Inhibition of Inhibitor of Nuclear Factor-κB Phosphorylation Increases the Efficacy of Paclitaxel in \textit{in Vitro} and \textit{in Vivo} Ovarian Cancer Models

Seiji Mabuchi,1,4 Masahide Ohmichi,1,2 Yukihiro Nishio,1 Tadashi Hayasaka,2 Akiko Kimura,1 Tsuyoshi Ohta,2 Jun Kawagoe,2 Kazuhiro Takahashi,2 Namiko Yada-Hashimoto,1 Hozumi Seino-Noda,1 Masahiro Sakata,1 Teiichi Motoyama,3 Hirohisa Kurachi,2 Joseph R. Testa,1 Keiichi Tasaka,1 and Yuji Murata1

1Department of Obstetrics and Gynecology, Osaka University Medical School, Osaka, Japan; Departments of 2Obstetrics and Gynecology and 3Pathology, Yamagata University, School of Medicine, Yamagata, Japan; and 4Human Genetics Program, Fox Chase Cancer Center, Philadelphia, Pennsylvania

ABSTRACT

We investigated whether inhibition of nuclear factor-κB (NFκB) increases the efficacy of paclitaxel in \textit{in vitro} and \textit{in vivo} ovarian cancer models. Treatment of paclitaxel-sensitive Caov-3 cells with paclitaxel transiently activated the phosphorylation of Akt, the phosphorylation of IκB kinase (IKK), and the phosphorylation of inhibitor of NFκB (IκBo). Paclitaxel also caused a transient increase in NFκB activity, followed by a decrease in NFκB activity. We show an association between Akt and IKK and show that the phosphorylation of IKK induced by paclitaxel is blocked by treatment with a phosphatidylinositol 3-kinase 3-kinase inhibitor (wortmannin or LY294002). Furthermore, interference of the Akt signaling cascade inhibits the transient induction of IκB phosphorylation and NFκB activity by paclitaxel. Inhibition of NFκB activity by treatment with an IκB phosphorylation inhibitor (BAY 11-7085) attenuated both basal and transient induction of IκBo phosphorylation by paclitaxel. Treatment with BAY 11-7085 also enhanced the inhibition of NFκB activity by paclitaxel for up to 24 hours. In addition, treatment with BAY 11-7085 decreased the viability of cells treated with paclitaxel. Moreover, treatment with BAY 11-7085 increased the efficacy of paclitaxel-induced inhibition of intraabdominal dissemination and production of ascites in athymic nude mice inoculated intraperitoneally with Caov-3 cells. These results suggest that paclitaxel transiently induces NFκB activity via the phosphatidylinositol 3-kinase/Akt cascade and that combination therapy with paclitaxel and an NFκB inhibitor would increase the therapeutic efficacy of paclitaxel.

INTRODUCTION

Paclitaxel, a natural product originally isolated from the bark of \textit{Taxus brevifolia}, has significant antitumor activity against several human cancers, particularly advanced ovarian and breast carcinomas (1, 2). Unlike other antimicrotubule agents that interfere with tubulin polymerization, paclitaxel increases tubulin polymerization, stabilizes microtubules, and prevents tubulin depolymerization, ultimately causing tubulin bundling (3–5). These effects of the drug are associated with cell cycle arrest in G2–M phase of the cell cycle, as well as cellular toxicity (5–7). The inclusion of paclitaxel in the treatment of patients with newly diagnosed ovarian cancer has led to improved response rates and prolonged median survival compared with the results of prior therapeutic regimens (8). Nevertheless, most of patients with advanced ovarian cancer are destined to relapse and develop resistance to initially active drugs such as paclitaxel (9).

The sensitivity of cells to chemotherapeutic drug-induced apoptosis seems to depend on the balance between proapoptotic and antiapoptotic signals. Therefore, it is possible that antiapoptotic signals such as the phosphatidylinositol 3-kinase (PI3K)/Akt survival cascade are involved in the sensitivity to chemotherapeutic drugs. We reported that Akt inactivation sensitizes human ovarian cancer cells to cisplatin (10) and paclitaxel (11), suggesting that Akt inactivation could be a hallmark of the sensitivity of cells to some chemotherapeutic drugs. Possible mechanisms by which Akt promotes cell survival include phosphorylation and inactivation of the proapoptotic proteins BAD and caspase-9 (12, 13). Akt also phosphorylates and inactivates the Forkhead transcription factors, resulting in reduced expression of the cell cycle inhibitor p27Kip1 and the Fas ligand (14–16). Via the phosphorylation of IκB kinase (IKK), Akt also activates nuclear factor-κB (NFκB), a transcription factor that has been implicated in cell survival (17, 18).

NFκB is activated in certain cancers and in response to chemotherapy and radiation. NFκB normally resides in the cytoplasm as an inactive form in a complex with inhibitor of NFκB (IκBo). Phosphorylation of IκBo by IKK promotes its degradation, allowing NFκB to translocate to the nucleus and induce target genes (15, 16). The transcriptional activation of genes associated with cell proliferation (19), angiogenesis (20, 21), metastasis (22, 23), and suppression of apoptosis (24)
seems to lie at the heart of the ability of NFκB to promote oncogenesis (25) and cancer therapy resistance (26, 27). Although it was proposed that NFκB might be required for paclitaxel-induced cell death (28), most reports suggest that paclitaxel-induced NFκB activity mediates survival signals that counteract apoptosis (29–31). It has been reported that intrinsically or constitutively activated NFκB may be critical in the development of drug resistance in cancer cells (29). Therefore, several agents that are able to inhibit NFκB function might be considered as an adjuvant approach in combination with paclitaxel for lung cancer (30), prostate cancer (31), pancreatic cancer (32), and breast cancer (33).

These considerations led us to examine whether the status of NFκB activity is involved in the sensitivity to paclitaxel in human ovarian cancer cells and whether agents that are able to inhibit NFκB function might be considered as an adjuvant approach in combination with paclitaxel for ovarian cancer. In the present study, we show that BAY 11-7085, a known pharmacologic inhibitor of IkBα phosphorylation (34), inhibits both basal and transient induction of IkBα phosphorylation and NFκB activity by paclitaxel for 3 hours. In addition, BAY 11-7085 enhances inhibition of NFκB activity by paclitaxel for up to 24 hours and increases the efficacy of paclitaxel in in vitro and in vivo ovarian cancer models.

EXPERIMENTAL PROCEDURES

Materials. Antiphospho-Iκκα (Ser-180)/Iκκβ (Ser-181), anti-Iκκα, Antiphospho-Iκκα, anti-Iκκα, anticleaved poly(ADP-ribose) polymerase (PARP) and anti-X-linked inhibitor of apoptosis protein (anti-XIAP) antibodies were obtained from Cell Signaling Technology (Beverly, MA). Anti-matrix metalloproteinase-9 (anti-MMP-9) antibody was purchased from Chemicon (Temecula, CA). Anti-f-actin antibody was purchased from Sigma. (St. Louis, MO). The IkBα phosphorylation inhibitor BAY 11-7085 was purchased from Alexis Biochemicals (San Diego, CA). Enhanced chemiluminescence Western blotting detection reagents were obtained from Amer sham (Arlington Heights, IL). The Cell Titer 96-well proliferation assay was obtained from Promega (Madison, WI).

Cell Cultures. The human ovarian papillary adenocarcinoma cell line Caov-3 was obtained from American Type Culture Collection (Rockville, MD). The cells were cultured at 37°C in DMEM with 10% fetal bovine serum in a water-saturated atmosphere of 95% air and 5% CO2.

Constructions. The NFκB reporter plasmid (pElam-luc) was a kind gift from Dr. J. Cheng (University of South Florida College of Medicine; ref. 35).

Proliferation Assay. Cell proliferation (36) was assessed by the addition of paclitaxel at the indicated concentrations for 48 hours 1 day after seeding test cells into 96-well plates. The number of surviving cells was determined 24 hours later by determination of A590 of the dissolved formazan product after 4 hours 1 day after seeding test cells into 96-well plates. The number of surviving cells was determined 24 hours later by determination of A590 of the dissolved formazan product after 4 hours 1 day after seeding test cells into 96-well plates. The number of surviving cells was determined 24 hours later by determination of A590 of the dissolved formazan product after 4 hours 1 day after seeding test cells into 96-well plates.
measured, tumor tissue was excised, fixed in 4% paraformaldehyde, and embedded in paraffin. Paraffin sections (5 μm) were used for histochemical analysis.

**Statistics.** Statistical analysis was done with one-way ANOVA followed by Fisher’s least significant difference test, and \( P < 0.05 \) was considered significant. Data are expressed as the mean ± SEM.

**RESULTS**

Effects of Paclitaxel on IKK Phosphorylation, IκBα Phosphorylation, and NFκB Activity.** We first examined the effect of paclitaxel on the NFκB signaling cascade. A putative Akt phosphorylation site (39) at amino acids 18–23 in IκBα has been reported (15). To examine whether phosphorylated Akt and IκBα were physically associated, cells were treated with the indicated concentrations of paclitaxel (B), 200 nmol/L paclitaxel for the indicated times (C), or pretreated with 50 μmol/L LY294002 for 20 minutes (Lane 3) or 100 nmol/L wortmannin for 15 minutes (Lane 4), and then treated with 200 nmol/L paclitaxel for 30 minutes (D). Cell lysates were subjected to Western blotting by antiphospho-IKKα (Ser-180)/IKKβ (Ser-181; ii), anti-IKKα (iii), antiphospho-Akt (iv), or anti-Akt (v) antibody. The positions of molecular weight markers are noted on the left. Relative densitometric units of the phospho-IKK bands (open bars) or the phospho-Akt bands (closed bars) are shown (in i) with the density of the control bands in Caov-3 cells set arbitrarily at 1. Values shown represent the mean ± SEM from at least three separate experiments. Significant differences are indicated by *asterisks*. **, \( P < 0.01 \).
1B) or 200 nmol/L paclitaxel for the indicated times (Fig. 1C) and used to prepare lysates that were analyzed by Western blotting with antiphospho-IKKα (Ser-180)/IKKβ (Ser-181), anti-IKKα, antiphospho-Akt, or anti-Akt antibody. Although each expression of IKKα and Akt was not changed (Fig. 1B and C, iii and iv), Caov-3 cells treated with paclitaxel showed a dose-dependent (Fig. 1B) and a transient (Fig. 1C) increase in the phosphorylation of Akt (i and iv) and IKK (i and ii). Pretreatment with either of the PI3K inhibitors LY294002 (Fig. 1D, Lane 3) or wortmannin (Fig. 1D, Lane 4) inhibited the paclitaxel-induced phosphorylation of both IKK (Fig. 1D, i and ii) and Akt (Fig. 1D, i and iv).

We next examined whether paclitaxel induces the phosphorylation and degradation of IkBα (Fig. 2A). Cells were treated with 200 nmol/L paclitaxel for the indicated times and used to prepare lysates that were analyzed by Western blotting with antiphospho-IkBα, anti-IkBα, or anti-β-actin antibody. Although the expression of β-actin was not changed (Fig. 2A, iv), paclitaxel-stimulated Caov-3 cells showed a transient increase in the phosphorylation of IkBα (Fig. 2A, i and ii) and in its subsequent degradation (Fig. 2A, i and iii). Pretreatment with either LY294002 (Fig. 2B, Lane 3) or wortmannin (Fig. 2B, Lane 4) inhibited both the paclitaxel-induced phosphorylation of IkBα (Fig. 2B, i and ii) and the degradation of IkBα (Fig. 2B, i and iii).

To assess NFκB activity, cells were transfected with an NFκB-luciferase reporter plasmid and treated with 200 nmol/L paclitaxel for the indicated times. Paclitaxel caused a transient increase in NFκB activity lasting 2 to 4 hours, followed by a decrease in NFκB activity thereafter (Fig. 2C). Pretreatment with LY294002 inhibited the transient up-regulation of NFκB activity by paclitaxel for 3 hours (Fig. 2D). Collectively, these results indicate that paclitaxel transiently induces phosphorylation of IKK and IkBα as well as activation of NFκB via a PI3K/Akt cascade, followed by the decrease in NFκB activity thereafter. As we reported previously (11), similar findings were also detected in other ovarian cancer cell lines (data not shown).

![Image](image_url) Fig. 2 Effect of paclitaxel on phosphorylation and degradation of IkBα, and on NFκB activity. Caov-3 cells were treated with 200 nmol/L paclitaxel for the indicated times (A) or were pretreated with 50 μmol/L LY294002 for 20 minutes (Lane 3) or 100 nmol/L wortmannin for 15 minutes (Lane 4), followed by treatment with 200 nmol/L paclitaxel for 30 minutes (B). Cell lysates were subjected to Western blotting with antiphospho-IkBα (ii), anti-IkBα (iii), or anti-β-actin (iv) antibody. The positions of molecular weight markers are noted on the left. Relative densitometric units of the phospho-IkBα bands (open bars) or the IkBα bands (closed bars) are shown in (i) with the density of the control bands in Caov-3 cells set arbitrarily at 1. Values shown represent the mean ± SEM from at least three separate experiments. Significant differences are indicated by asterisks. ***, P < 0.01. Caov-3 cells were transfected with pElam-luc. After transfection, the cells were incubated with 200 nmol/L paclitaxel for the indicated times (C) or pretreated with 50 μmol/L LY294002 for 20 minutes (Lanes 3 and 4), and then treated with 200 nmol/L paclitaxel for 3 hours (D). Cell pellets were collected and used to prepare lysates that were subjected to luciferase assays. The transcriptional activity of each plasmid was normalized with respect to that of the vehicle control of Caov-3 cells taken as 1. Values shown represent the mean ± SEM from at least three separate experiments. Significant differences are indicated by asterisks. ***, P < 0.01.
Inhibition of NFκB Activity Sensitizes Caov-3 Cells to Paclitaxel. The involvement of the NFκB signaling cascade in the paclitaxel-induced inhibition of cell viability was examined with an IκB phosphorylation inhibitor (BAY 11-7085; ref. 34). We first confirmed that treatment with BAY 11-7085 attenuated both basal and transient induction of IκB phosphorylation by paclitaxel (Fig. 3A). Treatment with BAY 11-7085 inhibited the transient induction of NFκB activity by paclitaxel for 3 hours (Fig. 3B, i) and enhanced the inhibition of NFκB activity by paclitaxel for 24 hours (Fig. 3B, ii). Whereas either treatment with paclitaxel for 24 hours (Fig. 3C, right) or treatment with BAY 11-7085 for 24 hours (Fig. 3C, left) inhibited cell viability, cotreatment with paclitaxel plus BAY 11-7085 for 24 hours further enhanced the inhibitory effects on cell viability (Fig. 3C, right).

We further examined the effect of treatment with paclitaxel for 24 hours and treatment with BAY 11-7085 for 24 hours alone and in combination on apoptosis by Western blotting with anticleaved PARP antibody (Fig. 3D). Paclitaxel induced cleavage of PARP. BAY 11-7085 seemed to enhance the ability of paclitaxel to induce cleavage of PARP, whereas the expression of β-actin was not changed by any of these treatments.

Effect of Inhibition of NFκB Activity on Paclitaxel-Induced Attenuation of the Expression of Survival Genes. NFκB regulates the expression of a number of antiapoptotic genes (40–42). Among them are the family of inhibitor of...
apoptosis proteins, which play a central role in repressing caspase-mediated cell death. It was reported that cisplatin inhibits the expression of XIAP (43, 44) and down-regulation of XIAP induces apoptosis and increases paclitaxel sensitivity (45–47), suggesting that XIAP is a determinant of paclitaxel sensitivity in ovarian cancer. Therefore, we examined the effect of paclitaxel and BAY 11-7085 alone and in combination on the expression of XIAP. Although paclitaxel treatment alone had no effect on XIAP expression under these conditions, BAY 11-7085 partially attenuated the expression of XIAP, and cotreatment with paclitaxel plus BAY 11-7085 almost completely abolished the expression of XIAP (Fig. 4).

**Effect of Inhibition of NFkB Activity on Paclitaxel-Induced Attenuation of Invasion of Caov-3 Cells through Matrigel.** Because it was reported that NFkB is involved in invasiveness and metastatic properties (48), we examined the effects of paclitaxel and BAY 11-7085 alone and in combination on the invasion of Caov-3 cells through Matrigel. Whereas either paclitaxel or BAY 11-7085 partially inhibited cell invasion through Matrigel, cotreatment with paclitaxel plus BAY 11-7085 almost completely inhibited cell invasion through Matrigel (Fig. 5A).

To confirm that inhibitory effects on invasion are not secondary to the cells simply being nonviable, the expression of MMP-9 was examined. Although either paclitaxel or BAY 11-7085 partially inhibited MMP-9 expression, cotreatment with paclitaxel plus BAY 11-7085 almost completely inhibited MMP-9 expression (Fig. 5B, i and ii). The expression of β-actin was not changed by any of these treatments (Fig. 5B, iii).

**Effect of BAY 11-7085 on Paclitaxel-Induced Inhibition of Intraabdominal Dissemination of Ovarian Cancer.** Ovarian cancer is initially clinically silent, and half of patients are first detected at an advanced stage with ascites and peritoneal dissemination (28). Because peritoneal dissemination is the main process of progression in ovarian cancer, and the amount of ascitic fluid and the size of the disseminated tumor are correlated with the patient’s prognosis (49), controlling ascitic fluid and peritoneal dissemination are crucial in the therapy of ovarian cancer. We therefore examined the effect of paclitaxel and BAY 11-7085 alone and in combination on the control of intraabdominal dissemination of ovarian cancer and ascites formation to assess whether combination therapy would increase the therapeutic efficacy of each agent. Athymic nude mice were inoculated intraperitoneally with Caov-3 cells or growth me-

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**Fig. 4** Effect of BAY 11-7085 on the expression of survival genes. Caov-3 cells were treated with the indicated concentrations of 200 nmol/L paclitaxel (Lane 2), 5 μmol/L BAY 11-7085 (Lane 4), or 5 μmol/L BAY 11-7085 + 200 nmol/L paclitaxel (Lane 6) for 24 hours. Lysates (50 μg of protein) were subjected to Western blotting by anti-XIAP (middle) or anti-β-actin (bottom) antibody. The positions of molecular weight markers are noted on the left. Relative densitometric units of the XIAP bands are shown in the top, with the density of the vehicle bands set arbitrarily at 1.0. Values shown represent the mean ± SEM from at least three separate experiments. Significant differences are indicated by asterisks. ***, P < 0.01.

![Image](https://example.com/image.png)

**Fig. 5** BAY 11-7085 enhances paclitaxel-induced attenuation of invasion. A, Caov-3 cells were treated with 200 nmol/L paclitaxel, 5 μmol/L BAY 11-7085, or 200 nmol/L paclitaxel + 5 μmol/L BAY 11-7085 for 24 hours. The cells (5 × 10⁴) were plated on Matrigel as described in “Materials and Methods.” The fraction of cells that penetrated through Matrigel is shown, with the fraction of penetrating cells in the vehicle control set arbitrarily at 100%. Values shown represent the mean ± SEM from at least three separate experiments. Significant differences are indicated by asterisks. ***, P < 0.01. B, Caov-3 cells were treated with 200 nmol/L paclitaxel and/or 5 μmol/L BAY 11-7085 for 24 hours. Lysates (50 μg of protein) were subjected to Western blotting by anti-MMP-9 (ii) or anti-β-actin (iii) antibody. The positions of molecular weight markers are noted on the left. Relative densitometric units of the MMP-9 bands are shown (in i), with the density of the vehicle bands set arbitrarily at 1. Values shown represent the mean ± SEM from at least three separate experiments. Significant differences are indicated by asterisks. ***, P < 0.01.
medium. Two weeks after inoculation, athymic nude mice were inoculated intraperitoneally with Caov-3 cells or growth medium (Control). Two weeks after inoculation, athymic nude mice inoculated intraperitoneally with Caov-3 cells were randomized into four groups treated with the following for 4 weeks: (a) vehicle alone (PBS); (b) paclitaxel (20 mg/kg) 3 times weekly; (c) BAY 11-7085 (5 mg/kg) 3 times weekly; and (d) paclitaxel (20 mg/kg) 3 times weekly plus BAY 11-7085 (5 mg/kg) 3 times weekly. The appearance of the mice is shown in Fig. 6A. The abdominal circumference (AC) was measured (B). At autopsy, the volume of ascites was measured (C).

**DISCUSSION**

The novel finding in this study is that an NFκB inhibitor, which enhanced paclitaxel-induced inhibition of IκBα phosphorylation and NFκB activity, increased the efficacy of paclitaxel in both *in vitro* and *in vivo* ovarian cancer models. Although adjuvant approaches in combination with paclitaxel have been reported for lung cancer (30), prostate cancer (31), pancreatic cancer (32), and breast cancer (33), there have been no reports about an adjuvant approach in combination with paclitaxel for ovarian cancer. In addition, *in vitro* models were used in previous reports, and there have been no reports using *in vivo* models.

It was previously proposed that NFκB might be required for paclitaxel-induced cell death (28). Thus, although NFκB is a transcription factor that has been implicated in cell survival (17, 18), there is a possibility that the function of NFκB depends on tumor type. However, most reports suggest that paclitaxel-induced NFκB activity mediates survival signals that counteract apoptosis (29–31).

We reported that Akt inactivation and inhibition of BAD phosphorylation sensitize human ovarian cancer cells to cisplatin (10) and paclitaxel (11). In addition, we showed recently that inhibition of Forkhead phosphorylation sensitizes human ovarian cancer cells to cisplatin (50). Although NFκB is a substrate of Akt, like BAD and Forkhead, NFκB activation is involved in
angiogenesis (20, 21) and metastasis (22, 23) as well as in suppression of apoptosis. Therefore, NFκB inhibitors might increase the efficacy of chemotherapy against both primary and metastatic lesions. It was reported that NFκB inhibitors induce adhesion-dependent colon cancer apoptosis (51). We showed in this study that treatment of athymic mice with BAY 11-7085 enhances the ability of paclitaxel to inhibit tumor implantation into the liver and peritoneum (Fig. 7). In addition, BAY 11-7085 increased the ability of paclitaxel to inhibit both cell proliferation in an MTS assay (Fig. 3C) and cellular invasion in an in vitro invasion assay (Fig. 5A). Thus, NFκB inhibitors might increase the efficiency with which paclitaxel inhibits both primary and metastatic lesions. Glycogen synthase kinase 3α (52) and endothelial nitric oxide synthase (53, 54) are also Akt substrates, and Akt is thus also involved in metabolic processes and vessel dilation, respectively. Therefore, it is possible that inhibition of PI3K/Akt activation is not a safe strategy for preventing chemoresistance. Accordingly, NFκB inhibitors might be more useful for sensitization to chemotherapeutic drugs than agents that are able to inhibit PI3K/Akt activity. Moreover, we also showed that the signaling cascade of NFκB is involved in the mechanism of maintaining the cell viability after cisplatin treatment of ovarian cancer cells (55), as in the case of paclitaxel. Thus, because NFκB inhibitors might augment the effects of both cisplatin and paclitaxel, which together constitute the first line regimen of treatment for ovarian cancer, the combination of NFκB inhibitors with cisplatin and paclitaxel might be useful for molecularly targeted therapy.

How do NFκB inhibitors cause the inhibition of growth of human ovarian cancer cells? It was reported that NFκB inhibitors reduced the expression of survival genes regulated by NFκB, such as c-IAP-2, TRAF-1, TRAF-2, XIAP, or IEX-1L (41, 42). We also showed that NFκB inhibitors inhibited the expression of survival genes in human ovarian cancer cells (Fig. 4). The fact that NFκB mediates the expression of multiple survival genes makes it an important and rational target for cancer chemotherapy.

Activation of NFκB via phosphorylation of an inhibitor protein (IκBα) leads to degradation of IκBα through the ubiquitin-proteasome pathway. Inhibition of IκBα degradation by proteasome inhibitors keeps NFκB in the cytoplasm, thereby preventing it from acting on nuclear DNA (56, 57). PS-341, which is a potent boronic acid dipeptide that is highly selective for proteasome inhibition, can be systemically administered clinically (58). PS-341 has been shown to enhance apoptotic response to chemotherapy in a variety of in vitro and in vivo models (59–62). A phase I trial of PS-341 and carboplatin in recurrent ovarian cancer is currently ongoing (63). A phase II trial of PS-341 for the treatment of recurrent platinum-sensitive ovarian or primary peritoneal cancer (GOG 146-N) is also being conducted. It was also reported that proteasome inhibitor 1 enhances paclitaxel-induced apoptosis in a human lung adenocarcinoma cell line (30). This is the first report that an NFκB inhibitor sensitizes human ovarian cancer cells to the effect of paclitaxel and the first to suggest that future clinical trials designed to examine the effect of NFκB inhibitors on the sensitivity to paclitaxel are warranted.

ACKNOWLEDGMENTS

We are grateful to Ayako Okamura and Tomoko Iwaki for technical and secretarial assistance.

REFERENCES
