$, respectively. Therefore, we determined the duration of incubation (2 h) of PBMCs.

### *Time-course of the effect of midazolam on IL-6 mRNA expression*

We investigated the time course of the effect of midazolam (10 μg/ml) and endotoxin-free 0.9% NaCl solution without midazolam (control) on IL-6 mRNA expression in the cells in the absence of LPS in a 2 h time-period. In the control, the IL-6 mRNA expression level increased at 30 min incubation, compared with the baseline value, but showed no difference from the baseline value at the incubation of 60 or 120 min. In the midazolam-treated cells, the IL-6 mRNA expression levels at incubation times of 30, 60, and 120 min were significantly lower than the values in the control (Fig. 2, $P<0.01$). Midazolam (10 μg/ml) time-dependently inhibited the IL-6 mRNA expression in PBMCs in the absence of LPS by 2 h.

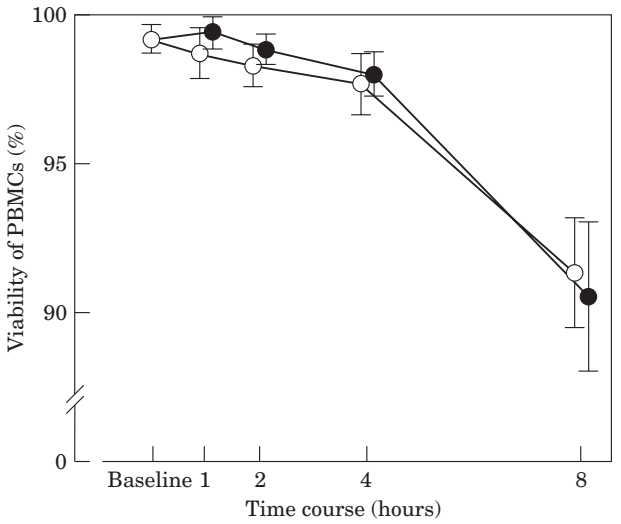


**Figure 1. Time course of PBMC viability in the presence of midazolam (10 μg/ml) (closed circles) and in 0.9% NaCl solution without midazolam (open circles) ($n=6$. mean ± SEM).**

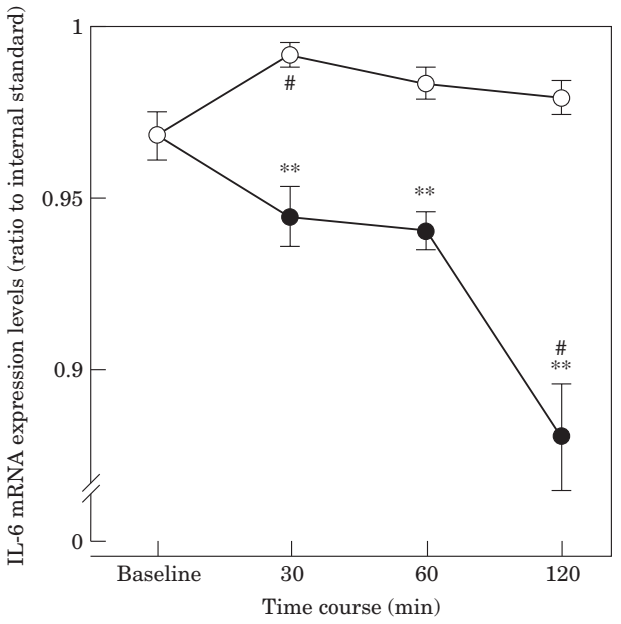


**Figure 2. Time-course of the effect of midazolam (10 μg/ml) (closed circles) and 0.9% NaCl solution without midazolam (control (open circles) on IL-6 mRNA expression levels in PBMCs in the absence of LPS ($n=5$, mean ± SEM).**

**$P<0.01$ compared with the control, analysed using the Mann–Whitney $U$-test. #$P<0.001$ compared with the baseline value, analysed using the Kruskal–Wallis test followed by Dunn's test.

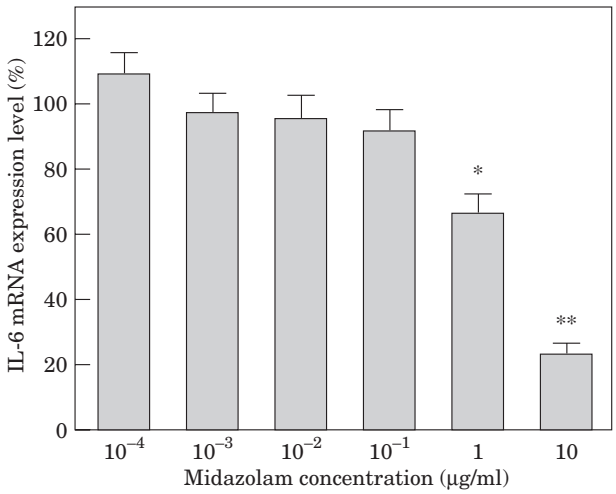


**Figure 3. The dose-dependent effect of midazolam on IL-6 mRNA expression level in the absence of LPS.**

Data are percentages of IL-6 mRNA expression level in PBMCs incubated for 2 h with each concentration of midazolam relative to the control values (only 0.9% NaCl solution) ($n=8$, mean ± SEM). *$P<0.05$, **$P<0.01$ compared with the control, analysed using the Kruskal–Wallis test followed by Dunn's test.

### *Dose-dependent effect of midazolam on IL-6 mRNA expression*

Midazolam was diluted with endotoxin-free 0.9% NaCl solution to $10^{-4}$, $10^{-3}$, $10^{-2}$, $10^{-1}$, 1, and 10 μg/ml. We incubated PBMCs for 2 h with each concentration of midazolam, and evaluated the effect of midazolam. Figure 3 shows the dose-dependent effect of midazolam on IL-6 mRNA expression level in the cells in the absence of LPS. Midazolam significantly inhibited the IL-6 mRNA expression at 1 μg/ml ($P<0.05$) or 10 μg/ml ($P<0.01$) in the absence of LPS, compared with the control (only 0.9% NaCl solution). This indicated a dose-dependent suppression of the IL-6 mRNA expression by midazolam.

### *The effect of a specific ligand of benzodiazepine receptors on IL-6 mRNA expression*

We incubated PBMCs with a specific agonist of PBRs, Ro5-4864 (10 μg/ml), and a specific agonist of CBRs, clonazepam (10 μg/ml), and evaluated the effect of each agonist on the IL-6 mRNA expression in the cells. Ro5-4864 had no effect on the IL-6 mRNA expression as well as clonazepam, and the suppression by midazolam was significantly great, compared with Ro5-4864 or clonazepam (Fig. 4).

Furthermore, we evaluated the reverse effect of a specific antagonist of PBRs, PK11195 (0.1, 1 and 10 μg/ml), on the suppression of IL-6 mRNA expression by midazolam for 2 h. No reverse effects of PK11195 on the suppression by midazolam were observed in the absence of LPS (Fig. 5).

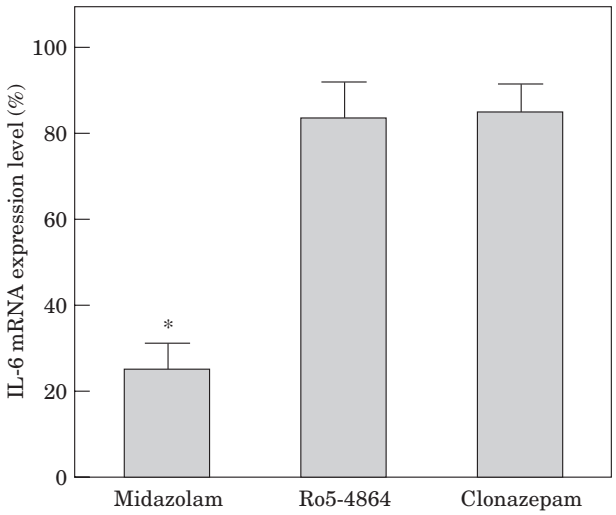


**Figure 4. The effects of midazolam (10 μg/ml), a specific agonist of PBRs, Ro5-4864 (10 μg/ml), and a specific agonist of CBRs, clonazepam (10 μg/ml), on IL-6 mRNA expression level in the absence of LPS.**

Data are percentages of IL-6 mRNA expression level in PBMCs incubated for 2 h with each drug, relative to the control values (only 0.9% NaCl solution) ($n=13$, mean ± SEM.). *$P<0.05$ compared with Ro5-4864 or clonazepam, analysed using the Kruskal–Wallis test followed by Dunn's test.

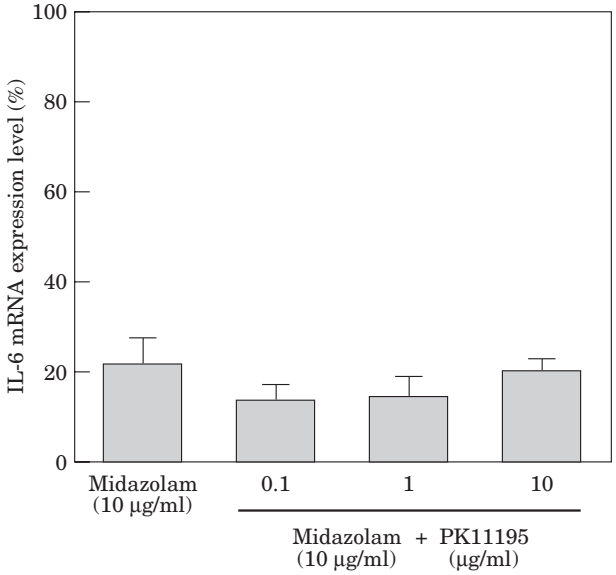


**Figure 5. The reverse effect of a specific antagonist of PBRs, PK11195 (0.1, 1 and 10 μg/ml), on the suppression of IL-6 mRNA expression by midazolam in the absence of LPS.**

Data are percentages of IL-6 mRNA expression level in PBMCs incubated for 2 h with each concentration relative to the control values (only 0.9% NaCl solution) ($n=6$, mean ± SEM).

## DISCUSSION

Cytokine activity was initially evaluated by the detection of cytokine proteins in a cell culture supernatant or in peripheral plasma as measured by a

bioassay or by an immunoassay. However, the measurements of cytokine proteins are not sensitive enough to fully evaluate the cytokine response. Recently, new molecular biology techniques have provided the opportunity to evaluate the cytokine response with a higher sensitivity than methods measuring proteins. Cytokine mRNA expression represents an early and potential part of the cascade of cytokine protein production and can be used as an indicator of cytokine response.[18] Amplification by PCR techniques preceded by reverse transcription of mRNA to cDNA has been shown to be a powerful technique for detecting the alterations of cytokine mRNA even in small tissue samples. Many studies[16,18–21] reported the cytokine response in various type of cells by using the PCR technique. The PCR technique, however, has difficulties obtaining accurate quantification for the initial amount of target gene before PCR, because the PCR product increases exponentially with each PCR cycle of amplification. To obtain the quantification for evaluating the initial amount of target gene, several studies[18,19] have calculated and used the ratio of the amplified products of a target gene to the amplified products of a housekeeping gene such as β-actin. However, the difference in the efficiency of amplification between the target gene and the housekeeping gene could produce discrepancies between the calculated amount and the initial amount of the target gene. Thus, the conditions for such a method, including concentrations of the target gene and number of PCR cycles, are limited for obtaining an accurate quantification.

Competitive PCR is one method for obtaining more accurate quantification, as described in a recent review.[17] Competitive DNA used as an internal standard in the competitive PCR is amplified in a single tube using similar primers to the target gene so that the efficiency of their amplification is thought to be similar. Thus, the ratio of the amplified target gene products to the amplified competitive DNA products, distinguished by size, remains precisely constant through the amplification, and accurate quantification of the initial amount of the target gene can be obtained.[14,15] In the present study, we established the best conditions of PCR for the quantification of IL-6 mRNA expression level in the preliminary study. Furthermore, because amplification can be performed over many cycles, competitive PCR is the most useful method for the quantification of mRNA from a small number of cells.[16] Amplification of 30–50 PCR cycles for competitive PCR was suggested to be most accurate.[17] In the present study, we were able to evaluate the innate IL-6 mRNA expression in the small number of PBMCs by amplifying at 45 PCR cycles.

Salts of midazolam can be prepared with hydrochloric, maleic, or lactic acid, and are easily soluble and very stable in water at a pH $\leqq$4. Furthermore, the hydrochloride solution, in particular, is known to be well-tolerated in both intravenous and intramuscular administration.[22] However, the solubility of the molecular form of midazolam is limited in a high pH medium and 55 μg/ml in pH 7.9.[23] Thus, to obtain a stable condition for the midazolam solution in the present study, we used 0.9% NaCl solution for the dilution of midazolam and the incubation of PBMCs, and 10 μg/ml as a maximum concentration of midazolam. In a defined culture medium, RPMI 1640, midazolam had the same effect on IL-6 response as 0.9% NaCl solution at a concentration of 1 μg/ml (data not shown). Therefore, it is thought that the use of 0.9% NaCl solution would not greatly influence the results in the present study.

Midazolam was used at a relatively high concentration in the present study. However, the viability of the midazolam-treated cells was not different from that of control cells after 2 h incubation. Furthermore, LPS-induced elevation of IL-6 mRNA expression levels was found in midazolam-treated cells as well as the control cells (data not shown). Therefore, the effect of midazolam is not thought to result from the toxicity of midazolam. The present study demonstrated that midazolam affected IL-6 response at a concentration of 1 μg/ml and that this effect was elicited during a short-term incubation (30 min). Clinically relevant plasma concentrations are variable but are thought to range between 0.04 and 0.85 μg/ml,[24–27] so that the effective concentrations of midazolam in the present study are a little higher than the plasma concentrations in clinical use. However, when midazolam was administered intravenously, the plasma concentration is thought to be high at the end of this range for a while after administration. Even if the elevation of midazolam concentration persists for a short time, the effect of midazolam on PBMCs may be elicited. Furthermore, relative high concentrations in this range of midazolam may have an inhibitory effect.

Benzodiazepines have been demonstrated to modulate the cytokine response in cells.[7–9,13,28] Taupin *et al.*[9,28] showed that the ligands of PBRs enhanced LPS-induced tumour necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) responses in vitro. In contrast, Matsumoto *et al.*[13] demonstrated the dose-dependent inhibitory effects of the ligands of PBRs on TNF-α activity in the presence of LPS. It was not clarified whether the discrepancy between these findings was due to the differences in the conditions of their methods. Similarly, the ligands of PBRs were reported to inhibit the LPS-induced IL-6 response in vivo,[7,8] whereas in another in vitro study[9] they enhanced the IL-6 response in the presence of LPS. With regards to the discrepancy between these findings results of IL-6, Zavala[29] suggested that the inhibition of the IL-6

response by the ligands of PBRs in vivo was not a direct effect but induced by the secondary actions of the drug at other sites in the body, and that benzodiazepines increased LPS-induced cytokine production at low concentrations and inhibited it at high concentrations. However, all their studies evaluated the cytokine responses in cells stimulated by LPS, which is a major inducer of not only IL-6 but also other cytokines, including TNF-α, IL-1β, and interleukin-10 (IL-10).[30] TNF-α and IL-1β induces the IL-6 production.[31] However, IL-10 inhibits TNF-α, IL-1β, and IL-6 production.[32] Thus, IL-6 production in a cell is induced by LPS, TNF-α and IL-1β, although in contrast, it is inhibited by IL-10. Furthermore, Zhong *et al.*[18] demonstrated that the cytokine responses by LPS stimulation itself were complicated, and are divided into two basic groups: an early and more LPS-sensitive group, including TNF-α and IL-1β, and a delayed and less LPS-sensitive group, including IL-6, and suggested that IL-6 activation in the presence of LPS might occur through a regulatory mechanism or be induced by the early-responding cytokines, such as TNF-α and IL-1β. These complicated responses appear to be induced by LPS stimulation in a cell or a culture during long-term incubation, resulting in different findings, depending on the conditions.

With regard to the mechanism of benzodiazepine action on cytokine response, Zavala[29] suggested that PBRs might be linked directly or indirectly to a second-messenger pathway that involves prostaglandin metabolism. Furthermore, benzodiazepines have been reported to inhibit cyclic AMP production[33], which is enhanced by prostaglandins and was shown to enhance IL-6 production.[34] However, a specific agonist of PBRs, Ro5-4864, was also found to have the effects on the prostaglandin metabolism and cyclic AMP production as well as diazepam.[33] This means that these effects were mediated with PBRs. In the present study Ro5-4864 had no effect on IL-6 response, and a specific antagonist of PBRs, PK11195, could not reverse the effect of midazolam. These findings suggest that midazolam induces the effect but not via benzodiazepine receptors. Other investigators[35–39] also reported that benzodiazepines had a peripheral effect that was independent of peripheral benzodiazepine receptors. However, the mechanism of action has not been fully clarified. Marti-Cabrera *et al.*[35] suggested that benzodiazepines had another low (micromolar)-affinity binding site. This binding site was postulated to be linked to L-type calcium channels. Although benzodiazepines have been suggested to be involved in modulation of calcium channels,[40] it seems that the effect of midazolam on IL-6 response in the present study was independent of the modulation of calcium channels because we used a calcium-free medium, 0.9% NaCl solution. Crocker *et al.*[36] suggested that an effect that was independent of peripheral benzodiazepine receptors might be involved in the inhibition of the calcium/protein kinase C-related pathway. Zhao *et al.*[38] showed that benzodiazepines at a submicromolar concentration potentiated the increase in free cytosolic calcium concentration in astrocytes. Furthermore, Yamakage *et al.*[39] demonstrated that calcium-activated and voltage-dependent potassium outward currents were inhibited by high concentrations of diazepam and midazolam in smooth muscle cells. These effects were seen at high concentrations in agreement with the finding in the present study. However, it is not clear whether these effects are linked directly or indirectly to IL-6 response in PBMCs. Further studies will be necessary to clarify the mechanism in the effect of midazolam on the IL-6 response in human PBMCs.

IL-6 has been suggested to be associated with post-operative complications in patients undergoing surgery.[2] In a previous study, we demonstrated that the elevation of plasma IL-6 level was correlated with post-operative fever.[41] Furthermore, prolonged increases of circulating IL-6 are associated with morbidity and mortality after cardiac operations.[42] IL-6 was demonstrated to have a negative inotropic effect on the heart, mediated though myocardial nitric oxide production.[43] Royblat *et al.*[44] indicated that ketamine, added to opioid-based anaesthesia, inhibited the increase in the plasma IL-6 level following coronary artery bypass surgery, and suggested that inhibition of cytokine responses might be clinically beneficial to improve outcome following surgery. In order to regulate the acute systemic inflammatory response including a post-operative state, further studies of inhibition of excessive cytokine response are important. Although the findings of the present study indicated a suppression of the IL-6 response in human PBMCs by midazolam in the absence of LPS, it will be necessary to evaluate the effect of midazolam on excessive IL-6 response, including injury-induced response and LPS-induced response, other cytokine responses, and the network of cytokine responses.

## MATERIALS AND METHODS

### *Drugs*

Midazolam (Dormicum) was purchased from Yamanouchi Pharmaceutical Co. (Tokyo, Japan). Ro5-4864 [4′-Chlorodiazepam: 7-chloro-5-(4-chlorophenyl)-1,3-dihydro-1-methyl-2H-1,4-benzodiazepine-2-one] was purchased from Fluka (Buchs, Switzerland). Clonazepam and PK11195 [1-(2-chlorophenyl)-N-methyl-N-(1-methylpropyl)-3-isoquinolinecarboxamide] were purchased from Sigma Chemical Co. (St Louis, MO, USA). Midazolam were dissolved with endotoxin-free 0.9% NaCl solution (S8776, Sigma, USA). Ro5-4864, clonazepam, and PK11195 were dissolved with ethanol (<0.1% final volume concentration) and the endotoxin-free 0.9% NaCl solution.

### *Separation and incubation of PBMCs*

The present study was approved by the ethics committee of our institution. We enrolled healthy volunteers aged between 25 and 40 years with their informed consent. From each, a venous blood sample (21 ml) was collected in a sterile tube containing EDTA (1 mg/blood 1 ml). PBMCs were separated by a density-gradient centrifugation using a Mono-Poly Resolving Medium (Dainippon Pharmaceutical Co. Osaka, Japan), followed by washing and placing at 3 to $4 \times 10^6$ cells/ml of the endotoxin-free 0.9% NaCl solution. In the absence of LPS, the separated PBMCs were incubated with midazolam, Ro5-4864, clonazepam, or PK11195 at 37°C immediately after the separation. The pH of each sample was measured and adjusted to the similar pH by adding a hydrochloride solution or a sodium hydroxide solution.

### *Evaluation of the viability of PBMCs*

We investigated the time course of viability of PBMCs in the absence and presence of midazolam in endotoxin-free 0.9% NaCl solution for 8 h. The viability of the cells was evaluated by the ability to exclude 0.2% trypan blue.

### *RNA isolation form PBMCs and IL-6 cDNA synthesis from isolated RNA*

Total RNA was isolated from the PBMCs by the single step acid guanidium-isothiocyanate-phenol-chloroform extraction method, using a TRIzol LS (GIBCO BRL, Life Technologies, Inc., Rockville, MD, USA). The amount of isolated RNA was measured using a spectrophotometer (model DU-640, Beckman, Tokyo, Japan). Isolated RNA was treated with amplification grade DNase I (GIBCO BRL, Life Technologies, Inc., Rockville, MD, USA). First-chain cDNA was synthesised from 1 μg RNA, using a Superscript Preamplification System (GIBCO BRL, Life Technologies, Inc., Rockville, MD, USA).

### *Competitive PCR*

Synthesized cDNA was amplified with primers specific for human IL-6 mRNA (Human Interleukin-6 Amplimer Sets, Clontech, Laboratories, Inc., Palo Alto, CA, USA) in the presence of $10^{-1}$ attomoles competitive DNA (PCR MIMICs human IL-6, Clontech, Laboratories, Inc., Palo Alto, CA, USA) as an internal standard by PCR with a DNA polymerase (AmpliTag Gold, PE Biosystems, Foster City, CA, USA) and a thermal cycler (PC-800 Programmable Temp Control System, Astec, Fukuoka, Japan). Housekeeping gene primers (Human G3PDH Control Amplimer Sets, Clontech, Laboratories, Inc., Palo Alto, CA, USA) were also used in PCR amplification of each sample for the uniformity of the RNA isolation and reverse transcription steps. The PCR mixture was heated at 95°C for 10 min, followed by 45 cycles for the IL-6 gene or by 35 cycles for the housekeeping gene, each consisting of denaturation for 45 s at 94°C, annealing for 45 s at 56°C, and elongation for 2 min at 72°C.

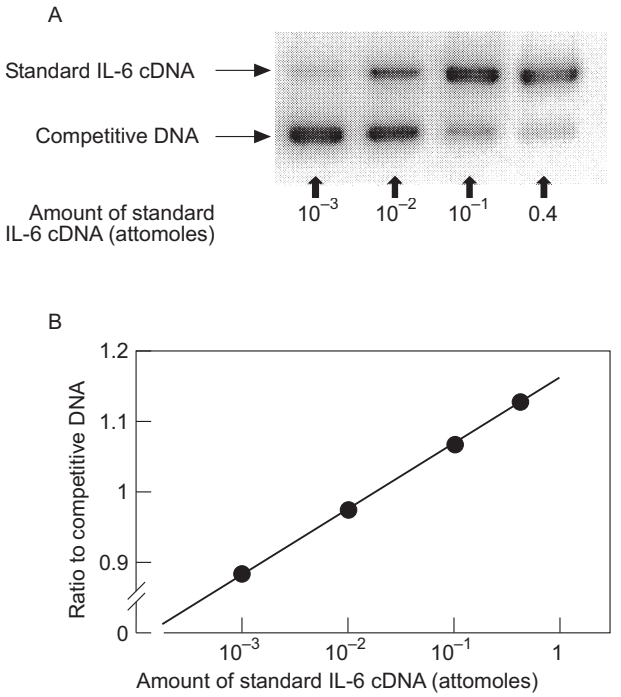


**Figure 6. (A) PCR product bands of standard IL-6 cDNA and competitive DNA, visualised on a transilluminator. (B) The proportionality between the initial amounts of IL-6 cDNA and the ratio of IL-6 cDNA to competitive DNA.**

Values are plotted as the ratio of log standard IL-6 cDNA intensity/log competitive DNA intensity vs the initial amounts of standard IL-6 cDNA ($10^{-3}$, $10^{-2}$, $10^{-1}$, and 0.4 attomoles) prior to PCR. It shows a linear relationship between the initial amounts of standard cDNA prior to PCR and the calculated ratio.

### *Quantitative evaluation of IL-6 mRNA expression*

PCR products were electrophoresed through 2% agarose and placed in ethidium bromide solution for 30 min. The size of the PCR product for IL-6 cDNA was clarified by a DNA size marker (φX174/Hae III digest, Toyobo Co., Ltd., Osaka, Japan) and electrophoresed. The band of competitive DNA was found to be located slightly below the IL-6 band. The intensity of the band generated by IL-6 cDNA or competitive DNA was quantified by a densitometer (Foto/Analyst Image Analysis System, Fotodyne Incorporated, Hartland, WI, USA). The ratio of log IL-6 cDNA intensity to log competitive DNA intensity was calculated. In a preliminary study, we established proportionality between the initial amounts of IL-6 gene and the calculated ratio. The preliminary study showed a linear relationship between the amounts of standard IL-6 cDNA prior to the PCR and the ratio of IL-6 cDNA to competitive DNA (Fig. 6). This finding indicates that this method is useful for quantification of IL-6 mRNA expression.

### *Statistical analysis*

Differences were determined using the Mann–Whitney *U*-test or the Kruskal–Wallis test followed by Dunn's test. Statistical significance was defined as $P<0.05$.

## Acknowledgements

This work was supported by a Grant-in-Aid for Scientific Research (C) 11671865 from the Ministry of Education, Science, Sports and Culture, Japan

## REFERENCES

1. Biffl WL, Moore EE, Moore FA, Peterson VM (1996) Interleukin-6 in the injured patient. marker of injury or mediator of inflammation? Ann Surg 224:647–664.

2. Baigrie RJ, Lamont PM, Kwiatkowski D, Dallman MJ, Morris PJ (1992) Systemic cytokine response after major surgery. Br J Surg 79:757–760.

3. Crozier TA, Muller JE, Quittkat D, Sydow M, Wuttke W, Kettler D (1994) Effect of anaesthesia on the cytokine responses to abdominal surgery. Br J Anaesth 72:280–285.

4. Gilliland HE, Armstrong MA, Carabine U, McMurray TJ (1997) The choice of anesthetic maintenance technique influences the antiinflammatory cytokine response to abdominal surgery. Anesth Analg 85:1394–1398.

5. Larsen B, Hoff G, Wilhelm W, Buchinger H, Wanner GA, Bauer M (1998) Effect of intravenous anesthetics on spontaneous and endotoxin-stimulated cytokine response in cultured human whole blood. Anesthesiology 89:1218–1227.

6. Reves JG, Fragen RJ, Vinik HR, Greenblatt DJ (1985) Midazolam: pharmacology and uses. Anesthesiology 62:310–324.

7. Taupin V, Jayais P, Descamps-Latscha B, Cazalaa JB, Barrier G, Bach JF, Zavala F (1991) Benzodiazepine anesthesia in humans modulates the interleukin-1β, tumor necrosis tumor-α and interleukin-6 responses of blood monocytes. J Neuroimmunol 35: 13–19.

8. Zavala F, Taupin V, Descamps-Latscha B (1990) In vivo treatment with benzodiazepines inhibits murine phagocyte oxidative metabolism and production of interleukin 1, tumor necrosis factor and interleukin-6. J Pharmocol Exp Ther 255:442–450.

9. Taupin V, Gogusev J, Descamps-Latscha B, Zavala F (1992) Modulation of tumor necrosis factor-α, interleukin-1β, interleukin-6, interleukin-8, and granulocyte/Macrophage colony-stimulating factor expression in human monocytes by an endogenous anxiogenic benzodiazepine ligand, triakontatetraneuropeptide: evidence for a role of prostaglandins. Mol Pharmacol 43:64–69.

10. Parola AL, Yamamura HI, Laird HE II (1993) Peripheral-type benzodiazepine receptors. Life Sci 52:1329–1342.

11. Ruff MR, Pert CB, Weber RJ, Wahl LM, Wahl SM, Paul SM (1985) Benzodiazepine receptor-mediated chemotaxis of human monocytes. Science 229:1281–1283.

12. Maeda S, Miyawaki T, Nakanishi T, Takigawa M, Shimada M (1998) Peripheral type benzodiazepine receptor in T lymphocyte rich preparation. Life Sci 63:1423–1430.

13. Matsumoto T, Ogata M, Koga K, Shigematsu A (1994) Effect of peripheral benzodiazepine receptor ligands on lipopolysaccharide-induced tumor necrosis factor activity in thioglycolate-treated mice. Antimicrob Agents Chemother 38: 812–816.

14. Gilliland G, Perrin S, Blanchard K, Bunn H.F (1990) Analysis of cytokine mRNA and DNA: detection and quantitation by competitive polymerase chain reaction. Proc Natl Acad Sci USA 87:2725–2729.

15. Siebert PD, Larrick JW (1992) Competitive PCR. Nature 359:557–558.

16. Zhu X-L, Zellweger R, Zhu X-H, Ayala A, Chaudry IH (1995) Cytokine gene expression in splenic macrophages and kupffer cells following haemorrhage. Cytokine 7:8–14.

17. Zimmermann K, Mannhalter W (1996) Technical aspects of quantitative competitive PCR. BioTechniques 21:268–279.

18. Zhong WW, Burke PA, Hand AT, Walsh MJ, Hughes LA, Forse RA (1993) Regulation of cytokine mRNA expression in lipopolysaccharide-stimulated human macrophages. Arch Surg 128:158–164.

19. Zhong WW, Burke PA, Drotar ME, Chavali SR, Forse RA (1995) Effects of prostaglandin $E_2$, cholera toxin and 8-bromo-cyclic AMP on lipopolysaccharide-induced gene expression of cytokines in human macrophages. Immunology 84:446–452.

20. Enk CD, Katz SI (1994) Extraction and quantitation of cytokine mRNA from human epidermal blister roofs. Arch Dermatol Res 287:72–77.

21. Agarwal S, PiescoJ N.P, Johns L.P, Riccelli AE (1995) Diffrential expression of IL-1β, TNF-α, IL-6, and IL-8 in human monocytes in response to lipopolysaccharides from different microbes. J Dent Res 74:1057–1065.

22. Gerecke M (1983) Chemical structure and properties of midazolam compared with other benzodiazepines. Br J clin Pharmac 16:11S–16S.

23. Andersin R (1991) Solubility and acid-base behaviour of midazolam in media of different pH, studied by ultraviolet spectrophotometry with multicomponent software. J Pharm Biomed Anal 9:451–455.

24. Allonen H, Ziegler G, Klotz U (1981) Midazolam kinetics. Clin Pharmacol Ther 30:653–661.

25. Crevat-Pisano P, Dragna S, Granthil C, Coassolo P, Cano JP, Francois G (1986) Plasma concentrations and pharmacokinetics of midazolam during anaesthesia. J Pharm Pharmacol 38: 578–582.

26. Lloyd-Thomas AR, Booker PD (1986) Infusion of midazolam in paediatric patients after cardiac surgery. Br J Anaesth 58:1109–1115.

27. Persson M.P, Nilsson A, Hartvig P (1988) Relation of sedation and amnesia to plasma concentrations of midazolam in surgical patients. Clin Pharmacol Ther 43:324–331.

28. Taupin V, Herbelin A, Descamps-Latscha B, Zavala F (1991) Endogenous anxiogenic peptide, ODN-diazepam-binding inhibitor, and benzodiazepines enhance the production of interleukin-1 and tumor necrosis factor by human monocytes. Lymphokine Cytokine Res 10:7–13.

29. Zavala F (1997) Benzodiazepines, anxiety and immunity. Pharmacol Ther 75:199–216.

30. Van der Poll T, Coyle SM, Moldawer LL, Lowry S.F (1996) Changes in endotoxin-induced cytokine production by whole blood after in vivo exposure of normal humans to endotoxin. J Infec Dis 174:1356–1361.

31. Shalaby MR, Waage A, Aarden L, Espevik T (1989) Endotoxin, tumor necrosis factor-α and interleukin 1 induce interleukin 6 production in vivo. Clin Immunol Immunopathol 53: 488–498.

32. Wang P, Wu P, Siegel MI, Egan RW, Billah MM (1994) IL-10 inhibits transcription of cytokine genes in human peripheral blood mononuclear cells. J Immunol 153:811–816.

33. Tenn CC, Neu JM, Niles LP (1996) PK1195 blockade of benzodiazepine-induced inhibition of forskolin-stimulated adenylate cyclase activity in the striatum. Br J Pharmacol 119:223–228.

34. Haynes DR, Whitehouse MW, Vernon-Roberts B (1992) The prostaglandin $E_1$ analogue, Misoprostol, regulates inflammatory cytokines and immune functions in vitro like the natural prostaglandins $E_1$, $E_2$ and $E_3$. Immunology 76:251–257.

35. Marti-Cabrera M, Llopis P, Abengochea A, Ortiz JL, Climent VJ, Cortijo J, Morcillo EJ (1994) Effect of $Ca^{2+}$ channel antagonists and benzodiazepine receptor ligands in normal and skinned rat urinary bladder. Eur J Pharmacol 255:157–165.

36. Crocker C, Niles LP (1996) Benzodiazepine-induced inhibition of human malignant melanoma (M-6) cell growth. Anticancer Res 16:1259–1264.

37. Raeburn D, Miller LG, Summer WR (1988) Peripheral type benzodiazepine receptor and airway smooth muscle relaxation. J Pharmacol Exp Ther 194:557–562.

38. Zhao Z, Hertz L, Code WE (1996) Effects of benzodiazepines on potassium-induced increase in free cytosolic calcium concentration in astrocytes: interactions with nifedipine and the peripheral-type benzodiazepine antagonist PK11195. Can J Physiol Pharmacol 74:273–277.

39. Yamagage M, Matsuzaki T, Tsujiguchi N, Honma Y, Namiki A (1999) Inhibitory effects of diazepam and midazolam on $Ca^{2+}$ and $K^+$ channels in canine tracheal smooth muscle cells. Anesthesiology 90:197–207.

40. Mestre M, Carriot T, Belin C, Uzan A, Renault C, Dubroeucq MC, Gueremy C, Doble A, Le Fur G (1985) Electrophysiological and pharmacological evidence that peripheral type benzodiazepine receptors are coupled to calcium channels in the heart. Life Sciences 36:391–400.

41. Miyawaki T, Maeda S, Koyama Y, Fukuoka R, Shimada M (1998) Elevation of plasma interleukin-6 level is involved in post-operative fever following major oral and maxillofacial surgery. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 85:146–152.

42. Deng MC, Dasch B, Erren M, Mollhoff T, Scheld HH (1996) Impact of left ventricular dysfunction on cytokines, hemodynamics, and outcome in bypass grafting. Ann Thorac Surg 62:184–190.

43. Finkel MS, Oddis CV, Jacob TD, Watkins SC, Hattler BG, Simmons RL (1992) Negative inotropic effects of cytokines on the heart mediated by nitric oxide. Science 257:387–389.

44. Roytblat L, Talmor D, Rachinsky M, Greemberg L, Pekar A, Appelbaum A, Gurman GM, Shapira Y, Duvdenani A (1998) Ketamine attenuates the interleukin-6 response after cardiopulmonary bypass. Anesth Analg 87:266–271.