Jpn. J. Cancer Res. 81, 536–543, May 1990

In vitro Sensitivity Test of Breast Cancer Cells to Hormonal Agents in a Radionucleotide-incorporation Assay

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Breast cancer cell lines (MCF-7, T47D, BT-20 and STT-11) and fresh cells from malignant effusions of eight breast cancer patients were examined for their *in vitro* sensitivity to 17β-estradiol (E_2), tamoxifen and toremifene in a miniaturized, improved nucleic acid precursor incorporation assay (MINI assay). Seven of the eight patients received either tamoxifen or toremifene following a MINI assay and the correlation was examined between *in vitro* sensitivity and clinical responses to the hormonal agents. In cell lines, E_2 stimulated thymidine incorporation by estrogen receptor (ER)-rich cells, MCF-7 and T47D, but not by ER-poor cells, BT-20 and STT-11. Tamoxifen induced both ER-mediated and -unmediated effects in ER-rich cells. The latter effect was also observed in ER-poor cells. Toremifene had less ER-unmediated effect in all of the cells tested than tamoxifen did. The ER-mediated effect of toremifene was weaker than that of tamoxifen in cell lines but was equipotent to tamoxifen in fresh cells. E_2 affected thymidine incorporation by cells withdrawn from patients who showed a partial response to the anti-estrogens. No clear correlation was demonstrated between *in vitro* sensitivity to anti-estrogens of fresh cells and clinical response to these agents. The present results suggest that 1) the MINI assay is a useful system to investigate hormonal effects on breast cancer cell lines; 2) clinical responses to anti-estrogens are not predicted by *in vitro* response to the agents but might be predicted by the *in vitro* response to E_2; and 3) toremifene has a smaller non-specific effect on breast cancer cells than tamoxifen and is equipotent to tamoxifen in the ER-mediated effect *in vitro*.

Key words: Breast cancer — *In vitro* sensitivity test — Tamoxifen — Toremifene

Tamoxifen, an anti-estrogen, is widely used in the treatment of patients with hormone-sensitive breast cancer. Hormonal sensitivity of breast cancer can be predicted by determining ER[5] and PgR concentrations in the cytosol of the tumor tissues obtained at the time of surgical operation for the treatment of the primary disease or at the time of recurrence of the disease.[1)] Although the significance of these assays has been established, the true positive rate of these assays is approximately 60% from the viewpoint of predictability of the clinical response to endocrine therapies.[2,3)] Attempts have been made to develop assay systems with improved precision utilizing cells obtained from breast cancer patients to predict hormonal sensitivity in individual patients.[4)] It has been suggested that the standard human tumor clonogenic assay requires modification before it can be employed to predict hormonal sensitivity of fresh human breast cancer specimens.[5)] The present study was conducted to examine whether a radionuclide-incorporation assay (the MINI assay)[6,7)] can predict hormonal sensitivity of breast cancer. To achieve this goal, we first searched for suitable experimental conditions under which the hormonal sensitivity of established breast cancer cell lines could be reproduced; then we assessed the value of the MINI assay in predicting clinical responses to hormonal agents using freshly obtained cancer cells from malignant effusion of patients with advanced breast cancer. In addition, we compared the *in vitro* potencies of tamoxifen and toremifene in terms of the growth-suppressive effect on established cell lines and clinical specimens.

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[5] The abbreviations used are: ER, estrogen receptor; E_2, 17β-estradiol; PgR, progesterone receptor; PR, partial response; PD, progressive disease; FCS, fetal calf serum; TCA, trichloroacetic acid; PBS, phosphate-buffered saline; MINI assay, miniaturized, improved nucleic acid precursor incorporation assay.

MATERIALS AND METHODS

Established breast cancer cell lines MCF-7, T47D and BT-20 were purchased from the American Type Culture Collection (Rockville, MD). STT-11 is a newly established cell line which was derived from a malignant pleural effusion withdrawn from a 49-year-old female who had received neither endocrine therapy nor chemotherapy (patient #5 in Table I). It has epithelioid ap-

Table I. Summary of the Patients whose Malignant Effusions Were Assayed for Hormonal Sensitivity in MINI Assay

Patient[a]	Age	Menopausal status	Evaluated lesion	Hormonal therapy		*In vitro* sensitivity		
					response	E_2	TAM	TOR
1	67	post	lymph node	TOR[b]	PD[d]	no	—[f, g]	50
2	82	post	skin	TOR	PR[e]	suppressed	—	20
3	55	post	bone	TOR	PD	no	10	8
4	46	pre	lung	TAM[c]	PD	no	8	10
5	49	pre	bone	TAM	PD	no	0	0
6	38	pre	bone	TOR	PD	no	—	30
7	56	post	—	—	—	no	50	32
8	43	pre	lymph node	TAM	PR	stimulated	20	10

a) Patient numbers correspond to those in the text.
b) Toremifene.
c) Tamoxifen.
d) Progressive disease.
e) Partial response.
f) Sensitivities to tamoxifen or toremifene are expressed as percent suppression of thymidine incorporation by these anti-estrogens at 10^{-7} *M* with respect to the respective control.
g) (—) indicates the thymidine incorporation at 10^{-7} *M* was higher than that of the control.

pearance and grows in a semi-attached monolayer. These cell lines were maintained in RPMI 1640 (Gibco Laboratories, Long Island, NY) supplemented with 10% FCS (Boehringer-Mannheim GmbH, Mannheim) in a humidified atmosphere of 95% air: 5% CO_2 at 37°C. Cells were digested with 0.25% trypsin in 0.01% EDTA (Gibco) and dispersed by repeated gentle pipetting to obtain a single cell suspension at the time of splitting the maintenance cultures and setting up the MINI assay.

Cancer cells from malignant effusions from patients with advanced breast cancer One sample of peritoneal and seven samples of pleural effusion were obtained from eight patients with advanced breast cancer. The characteristics of the patients are presented in Table I. One hundred units of preservative-free heparin were added to each ml of aspirated fluid. Samples were centrifuged at 400*g* for 5 min at 25°C. Each pellet was resuspended in 5 ml of the supernatant solution, layered on top of 35 ml of Ficol-Conray mixture (specific gravity 1.080) and centrifuged at 1,000*g* for 45 min at 25°C to obtain nucleated cells. A portion of each nucleated cell suspension was microscopically examined after May-Gimsa staining and cancer cells were observed in all of the specimens.

Hormone receptor assays of the breast cancer cell lines Confluent cells grown in RPMI 1640 supplemented with 10% FCS in a 75-cm^2 culture flask were washed three times with Ca^{2+}, Mg^{2+}-free PBS, harvested with a scraper and homogenized in 10 m*M* Tris; 1 m*M* EDTA buffer, pH 7.4 containing 12 m*M* thioglycerol and 10% (v/v) glycerol with a Polytron homogenizer (Kinematica GmbH, Lucerne) with three 15-s bursts with 45-s intervals. The homogenate was centrifuged at 105,000*g* at 4°C for 60 min to obtain a cytosol fraction which was assayed for ER, PgR and protein concentrations. ER and PgR in the cytosol were determined using enzyme immunoassay kits from Abbott Laboratories (Dinabot Co., Tokyo).[8] Protein determinations were performed with the Bio-Rad kit (Coomassie brilliant blue method) (Bio-Rad Laboratories, Richmond, CA) with human serum albumin as a reference.

MINI assay The effects of various hormonal reagents on the *in vitro* growth of breast cancer cells were examined by means of the MINI assay.[6, 7] Culture medium used in the MINI assay was phenol red-free RPMI 1640 (Gibco) supplemented with 10% FCS treated with dextran-coated charcoal. First, 500 μl of the medium containing 0.6% agarose (Sea Plaque; FMC Corporation, Rockland, ME) was added to each well of a 24-well multiwell plate to prepare an underlayer. After hardening of this layer, 500 μl of the medium containing 0.4% agarose and dispersed cells, which had been washed three times with the medium, was overlayered on each underlayer to make a top layer and allowed to harden. Then 100 μl of medium containing the test substance, at eleven times the designated concentration, was added onto the top of these two agarose layers. After incubation for 96 h in a humidified CO_2 incubator (5% CO_2: 95% air) at 37°C, 185 kBq of tritiated thymidine (TRK758, Amersham Laboratories, Buckinghamshire) in 250 μl of the medium was added to each well and the plates were incubated for an additional 24 h. Incorporation of tritiated thymidine

by the cells was terminated by addition of 6 ml of Ca^{2+}, Mg^{2+}-free PBS at 85°C. The pellet was collected after immediate centrifugation at 450*g* for 30 min, washed once with 4 ml of Ca^{2+}, Mg^{2+}-free PBS at 85°C, and then sedimented by addition of 4 ml of 10% TCA in the presence of 50 μl of human serum albumin (1%, w/v). After storage for 12 to 16 h at 4°C followed by centrifugation, the precipitate was washed once with 4 ml of 5% TCA, and completely dissolved by addition of 100 μl of 1 *M* potassium hydroxide. The solution was transferred to a scintillation vial containing 4 ml of ACS II (Amersham Laboratories). Radioactivity in each vial was determined by the use of a liquid scintillation counter (Model LS-3801, Beckman Instruments, Irvine, CA). In all specimens, thymidine incorporation in the control well was more than 1500 dpm.

Reagents E_2 was purchased from Sigma Chemical Co. (St. Louis, MO); tamoxifen citrate {2-[4-(1,2-diphenyl-1-butenyl)phenoxy]-N,N-dimethylethanamine citrate} and toremifene citrate {2-[4-((Z)-4-chlor-1,2-diphenyl-1-butentyl) phenoxy]-N, N-dimethylethylamine citrate} were generously provided by ICI-Pharma Manufacturing, Ltd. (Tokyo) and Nippon Kayaku Co., Ltd. (Tokyo), respectively. Aliquots of these reagents were dissolved in ethanol at a concentration of 10^{-3} *M* and stored at −20°C. These aliquots were diluted with the medium immediately before addition to the MINI assay wells. The final concentration of ethanol was less than 1% in the assay wells. Ethanol at this concentration had no effect on the incorporation of tritiated thymidine by the cell lines tested.

Statistics Statistical analyses were performed by one-way analysis of variance, followed by Duncan's multiple range test. $P<0.05$ was considered significant. Calculations were performed with a statistical package, SPSS PC plus (SPSS Japan, Inc., Tokyo) on an NEC PC98XL^2 personal computer (Nippon Electronics Corporation, Tokyo).

Table II. ER and PgR Concentrations in the Cytosol of Breast Cancer Cell Lines

Cell line	ER[a]	PgR[a]
MCF-7	175.2±33.5[b]	368.7±31.9
T47D	67.6±6.2	4374.7±134.2
BT-20	6.2±1.2	3.9±0.7
STT-11	1.1±0.3	1.9±0.8

a) Values are expressed as fmol/mg cytosol protein.
b) Mean±standard error of the mean of three means of five determinations.

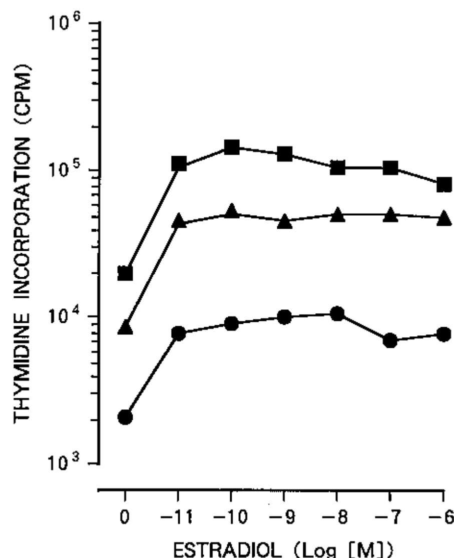

Fig. 1. Thymidine incorporation by MCF-7 responding to increasing concentrations of E_2 in the MINI assay. Cells were plated at three different densities (0.25×10^4/well; ●, 10^4/well; ▲, 5×10^4/wells; ■) and E_2 (10^{-11}–10^{-6} *M*) was added. Thymidine incorporation by the cells was determined after a 96 h incubation. Each point represents the mean of triplicate wells.

RESULTS

Steroid hormone receptor assays ER and PgR contents in the cytosol fraction of the four breast cancer cell lines are shown in Table II. The receptor contents of MCF-7, T47D and BT-20 are consistent with the findings of other authors.[9] Both ER and PgR were very low in the cytosol of the newly established breast cancer cell line, STT-11, which is categorized as an ER- and PgR-poor cell line.

Effects of increasing concentrations of E_2 on thymidine incorporation by breast cancer cell lines E_2 (10^{-11}–10^{-6} *M*) was added to the MINI assay wells and the effects of the hormone on the thymidine incorporation by the breast cancer cells were examined. Thymidine incorporation by MCF-7 cells was enhanced by E_2 at concentrations as low as 10^{-11} *M* and the maximum 6-fold increment was observed irrespective of the cell number added to the top layer (Fig. 1). Thymidine incorporation was enhanced by E_2 in another ER-rich cell line, T47D, but the increment was only 2.3-fold over the control (Fig. 2-A). BT-20 and STT-11, ER-poor cell lines, did not show enhanced thymidine incorporation in response to E_2 at concentrations as high as 10^{-6} *M* (Fig. 2-A, B). Thus, the growth-stimulatory effect of E_2 on ER-rich breast cancer cell lines was demonstrated in the MINI assay.

Effects of anti-estrogens on E_2-stimulated thymidine incorporation by breast cancer cell lines Tamoxifen or

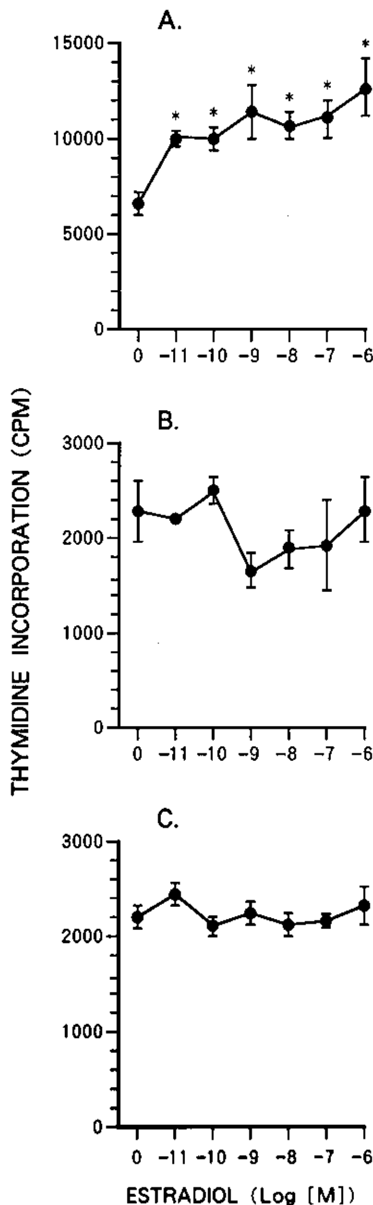

Fig. 2. Effects of increasing concentrations of E_2 on thymidine incorporation by three breast cancer cell lines. T47D (A), BT-20 (B) and STT-11 (C) were plated (10^4 cells/well) for MINI assay and E_2 (10^{-11}–10^{-6} *M*) was added. Thymidine incorporation by the cells was determined after a 96 h incubation. Each point represents the mean of triplicate wells; the brackets indicate the SEM. * indicates $P < 0.05$ with respect to the control (0).

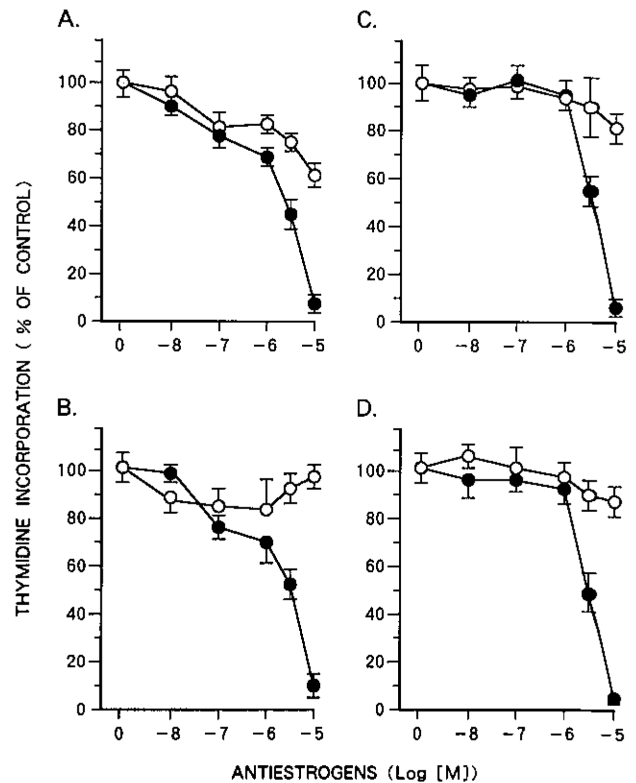

Fig. 3. Effects of increasing concentrations of antiestrogens on thymidine incorporation by four breast cancer cell lines. MCF-7 (A), T47D (B), BT-20 (C) and STT-11 (D) were plated (10^4 cells/well) for MINI assay and the effects of tamoxifen (●) and toremifene (○) in the presence of 10^{-10} *M* E_2 on thymidine incorporation by the cells were determined after a 96 h incubation. Values are expressed as percentages of the respective control. Each point represents the mean of triplicate wells; the brackets indicate the SEM.

toremifene at 10^{-8} to 10^{-5} *M* was added to the MINI assay wells and thymidine incorporation by the four cell lines was determined. The concentration of E_2 in the wells was 10^{-10} *M*. Thymidine incorporation by both MCF-7 and T47D was suppressed to 75% and to 10% of the control by 10^{-7} *M* and 10^{-5} *M* tamoxifen, respectively (Fig. 3-A, B). Addition of 10^{-6} *M* E_2 reversed the suppressive effect of tamoxifen at 10^{-7} *M* but not at 10^{-5} *M* (data not shown). Although thymidine incorporation by MCF-7 was suppressed by toremifene dose-relatedly, 50% suppression was not observed even at 10^{-5} *M*. Suppression of thymidine incorporation by T47D with toremifene, unlike that with tamoxifen, was not observed at concentrations lower than 10^{-5} *M*. In ER-poor BT-20 and STT-11, thymidine incorporation was not suppressed at all by either tamoxifen or toremifene at concentrations lower than 10^{-6} *M*; but tamoxifen, not toremifene, at more than 10^{-6} *M* suppressed thymidine incorporation in a manner very similar to its effect observed with the

ER-rich cell lines (Fig. 3-C, D). Thus, the suppression of thymidine incorporation by ER-rich cells induced by a lower concentration than 10^{-6} M seems to be a result of the anti-estrogenic effect of tamoxifen because it was not observed with the ER-poor cell lines, and was reversed by addition of 10^{-6} M E_2. The suppressive effect of tamoxifen at concentrations higher than 10^{-6} M on the thymidine incorporation seemed to be unrelated to the ER-mediated reaction. This effect was not observed with toremifene at the high dose in any of the cell lines tested.

Effect of E_2 and anti-estrogens on the thymidine incorporation by breast cancer cells from patients with advanced breast cancer The effects of increasing concentrations of E_2, tamoxifen and toremifene on thymidine incorporation by breast cancer cells obtained from eight patients with malignant effusions were examined in the MINI assay under identical experimental conditions to those employed for the breast cancer cell lines described above. Culture medium was supplemented with 10^{-10} M E_2 when the effects of anti-estrogens were to be examined. Thymidine incorporation by cells was suppressed by 10^{-10} M E_2 in a specimen obtained from patient #2 in Table I (Fig. 4-A). Another specimen (from patient #8)

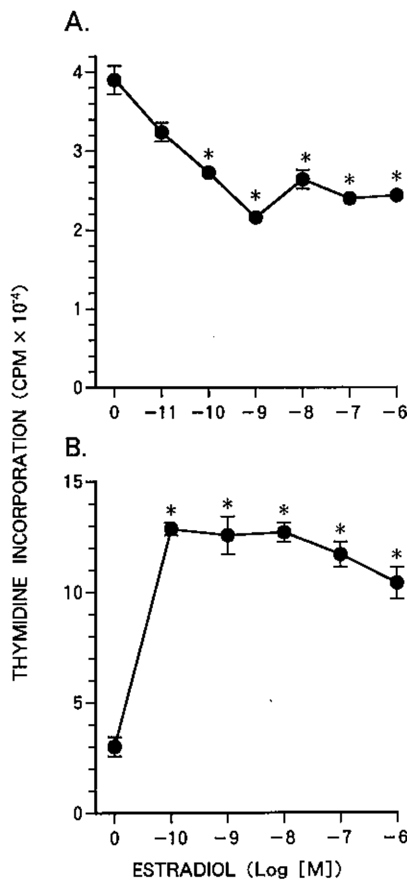

Fig. 4. Effects of increasing concentrations of E_2 on thymidine incorporation by two clinical specimens. Nucleated cells in the pleural effusions of two patients (A; patient #2, B; patient #8) were plated (10^5 cells/well) for the MINI assay and the effect of E_2 on thymidine incorporation by the cells was determined after a 96 h incubation. Each point represents the mean of triplicate wells; the brackets indicate the SEM. * indicates $P<0.05$ with respect to the respective controls (0).

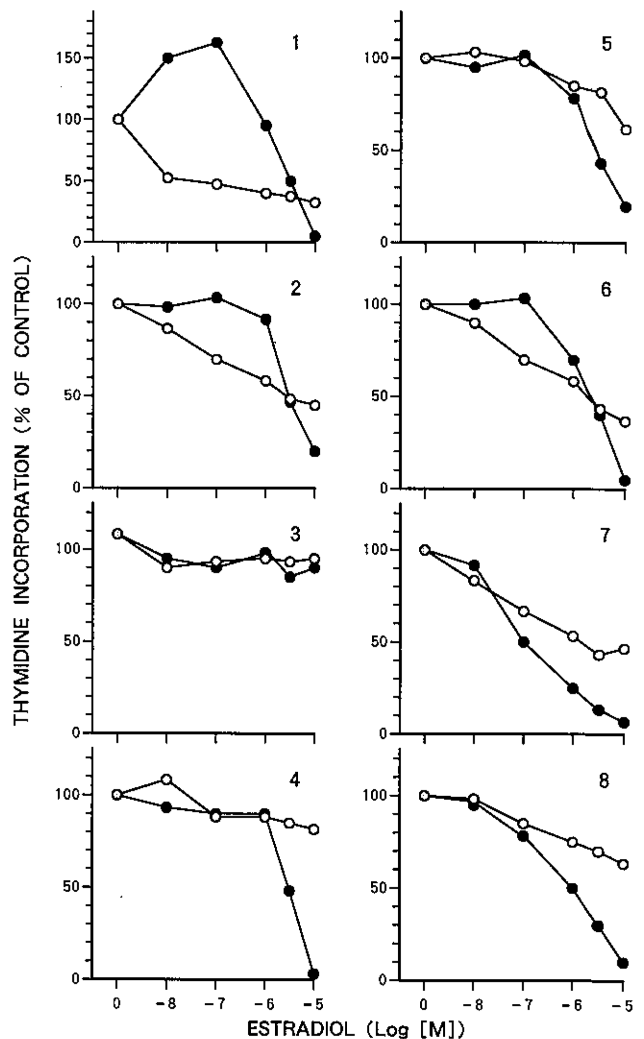

Fig. 5 Effects of increasing concentrations of antiestrogens on thymidine incorporation by eight clinical specimens. Nucleated cells in the malignant effusions of patients with advanced breast cancer were plated (10^5 cells/well) for the MINI assay and the effects of tamoxifen (●) and toremifene (○) in the presence of 10^{-10} M E_2 on thymidine incorporation by the cells was determined after a 96 h incubation. Each point represents the mean of triplicate wells.

showed enhanced thymidine incorporation by E_2 at the concentration of 10^{-10} *M* (Fig. 4-B). In six other specimens, thymidine incorporation was not affected by E_2 at the concentrations tested. The effects of anti-estrogens on the thymidine incorporation by these primary cells were quite different from those observed with the established cell lines described above (Fig. 5). The specimen #1 showed higher sensitivity to toremifene than to tamoxifen, with which thymidine incorporation was rather stimulated. Higher sensitivity to toremifene than to tamoxifen at concentrations lower than 10^{-6} *M* was also observed in patients #2 and #6. In all the specimens tested, toremifene had a weaker effect than tamoxifen at concentrations higher than 10^{-6} *M*. This is a similar phenomenon to that observed with established breast cancer cell lines. Specimen #7, from which STT-11 was established, responded to both tamoxifen and toremifene at concentrations lower than 10^{-6} *M*. These effects were not identical with that observed with STT-11 where anti-estrogens lower than 10^{-6} *M* had no effect on thymidine incorporation (Fig. 3-D).

Clinical response to anti-estrogens in patients with advanced breast cancer Clinical response to either of the anti-estrogens was examined in the seven patients with advanced breast cancer. These patients received either a daily oral dose of 20 mg tamoxifen or 240 mg toremifene, regardless of the result of the *in vitro* sensitivity test. Two patients (#2 and #8) whose cancer cells responded to E_2 in the *in vitro* sensitivity test showed PR to the anti-estrogens (Table I). Clinical response was not observed in other patients even though some of the specimens showed higher *in vitro* sensitivities to anti-estrogens than specimens from the responders. Thus, we failed to correlate the results of the *in vitro* sensitivity assay and the clinical response to the anti-estrogens.

DISCUSSION

Our present results demonstrate that hormone dependency of breast cancer cell lines is reproducible in the MINI assay since E_2 stimulated thymidine incorporation by ER-rich cell lines but not by ER-poor cell lines. The growth-inhibitory effect of tamoxifen was also demonstrated in this assay in four breast cancer cell lines. As previously described by others,[10] the inhibitory effects of tamoxifen on thymidine incorporation by the four cell lines described above can be separated into two components; one is the non-specific effect at concentrations higher than 10^{-6} *M* which occurred in all cell lines irrespective of ER status and was not reversed by addition of a high concentration of E_2. The other is the effect at concentrations lower than 10^{-6} *M* which was confined to the two ER-rich cell lines and was reversed by addition of a high concentration of E_2. The latter effect, however, resulted in only 25% suppression of thymidine incorporation with respect to the control in both of the cell lines (Fig. 3-A, B). If the stimulatory effects of E_2 had been suppressed completely, the expected magnitude of suppression by anti-estrogens would be 85% in MCF-7 and 35% in T47D, based on the results shown in Fig. 2. This incomplete suppression by tamoxifen is probably because of the estrogenic effect of this compound.[11]

It is worth noting that toremifene had less suppressing effect than tamoxifen on thymidine incorporation at concentrations higher than 10^{-6} *M* in all of the cell lines tested. The estrogen-irreversible cytotoxicity induced by tamoxifen has been suggested to be mediated by mechanisms independent of the anti-estrogenic effect.[12] There is evidence that tamoxifen either inhibits protein kinase C, which mediates signals for cellular proliferation,[13] or interacts with calmodulin to induce estrogen-independent inhibition of breast cancer cell growth.[14, 15] The observation that toremifene has little non-specific cytotoxicity may explain the result of clinical trials where relatively low toxicity of toremifene as compared with tamoxifen was demonstrated.[16, 17]

Tamoxifen is the treatment of choice for patients with ER-positive breast cancer, the response rate being 50–70% in this patient population. Low incidence of side effects of tamoxifen at an oral daily dose of 20–40 mg facilitates the use of this anti-estrogen also in the postoperative adjuvant therapy of postmenopausal patients with ER-positive breast cancer.[18] It was suggested that high-dose administration of tamoxifen or toremifene was effective in patients with advanced breast cancer who progressed after successful treatment with tamoxifen at a standard dose.[19, 20] According to recent reports on the pharmacokinetics of toremifene, the clinically achievable plasma concentration of toremifene at the steady state after oral daily administration of 200–400 mg is $2–4 \times 10^{-6}$ *M*.[21, 22] We observed that toremifene is less toxic than tamoxifen in this concentration range. Because the pharmacokinetic behavior of toremifene following chronic oral administration is reported to be very similar to that for tamoxifen,[22] our observation presented above suggests that toremifene may be a more suitable choice of drug for administering at high dose than tamoxifen owing to its lower non-specific cytotoxic effect on breast cancer cells.

E_2 affected the thymidine incorporation *in vitro* by two clinical specimens; in one it was suppressed and in the other, enhanced. It is generally recognized that E_2 stimulates the growth of hormone-sensitive breast cancer cells at lower concentrations and suppresses it at higher concentrations, as exemplified by the use of diethylstilbestrol for the treatment of postmenopausal women with advanced breast cancer.[23] However, an exception for this rule is T61, a human breast cancer cell line transplantable

in athymic mice, whose growth is inhibited by E_2 dose-relatedly.[24] Therefore, the suppression by E_2 of thymidine incorporation observed in a freshly obtained specimen from patient #8 can be regarded as evidence supporting the hormone dependency of the specimen. Although the *in vitro* hormonal sensitivity of breast cancer in this regard showed coincidence with the clinical response to anti-estrogens, no clear correlation between the *in vitro* sensitivity of freshly obtained cells to anti-estrogens and the clinical response to these drugs could be demonstrated in this study. The problem is that the clinical responses to the anti-estrogens in the eight patients were evaluated with metastatic sites other than the malignant effusion from which the cells were obtained, because malignant effusion is routinely controlled with local therapies. One possible explanation for the failure of prediction with the *in vitro* sensitivity test of clinical response to the anti-estrogens is the heterogeneous hormonal sensitivities among different metastatic sites.[25] Further investigation is required to confirm the relationship between *in vitro* response to E_2 and clinical effect of anti-estrogens, and the reliability of the MINI assay to predict clinical effects of anti-estrogens.

ACKNOWLEDGMENTS

We thank Drs. Koichi Minato, Yasutsuna Sasaki and Nagahiro Saijo, Pharmacology Division and Department of Medicine, National Cancer Center for their generous assistance and valuable advice concerning the MINI assay. This work was supported in part by a Grant-in-Aid for Cancer Research (62S-1) from the Ministry of Health and Welfare and by special coordination funds for promoting science and technology from the Science and Technology Agency.

(Received January 5, 1990/Accepted March 8, 1990)

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