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HIGH *IN VITRO-IN VIVO* CORRELATION OF DRUG RESPONSE USING SPONGE-GEL-SUPPORTED THREE-DIMENSIONAL HISTOCULTURE AND THE MTT END POINT

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The *in vitro* sponge-gel-supported three-dimensional histoculture chemosensitivity assay (Hoffman assay) allows the *in vivo*-like culture of human tumors. In this study, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H tetrazolium bromide (MTT) end point was applied to the Hoffman assay in an attempt to increase *in vitro-in vivo* correlation. The chemosensitivities of 16 human tumor lines were determined *in vitro* by the histoculture assay, and retrospectively correlated to their *in vivo* chemosensitivity as xenografts in nude mice. The *in vitro* test was considered to be positive if tumor-cell MTT reduction activity was lowered by more than 50%. The cutoff drug concentrations to determine sensitivity *in vitro* were determined for mitomycin C, doxorubicin, 5-fluorouracil and cisplatin. Using these cutoff drug concentrations *in vitro* we found, as a function of time of exposure, a strong correlation between serum drug concentrations found in nude mice given maximum tolerated doses and drug concentrations found in the histoculture media *in vitro*, thereby establishing a relationship between the amounts of drugs to which tumors were exposed *in vivo* and *in vitro*. The overall correlation rate of the efficacy results of the drug-response assay to *in vivo* chemosensitivities was 89.8%, with 90.0% true-positive and 89.7% true-negative rates, 81.7% sensitivity and 94.6% specificity, thereby indicating potential clinical use for tumor histoculture with the MTT end point.

Over the past few decades a great deal of research has been devoted to the development of *in vitro* methods to predict the chemosensitivity of human tumors *in vivo* accurately (Hoffman, 1991). In studies with the human tumor clonogenic assay developed by Hamburger and Salmon (1977), predictabilities of 91% for clinical chemoresistance and 69% for chemosensitivity have been achieved (Von Hoff, 1988). However, the assay is highly labor-intensive, results are not available for 2 to 3 weeks, and 30 to 70% of the tumor cultures initiated in the assay are found to be unevaluable for drug response (Twentyman, 1985; Von Hoff, 1988).

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H tetrazolium bromide (MTT) end point is a simple colorimetric test of cell proliferation and survival which was developed by Mosmann (1983), and a number of groups have used this end point in different assays for drug-sensitivity testing of human cancers. Because of its simplicity, the MTT end point has the potential to overcome a number of problems that have prevented routine use of other chemosensitivity end points in clinical practice. However, predictabilities of the MTT end point for clinical chemoresponses have not been established at the present time. We have adopted the MTT end point for chemosensitivity testing of human surgical specimens in suspension culture, and reported a relatively high incidence of overestimating clinical chemoresponsiveness, in spite of its excellent predictability for clinical chemoresistance (Furukawa *et al.*, 1991).

Kern and Weisenthal (1990), with agar-based and suspension-culture systems, were able to predict chemoresistance, but not chemosensitivities, accurately. Cell-suspension models employed in these methods eliminate the cellular interaction which may affect chemosensitivity *in vitro* (Hoffman, 1991). At present, the clinical efficacy of any single chemotherapeutic agent is still only 10 to 25% which is reflected by a high true-negative rate in most *in vitro* methods. Therefore it is important to develop methods which will enable the relatively infrequent positive responses to chemotherapy to be determined accurately. The major problem encountered with most current methods may be that of mimicking *in vitro* the drug exposure conditions which cells experience *in vivo* (Twentyman, 1985).

The three-dimensional histoculture assay developed by Hoffman and co-workers (Vescio *et al.*, 1991; Hoffman, 1991) (Hoffman assay) allows fresh surgical specimens to maintain their cell-to-cell contact and three-dimensional native tissue architecture in culture. Furthermore, this assay was found to detect *in vivo*-like drug responses of human tumors (Vescio *et al.*, 1991). The histoculture assay, with the end-point of ^{3}H-thymidine (^{3}H-TdR) incorporation measured by histological autoradiography, had an 86% correlation rate for chemoresistance when used to compare the drug responses of human tumor lines *in vitro* to suprapharmacological drug doses and with maximum tolerated doses (MTDs) in nude mice. The *in vitro-in vivo* correlation of drug response varied, however, depending on the drug, from a rate of 53% for 5-fluorouracil to 78% for doxorubicin (Vescio *et al.*, 1991). In addition, the histoculture assay with the ^{3}H-TdR incorporation end-point measured by histological autoradiography requires some complicated techniques including histological preparation of the histocultured specimens for autoradiography and approximately 2 weeks to obtain data.

In this study we used the Hoffman histoculture assay with the MTT end-point in an attempt to increase the correlation rate with *in vivo* drug responses and to simplify the assay. For the *in vivo* arm, we used nude mice with human tumor xenografts which provides a constant and reproducible cohort.

MATERIAL AND METHODS

Mice

BALB/c nu/nu mice, which originated from the Central Institute for Experimental Animals, Kawasaki, Japan, were obtained from CLEA, Tokyo, Japan. Animals which were 6 to 8 weeks old and weighed 20 to 22 g were used for human tumor transplantation.

Human tumor xenografts

The tumors evaluated were the gastric cancer lines St-4, St-15, St-40, H-111 and SC-2-JCK; the colonic cancer lines Co-3, Co-4, Co-6 and Co-8; the breast cancer lines MX-1 and MCF-7; the small-cell lung cancer lines Lu-24, Lu-130 and H-69; hepatoma line Li-7 and neuroblastoma line CR-NB9.

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The H-111 tumor line was kindly provided by Dr. M. Fujita, Osaka University, SC-2-JCK was established at the Central Institute for Experimental Animals, Kawasaki, Japan, and provided by Dr. K. Maruo, MX-1 was established by Ovejera *et al.* (1978) and was provided by Dr. K. Inoue, Cancer Chemotherapy Center, Tokyo. The other lines were established in the Pathology Division, of the National Cancer Center Research Institute, and Keio University School of Medicine, Tokyo. All the tumors were maintained by serial transplantation into nude mice at Keio University School of Medicine.

Drugs

Four anti-cancer agents were used for the assay: mitomycin C (MMC), doxorubicin (DXR) and 5-fluorouracil (5-FU) were purchased from Kyowa Hakko, Tokyo, and cisplatin (DDP) was purchased from Bristol-Myers Squibb K. K., Tokyo.

In vivo *chemosensitivity test*

Tumor fragments were inoculated into the subcutaneous tissue of either side of the backs of nude mice. The female nude mice inoculated with the MCF-7 human breast-cancer line were treated once with 17 beta-estradiol dipropionate at a dose of 5 mg/kg intramuscularly. The length and width of the tumors were measured with sliding calipers 3 times a week by the same person. The tumor weight was estimated according to the formula: tumor weight (mg) = length (mm) × [width (mm)]^2/2. When tumors reached 100 to 300 mg, usually 2 to 3 weeks after tumor inoculation, tumor-bearing mice were randomized into control and treated groups, which consisted of at least 4 mice each, and treatment was initiated. All the drugs were dissolved in 0.2 ml of 0.9% NaCl per 20 g body weight and administered as i.p. boluses for MMC and DDP, as i.v. bolus for DXR, and 5-FU was given as 3 i.p. injections at 4-day intervals. The doses administered were 6, 9, 8 and 60 mg/kg for MMC, DDP, DXR and 5-FU respectively, which had been determined to be MTDs in nude mice in our previous reports (Kubota *et al.*, 1986). A split schedule of 5-FU administration was determined from the fact that 3 i.p. injections at a dose of 60 mg/kg at 4-day intervals demonstrated a greater anti-tumor effect than an i.p. bolus at a dose of 180 mg/kg.

Mice and tumors were observed 3 times a week for 3 weeks after the initial treatment and the relative mean tumor weight (RW) was calculated as Wi/Wo, where Wi was the mean tumor weight of a group at any given time and Wo was the mean initial tumor weight at the start of treatment. The anti-tumor effects of drugs were evaluated in terms of the lowest Trw/Crw during the experiment, where Trw was the relative mean tumor weight of the treated group and Crw was that of the control group at the same time. The anti-tumor activity was evaluated as positive when the lowest Trw/Crw during the experiment was less than 42%, which is equivalent to a cubic value of 75%, reflecting a 25% reduction of tumor diameter (Kubota *et al.*, 1986).

Histoculture

Tumors were explanted using the three-dimensional *in vitro* histoculture system, developed by Hoffman and co-workers (Vescio *et al.*, 1991; Hoffman, 1991) based on the early work of Leighton (1951).

Specialized collagen gels manufactured from pig skin were purchased from Health Design, Rochester, NY. Gels were removed from their sterile packages and cut with scissors into 1-cm^3 pieces and one piece was placed into each well of 24-well plates. Continuous drug exposures from the time of initiation of histoculture were used for *in vitro* chemosensitivity testing. The anti-cancer drugs were dissolved, at various concentrations, in RPMI-1640 medium (Nissui, Tokyo, Japan) which contained 20% fetal calf serum (Life Technologies, Grand Island, NY), and 1 ml per well of the required solutions was added to the wells, which reached, but not covered the upper part of the gels. At least 4 concentrations of each drug were prepared for each single assay.

Tumors at the exponential growth phase in nude mice were resected aseptically, necrotic tissues were cut away, and remaining healthy tumor tissues were scissor-minced into pieces about 2 mm in diameter in Hanks' balanced salt solution. Each piece of tumor was weighed on an AM 100 balance (Mettler Toledo, Nanikon-Uster, Switzerland), minced further into 5 to 10 pieces about 1 mm in diameter, and all the pieces were placed on one of the prepared collagen surfaces in 24-well plates. The plates were incubated for 3 to 11 days at 37°C in a humidified atmosphere containing 95% air/5% CO_2.

In vitro *histoculture assay with the MTT end point*

The activity of the living cells in and on the collagen gels was evaluated using the MTT end-point (Shimoyama *et al.*, 1989); 100 μl per well of Hanks' balanced salt solution, containing 0.6 mg/ml collagenase (type I: Sigma, St. Louis, MO) and 100 μl per well MTT (Dojindo, Kumamoto, Japan) solution prepared by dissolving the solid (5 mg/ml) in phosphate-buffered saline and filtering it through a 0.45-μm membrane filter (Millipore, Bedford, MA), were added to each well and the plates were incubated for a further 8 hr. Collagen gels were dissolved within less than 30 min and the tumor pieces, which by then were floating in the medium, turned violet. The medium was aspirated completely from each well by careful use of micropipets and 1 ml per well dimethyl sulfoxide (Nacalai Tesque, Kyoto, Japan) was added to dissolve the MTT-formazan product. After 2 hr the solutions were transferred to 96-well microplates (100 μl per well) and the absorbance of the solutions in each well were read at 540 nm on a Model EAR 340 AT reader (SLT, Gzoedig/Salzburg Austria).

The absorbance/g of each tumor was calculated from the mean absorbance from 4 wells and initial tumor weight which was estimated prior to culture. All drug concentrations were tested in triplicate wells and the inhibition rate at each concentration was calculated as follows: inhibition rate (%) = (1 − mean absorbance/g tumor of the treated wells/mean absorbance/g tumor of the control wells) × 100. The drug concentrations which lowered tumor-cell MTT-reduction activity by 50% (IC_{50}) were determined from the concentration-effect data with the slope of steepest descent determined by linear regression.

In vivo *and* in vitro *pharmacokinetics*

After drug administration at MTD, mice were killed at intervals for 4 hr by taking, under ether anesthesia, blood from the femoral vessels. The collected blood was separated into serum with a freezing centrifuge. The collected sera were then immediately stored at −80°C for MMC, DXR and 5-FU. The serum samples were centrifuged through a Centrifree Micropartition System (Amicon, Beverly, MA) before being stored for quantitation of free (non-protein-binding and active) DDP. Blood was collected from at least 3 mice for a single measurement of each of the above drugs.

For determination of drug levels in the culture media and the histocultured tumors, media in the culture wells and tumor pieces on collagen gels were collected on days 1, 2, 3, 5 and 7, and were stored at −80°C. For determination of DDP levels, part of the medium sample was centrifuged as described above before being stored. Media from 3 wells and tumor pieces from at least 20 wells were collected for a single measurement of each of the above drugs. The MX-1 human breast-cancer line was used for this particular study.

The concentrations of drugs in the sera, the media and the tumor pieces were measured by high-performance liquid chromatography (HPLC) for MMC, DXR and 5-FU (Masuike *et al.*, 1984, 1985, 1991), and by flameless atomic absorption spectrophotometry (FAAS) for DDP (LeRoy *et al.*, 1977).

Since free DDP could not be isolated for measurement in the tumor pieces, only total DDP was measured in this case.

In the case of quantitation of MMC, 6 ml ethylacetate was added to 500 μl of the serum or the medium sample and the mixture was shaken for 5 min followed by centrifugation at 450 *g* for 10 min. For the tumor samples, 200 μg of the sample was homogenized in 3 ml chilled acetonitril and 100 mg silica gel (60–80 mesh, Kanto Kagaku, Tokyo, Japan), and the homogenate was centrifuged after the homogenizer was washed with 2 ml acetonitril. Then, after addition of 50 μl of anti-oxigenating solution, prepared by dissolving 400 mg 3-*t*-butyl-4-hydroxyanisole and 250 mg *t*-butyl hydroxytolene in 1 ml of acetonitril, the organic layer in the case of the serum and the medium sample, or the supernatant for the tumor sample was evaporated under vacuum at 40°C. The residues were dissolved in 1 ml of a chloroform and methanol (98: 2) mixture, and the solutions were injected onto a silica gel column (Nucleosil 100-5, Kemuko, Osaka, Japan) and eluted with a mobile phase consisting of chloroform, methanol and water (90: 10: 0.15). The eluate was monitored at 360 nm.

In the case of quantitation of DXR, 1 ml 0.1 M Kolthoff buffer (pH 8.0) was added to 1 ml of the serum or the medium sample, or 100 μg of the tumor sample was homogenized in 2 ml of the buffer saturated with NaCl. Then 8 ml of a mixture of butanol and toluene (1: 1) was added in each case, and the mixture was shaken for 15 min followed by centrifugation at 450 *g* for 10 min. Then 6 ml of the organic layer was evaporated under vacuum, and the residue was dissolved in 1 ml of a mixture of phosphate buffer and methanol (1: 1) followed by centrifugation. The supernatant was injected onto a reversed-phase column (TSK gel ODS-120A, Toyo Sotatsu, Tokyo, Japan) and eluted with a mixture of 1 N formic acid and methanol (55: 45). The fluorescence signal was monitored at 470 nm and 585 nm.

In the case of quantitation of 5-FU, 25 μl 0.5 M phosphate buffer (pH 8.0) and 6 ml ethylacetate was added to 100 μl of the serum or the medium sample and the mixture was shaken for 5 min followed by centrifugation at 450 *g* for 10 min. For the tumor samples, 200 μg of the sample was homogenized in 3 ml chilled acetonitril and 100 mg silica gel (60 to 80 mesh, Kanto Kagaku, Japan), and the homogenate was centrifuged after the homogenizer was washed with 2 ml acetonitril. After centrifugation, the organic layer in the case of the serum and the medium sample, or the supernatant for the tumor sample, was evaporated under vacuum. The residue in the case of the tumor sample was purified using column chromatography, prepared by packing 500 mg of silica gel (80 to 100 mesh, Kanto Kagaku) into a disposable syringe and washed with acetone before use. Then the residue in each case was dissolved in a mixture of mobile phase for HPLC, consisting of ethylacetate, *n*-hexane, 88% formic acid and water (60: 40: 0.5: 0.2), and *n*-hexane (3: 2). The solution was analyzed by HPLC using a silica gel column (Develosil 60-3, Nomura Kagaku, Tokyo, Japan) and the mobile phase with the eluate monitored at 264 nm.

In the case of quantitation of DDP, the serum or the medium sample was directly applied to FAAS. The tumor sample was weighed, freeze-dried and triturated to yield a homogenous sample, and then liquified and digested by heating with the addition of approximately 20 ml per gram of tissue of a mixture of 70% $HClO_4$ and 60% HNO_3 (3: 2). When the digest was almost dry, the residue was taken up in 10% HCl, evaporated, and resolubilized in a known volume of the HCl. Then the solution was applied to FAAS.

Drug concentrations of each sample was calculated from the calibration curves of which linearity had been established with correlation constants of over 0.999 for each drug (Masuike *et al.*, 1984, 1985, 1991; LeRoy *et al.*, 1977).

Pharmacokinetic data in nude mice were analyzed by computer-assisted fitting of the data to a most adapted one- or two-compartment model. Peak drug concentrations (C_{max}) and the area under the curve (AUC) of each drug were calculated as the index. In case of 5-FU, the AUC in nude mice was calculated as 3-fold of that obtained from single administration of 60 mg/kg, according to its administration schedule in nude mice.

RESULTS

Relationship in histoculture between total MTT-reduction and tumor weight

The relationship between initial tumor weight and MTT-reduction activity was analyzed using the MX-1 human breast-cancer line. The absorbance of the extracted formazan produced by MTT reduction was proportional to tumor weight in both control and treated cultures (10 μg/ml of MMC) as shown in Figure 1.

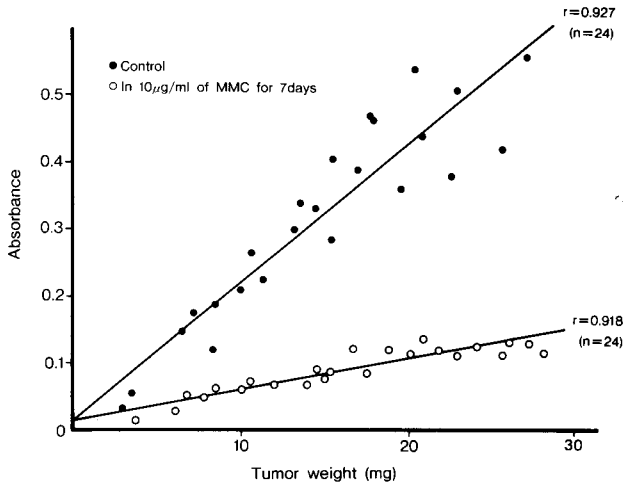

FIGURE 1 – Relationship between initial tumor weight and MTT-reduction activity of the MX-1 human breast-cancer line in histoculture. These tumor specimens were incubated for 7 days on collagen gels after being cut into 5 to 10 1-mm^3 pieces, and evaluated subsequently utilizing the MTT end point determined by optical density. Both untreated and MMC-treated cultures were evaluated.

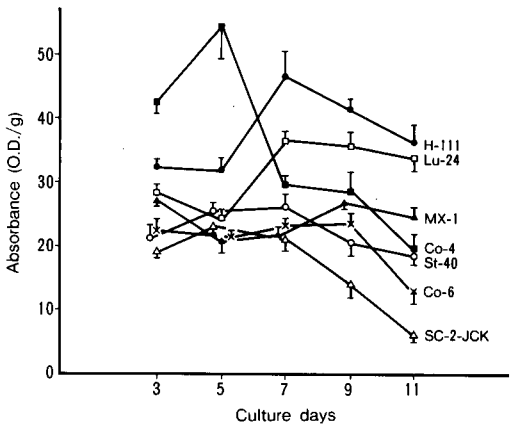

FIGURE 2 – Change in MTT-reduction activity determined by optical density of untreated histocultures as a function of culture time for 7 tumor lines. Each point represents the mean of the results from 3 culture wells. Bars, standard deviations.

MTT-reduction activity of tumors as function of time in histoculture

The change of MTT-reduction activity determined by absorbance measurements at 540 nm of extracts of the resulting formazan from untreated cultures as a function of culture time for each tumor type are shown in Figure 2. The MTT-reduction activity of the H-111 human gastric-cancer line, Co-4 human colonic-cancer line and Lu-24 human small-cell lung-cancer line increased temporarily on days 7, 5 and 7 respectively after the initiation of histoculture, whereas those of the other 4 tumors were almost constant for 9 days. A considerable decline in MTT-reduction activity was shown on day 11 by the SC-2-JCK human gastric-cancer line and Co-6 human colonic-cancer line histocultures.

Drug sensitivity of tumors as function of time in histoculture

Fig. 3, *a, b, c and d* show the changes in the concentration-effect curves at different culture periods where there was continuous drug exposure for MMC on the Co-6 human colonic-cancer line, DXR on the MX-1 human breast-cancer line, 5-FU on the H-111 human gastric-cancer line and DDP on the St-40 human gastric-cancer line. The linear portions of these curves were restricted to 1 to 2 log units of concentration, which shifted to lower concentrations later in the culture period.

Period of drug exposure in vitro *to determine* IC_{50}

Changes in IC_{50} values of drugs with respect to time in histoculture and hence to time of exposure to drugs in 10 combinations of drugs and tumor lines are shown in Table I. IC_{50} values decreased with time in histoculture and exposure to drugs in all 10 combinations of drugs and tumor lines. The IC_{50} values of both MMC and DDP on the SC-2-JCK human gastric-cancer line showed exceptional decreases on day 5, whereas the IC_{50} values of DXR on the Lu-24 human small-cell lung-cancer line stayed high until day 11. In 7 other combinations of drugs and tumor lines, the main decreases in IC_{50} values of drugs were observed on day 7, which therefore was chosen for the assay period for correlation with *in vivo* drug responses.

In vivo *response of tumors xenografted in nude mice to drugs*

The anti-tumor activities of the drugs in nude mice are shown in Table II. Mitomycin C and DDP showed positive anti-tumor activity in 12 of 16 and 6 of 14 lines respectively, with corresponding efficacy rates of 75.0% and 42.9%. On the other hand, DXR and 5-FU were effective against only 2 of 15 and 14 lines respectively, with corresponding efficacy rates of 13.3% and 14.3%.

Determination of the in vitro *cutoff concentrations of drugs used for correlation to* in vivo *drug response*

The 7-day Hoffman-histoculture assay with the MTT end point was carried out in triplicate for the tumor lines in this study to determine the correlation with the *in vivo* chemosensitivity at MTD of the same tumors growing as xenografts in nude mice. IC_{50} values of the drugs determined from triplicate assays for the 7-day drug exposure period are shown in Table

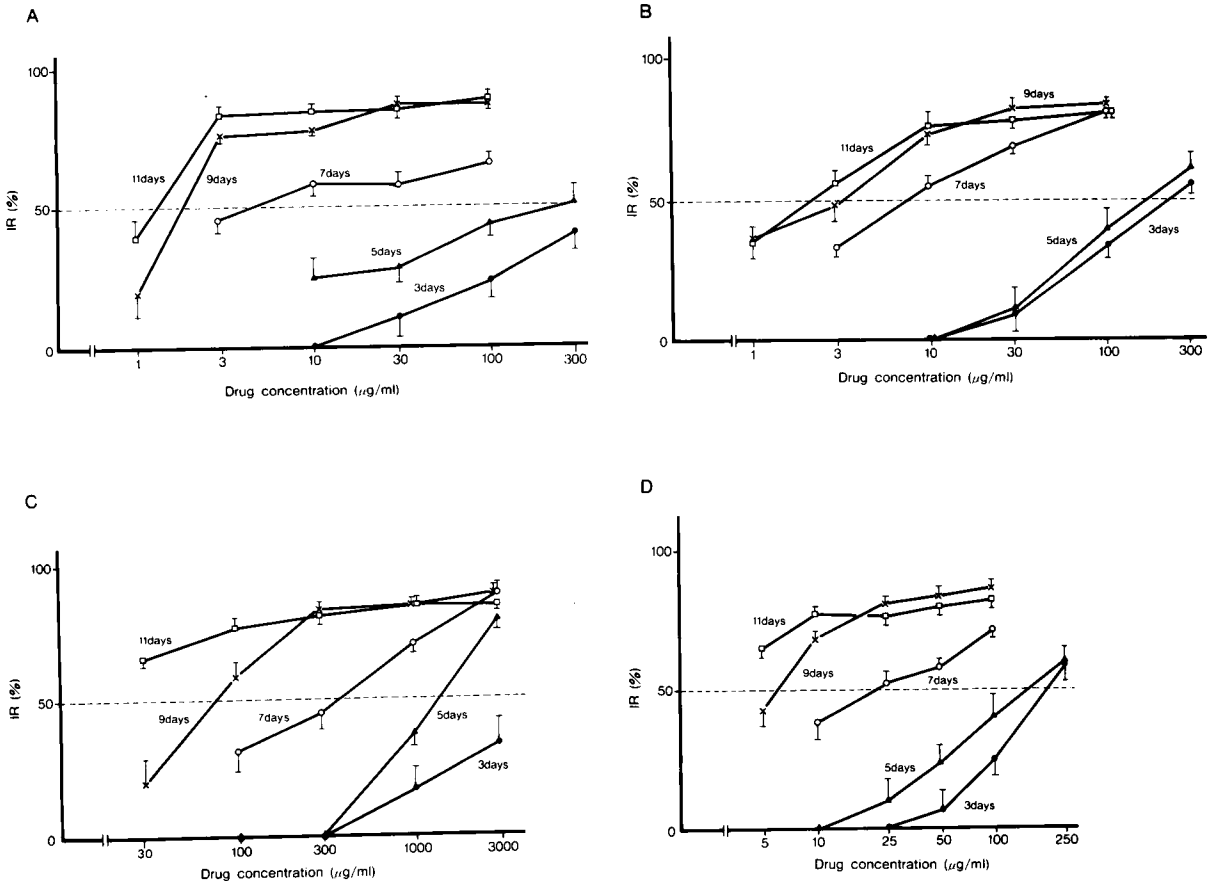

FIGURE 3 – Change in the concentration-effect curves of *(a)* MMC on Co-6, *(b)* DXR on MX-1, *(c)* 5-FU on H-111 and *(d)* DDP on St-40 with respect to time in histoculture and drug exposure. Tumor histocultures were exposed continuously to drugs at various concentrations for 3, 5, 7, 9 and 11 days. Each point represents the mean of the results from 3 culture wells. Bars, standard deviations.

TABLE I – CHANGES IN IC_{50} VALUES OF DRUGS WITH RESPECT TO DRUG-EXPOSURE PERIOD IN HISTOCULTURE

Drug	Tumor line	Histoculture and drug-exposure period (days)				
		3	5	7	9	11
MMC	St-40	412[1]	230	2.51	1.14	<1
	SC-2-JCK	332	7.05	5.04	1.24	<1
	Co-6	>300	245	3.94	1.80	1.40
DXR	MX-1	219	151	8.21	2.88	2.43
	Lu-24	>300	>300	>100	>100	37.0
5-FU	H-111	>3000	1355	340	77.8	<30
	Co-4	2646	631	37.0	<30	<30
DDP	St-40	202	160	23.3	5.93	<5
	SC-2-JCK	131	12.2	9.16	6.06	<5
	MX-1	144	115	8.02	<2.5	<2.5

[1]Data are shown in μg/ml.–Tumors were histocultured on collagen-gel surfaces as described in the text. Drugs were added at time zero and the MTT end-point was evaluated on the days indicated as described. Results for the drug-treated histocultures were compared with untreated controls to determine the IC_{50}. The highest and the lowest concentrations of each drug prepared in each assay performed for each tumor line on each day are not the same throughout experiments.

TABLE II – CHEMOSENSITIVITY OF HUMAN TUMOR XENOGRAFTS IN NUDE MICE

Xenograft	MMC[1]	DXR	5-FU	DDP
St-4	65.0 (−)	48.3 (−)	87.6 (−)	91.7 (−)
St-15	22.7 (+)	66.2 (−)	61.4 (−)	27.2 (+)
St-40	7.3 (+)	64.9 (−)	98.1 (−)	23.6 (+)
H-111	45.5 (−)	54.4 (−)	31.3 (+)	67.8 (−)
SC-2-JCK	18.0 (+)	69.4 (−)	56.2 (−)	29.4 (+)
Co-3	71.9 (−)	55.4 (−)	44.8 (−)	92.0 (−)
Co-4	13.8 (+)	52.1 (−)	13.4 (+)	15.8 (+)
Co-6	2.6 (+)	69.4 (−)	44.2 (−)	52.9 (−)
Co-8	59.1 (−)	64.6 (−)	52.4 (−)	72.6 (−)
MX-1	7.9 (+)	39.8 (+)	52.4 (−)	3.4 (+)
MCF-7	23.4 (+)	64.5 (−)	63.5 (−)	ND[2]
Lu-24	20.4 (+)	33.3 (+)	68.0 (−)	65.6 (−)
Lu-130	6.4 (+)	66.1 (−)	90.6 (−)	86.8 (−)
H-69	28.3 (+)	ND	56.0 (−)	84.0 (−)
Li-7	21.7 (+)	84.3 (−)	ND	ND
CR-NB9	19.4 (+)	46.9 (−)	ND	32.3 (+)
Efficacy rate[3]	12/16 (75.0%)	2/15 (13.3%)	2/14 (14.3%)	6/14 (42.9%)

[1]Data indicate the lowest value of relative mean tumor weight of the treated group/relative mean tumor weight of control group (Trw/Crw) (%). (+) indicates positive anti-tumor activity (= <42%). (−) indicates negative anti-tumor activity (>42%).–[2]ND: not done.–[3]Efficacy rate: sensitive cases/total cases.–Human xenografts were established and transplanted S.C. in nude mice as described in the text. Maximum tolerated doses of drugs were administered as described. Tumor size was determined 3 times a week with calipers, as described in "Materials and methods".

III. The cutoff concentrations of the IC_{50} values of drugs, which were to be used to distinguish positive and negative *in vitro* anti-tumor activity, were determined retrospectively to be 7.5 μg/ml for MMC, 15 μg/ml for DXR, 300 μg/ml for 5-FU and 20 μg/ml for DDP as shown in Fig. 4, *a, b, c and d* respectively.

Correlation of in vivo *and* in vitro *drug response*

With the cutoff criteria determined retrospectively as described above, the results of the *in vitro* histoculture assay correlated with the *in vivo* chemosensitivities to MMC, DXR, 5-FU and DDP with correlation rates of 87.5% (14/16), 86.7% (13/15), 92.9% (13/14) and 92.9% (13/14) respectively, with an overall correlation rate of 89.9% (53/59) which consisted of 90.0% (18/20) true-positive and 89.7% (35/39) true-negative rates, 81.8% (18/22) sensitivity and 94.6% (35/37) specificity (Table IV). There were 2 false-positive cases, DXR and 5-FU on the St-15 human gastric-cancer line, and 4 false-negative cases, MMC on the Lu-130 human small-cell lung-cancer line, MMC and DXR on the Lu-24 human small-cell lung-cancer line and DDP on the St-40 human gastric-cancer line. The correlation rates for the St-15 and the Lu-24 tumors were 50% (2/4) each, which were lower than the 96.1% (49/51) correlation rate for the rest of the tumor lines.

Comparison of in vitro *and* in vivo *pharmacokinetics*

Figure 5*a* demonstrates the determination of drug concentrations, on a μg/ml basis, in the medium as a function of time in histoculture of the tumors. It can be seen that the concentrations of 5-FU and DDP were not reduced to any apparent degree, whereas those of DXR and MMC decreased slowly in the medium with time of histoculture. Figure 5*b* demonstrates the amount of drugs measured on a μg/g basis in the histocultured tumors themselves. As can be seen, the amount of 5-FU in the tumors is very high by day 1 and stays constant. DDP is also constant with time after day 1. For DXR and MMC there is a slight decrease in concentration found in the tumors with time in histoculture.

Figure 6, *a* and *b,* demonstrate high *in vitro-in vivo* correlations in C_{max} and AUC respectively. For both parameters C_{max} and AUC, when the *in vitro* amounts of drugs in the media in

TABLE III – IC_{50} VALUES ON THE MTT END POINT FOR DRUGS ON HUMAN TUMOR LINES AFTER 7 DAYS OF HISTOCULTURE AND DRUG EXPOSURE

Tumor line	MMC[1]	DXR	5-FU	DDP
St-4	12.2 ± 2.1	77.3 ± 5.0	839 ± 159	63.0 ± 9.7
St-15	4.64 ± 0.12	6.40 ± 0.83	186 ± 49	10.4 ± 0.9
St-40	2.36 ± 0.16	43.3 ± 4.7	1976 ± 336	21.8 ± 2.7
H-111	23.1 ± 4.4	87.5 ± 12.8	251 ± 96	56.4 ± 10.4
SC-2-JCK	5.57 ± 0.72	50.2 ± 10.2	743 ± 130	14.7 ± 4.7
Co-3	75.7 ± 9.3	57.3 ± 13.9	1528 ± 364	> 100
Co-4	1.78 ± 0.11	18.5 ± 6.0	55.9 ± 14.5	12.4 ± 4.7
Co-6	5.62 ± 1.97	> 100	816 ± 110	81.6 ± 7.7
Co-8	8.61 ± 1.51	> 100	642 ± 49	23.6 ± 1.0
MX-1	1.97 ± 0.31	11.9 ± 2.8	656 ± 198	7.19 ± 0.58
MCF-7	2.42 ± 0.20	> 100	633 ± 184	53.1 ± 5.5
Lu-24	76.4 ± 15.1	> 100	> 3000	> 100
Lu-130	11.7 ± 1.1	> 100	> 3000	> 100
H-69	2.30 ± 0.41	75.9 ± 9.2	2412 ± 268	> 100
Li-7	3.01 ± 0.23	> 100	> 3000	77.6 ± 13.2
CR-NB9	4.36 ± 1.50	51.0 ± 13.0	1405 ± 304	12.8 ± 3.7

[1]Data indicate mean ± standard deviation (μg/ml) of IC_{50} values determined in triplicate assays.–See Table I for explanation of methods.

the histoculture measured at intervals for 7 days are plotted against the *in vivo* sera amounts of drugs measured at intervals for 4 hr for all 4 agents used, a very straight line is formed with correlation constants of over 0.999 ($p < 0.001$) in each case. Thus there seems to be a relationship between *in vitro* and *in vivo* pharmacokinetics in the conditions of our experiments.

DISCUSSION

Vescio *et al.* (1991) reported a relatively high *in vitro-in vivo* correlation of drug response using human tumor lines in the histoculture assay with histological autoradiography of ^{3}H-TdR incorporation as an end point. The MTT end point in the Hoffman assay appears to have 2 advantages compared with the ^{3}H-TdR-incorporation end point which were shown in this study: (1) it can be used to evaluate both proliferating and non-proliferating cells; (2) it is very simple and rapid. Both the MTT- and ^{3}H-TdR-incorporation end points in the Hoffman histoculture assay have high probabilities of correlating with chemoresistance *in vivo.* However, the MTT end point in this assay appears to have a higher ratio of correlating with chemoresponsiveness *in vivo* than the ^{3}H-TdR-incorporation end point. In our previous study, growth fractions of the majority of the tumors used in this study as xenografts were found to be approximately 100%, while the others had a growth fraction of approximately 40% (Kubota *et al.,* 1986). In the latter cases, where the growth fraction is relatively low, the MTT end point may be advantageous compared with the ^{3}H-TdR-incorporation end point in correlating with chemoresponsiveness *in vivo,* since the MTT end point evaluates resting tumor cells, which may be less sensitive to drugs than proliferating cells, which the MTT end point also measures (Kubota *et al.,* 1986). This may be a possible explanation for the observed higher *in vitro-in vivo* correlation when the histoculture assay is evaluated by the MTT end point.

The decline of the control MTT-reduction activity in some but not all tumor lines after 11 days of histoculture suggested that tumor viability in 3-dimensional histoculture varies depending on the individual tumor. Although other workers reported reduced production of formazan crystals in cells treated with old media in which there was previous significant cell production (Jabbar *et al.,* 1989), we observed little difference between old and fresh media in the production of formazan crystals in the histocultured tumors (data not shown). Therefore a shorter period was chosen for the histoculture assay with the MTT end point, since the decline of control MTT-reduction activity in some tumor lines was considered to reflect the decline of viability in these tumor lines. Although 5 days of culture was sufficient for the IC_{50} values for each tumor line to be determined, the IC_{50} values were extremely high, resulting in a negative chemoresponse *in vitro* in some tumor lines which were chemosensitive *in vivo* (data not shown). We estimated that the optimal histoculture period with the MTT end point was 7 days, at which time cutoff concentrations to distinguish positive and negative anti-tumor responses were determined retrospectively to be 7.5 μg/ml for MMC, 15 μg/ml for DXR, 300 μg/ml for 5-FU and 20 μg/ml for DDP, which resulted in the highest *in vitro-in vivo* correlation of drug response. Although the base-line MTT-reduction activity decreased by day 7 for some of the histocultured tumor lines, this was not the case for all, since for example the H-111 human gastric-cancer line actually peaked in MTT-reduction activity on day 7 (Fig. 2). However, since all tumor lines became more sensitive to drugs with time of drug exposure in histoculture, we therefore concluded that it was the continuous exposure to drugs, not the diminishing baseline MTT-reduction activity of some tumor lines, that actually was the basis of the increased sensitivity to drugs with time of drug exposure in histoculture.

Determination of cutoff drug concentration is very important in any *in vitro* drug-response assay. Hamburger and Salmon (1977) used 1/10 peak plasma concentrations as the cutoffs in the human tumor clonogenic assay and Vescio *et al.* (1991) also adopted these concentrations as their cutoffs in the histoculture assay with the ^{3}H-TdR incorporation end point. The cutoff concentrations determined in our study were supra-pharmacological and could not be achieved under physiological conditions. We attempted to use the peak plasma concentrations of the chemotherapeutic drugs as cutoffs in our initial experiments, but they all resulted in negative responses *in vitro* (data not shown). On the other hand, since the number of false-positive cases increase when the peak plasma concentrations were used as cutoffs in the histoculture assay with the ^{3}H-TdR incorporation end point (Vescio *et al.,* 1991), the supra-pharmacological cutoff concentrations determined as optimal for the MTT end point in this study would result in a higher false-positive rate with *in vivo* drug responses when applied to the ^{3}H-TdR incorporation end point. Therefore, it appears that optimal cutoff drug concentrations should be determined independently with each assay and with each end-point used, since the cutoff drug concentrations differ depending on the end point, as different end points evaluate different functions or morphological aspects of the tumor cells.

In our previous study of the cell-suspension assay using the same tumor lines and the MTT end point, the cutoff concentra-

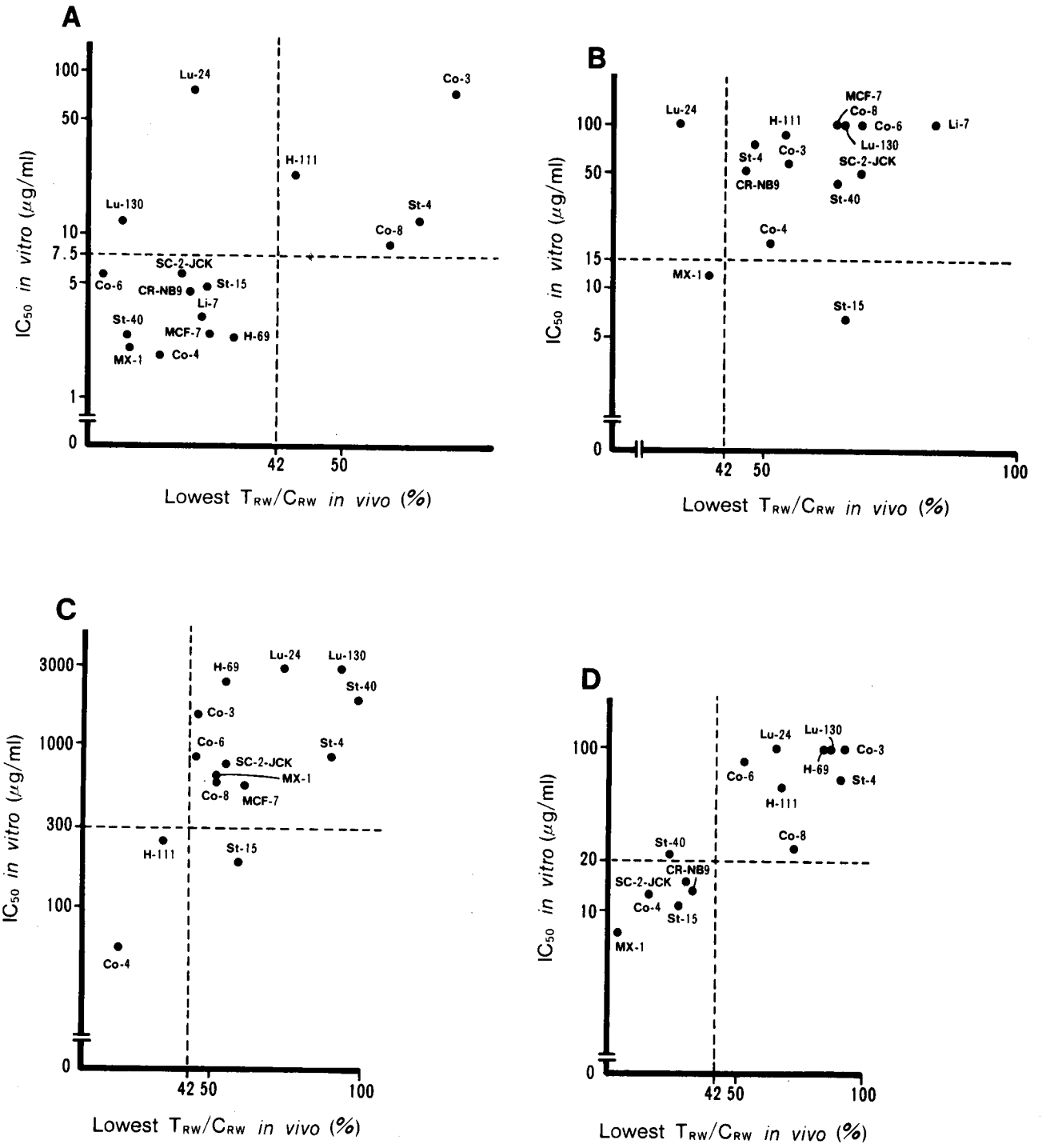

FIGURE 4 – Relationship between *in vivo* chemosensitivity and *in vitro* histoculture chemosensitivity, with the MTT end point for *(a)* MMC, *(b)* DXR, *(c)* 5-FU and *(d)* DDP. Each point represents the lowest Trw/Crw *in vivo* and the mean IC_{50} *in vitro* for each tumor. The perpendicular dotted lines indicate 42% of the lowest Trw/Crw, which is the criterion for an anti-tumor effect *in vivo*. The horizontal dotted lines indicate *(a)* 7.5, *(b)* 15, *(c)* 300 and *(d)* 20 μg/ml as the *in vitro* IC_{50} cutoff concentrations for MMC, DXR, 5-FU and DDP respectively, which are the criteria for an anti-tumor effect *in vitro*.

tions were determined as 10 μg/ml for MMC and DXR, 50 μg/ml for 5-FU and 20 μg/ml for DDP (Shimoyama *et al.*, 1989). The cutoff concentrations for MMC, DXR and DDP are comparable to those of the histoculture assay determined in the present study, whereas the cutoff concentration for 5-FU is 6-fold higher in the histoculture assay. Since the optimal culture periods and drug-exposure times were determined to be 2 days for the cell-suspension assay and 7 days for the histoculture assay, the cutoff concentration × time are estimated to be increased for 4 drugs in the histoculture assay. These results suggest the possibility that intact tissue architecture and the cellular reactions within the intact tissue increase the drug resistance of both sensitive and relatively insensitive tumor lines, as observed previously in a number of tumor types

TABLE IV – SUMMARY OF CORRELATION OF *IN VITRO* AND *IN VIVO* SENSITIVITY TO ANTI-CANCER DRUGS

	MMC	DXR	5-FU	DDP	Total
True-positive[1]	10	1	2	5	18
False-positive[2]	0	1	1	0	2
True-negative[3]	4	12	11	8	35
False-negative[4]	2	1	0	1	4
True-positive rate[5]	10/10	1/2	2/3	5/5	18/20
True-negative rate[6]	4/6	12/13	11/11	8/9	35/39
Sensitivity[7]	10/12	1/2	2/2	5/6	18/22
Specificity[8]	4/4	12/13	11/12	8/8	35/37
Correlation rate[9]	14/16	13/15	13/14	13/14	53/59

[1]True-positive (TP): tumor sensitive both *in vivo* and *in vitro*.–[2]False-positive (FP): tumor resistant *in vivo* and sensitive *in vitro*.–[3]True-negative (TN): tumor resistant both *in vivo* and *in vitro*.–[4]False-negative (FN): tumor sensitive *in vivo* and resistant *in vitro*.–[5]True-positive rate: TP/(TP + FP).–[6]True-negative rate: TN/(TN + FN).–[7]Sensitivity: TP/(TP + FN).–[8]Specificity: TN/(FP + TN).–[9]Correlation rate: (TP + TN)/(TP + FP + TN + FN).

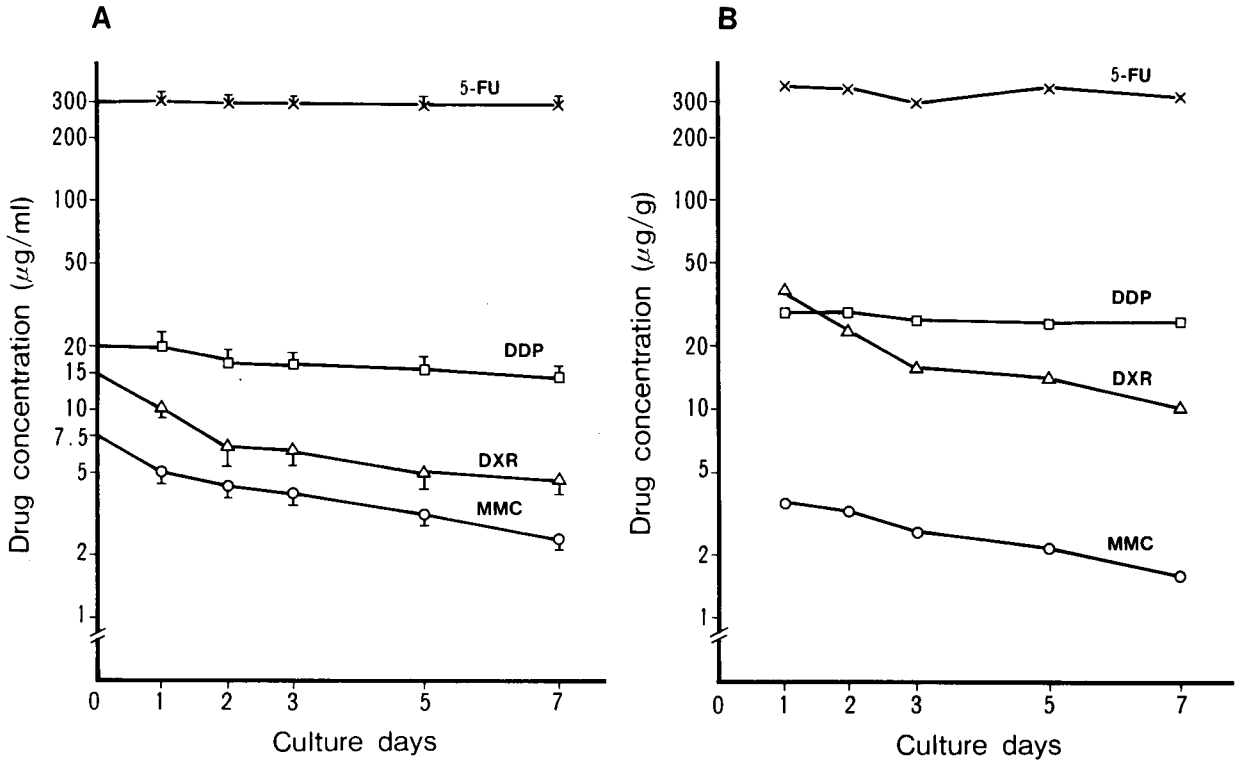

FIGURE 5 – *In vitro* histoculture pharmacokinetics *(a)* in media and *(b)* in tumor pieces. *(a)* The initial concentrations in media are the cutoffs determined in the study: 7.5 μg/ml for MMC, 15 μg/ml for DXR, 300 μg/ml for 5-FU and 20 μg/ml for DDP. Each point represents the mean of results from triplicate determinations measured in the media at the indicated days. Bars, standard deviations. *(b)* Tumor pieces from about 20 collagen-gel surfaces were collected for each determination at the indicated days. Since free DDP could not be separated in tumor pieces, data are shown as total DDP. See "Material and methods" for techniques of drug concentration determinations.

(Sutherland *et al.*, 1979; Hoffman, 1991). Increased drug resistance in the histocultured tumors may also be due to reduced amounts of drugs, nutrients and oxygen in the inner parts of the histocultured tumors (Nederman, 1984).

Although the drug concentration in the tumors (μg/g) might not be directly comparable to those in the media (μg/ml), the drug concentrations measured in histocultured tumor pieces in this study (μg/g) were as high or higher than the drug concentrations measured in the media (μg/ml) (Fig. 5, *a, b*). This result seems consistent with data from other laboratories where it can be estimated that total drug amounts in spheroids, monolayer tumor cell lines and patient tumors (μg/g) apparently surpass the drug concentrations in the media or serum (μg/ml) when compared using these units (Sutherland *et al.*, 1979; Andrews *et al.*, 1988; Mattox *et al.*, 1983). The actual intracellular drug concentrations may be surpassing the extracellular drug concentrations by binding to intracellular constituents (Andrews *et al.*, 1988).

Since statistically significant *in vitro-in vivo* correlations were observed both in C_{max} and AUC of 4 drugs at MTDs and the cutoff criteria (Fig. 6, *a, b*), the high *in vitro-in vivo* correlation of chemoresponsiveness in this study was considered to reflect the high *in vitro-in vivo* correlation of pharmacokinetics, in spite of the suprapharmacological drug concentrations used *in vitro* which resulted in apparent supra-pharmacological drug concentrations in the histocultured tumor pieces. In addition,

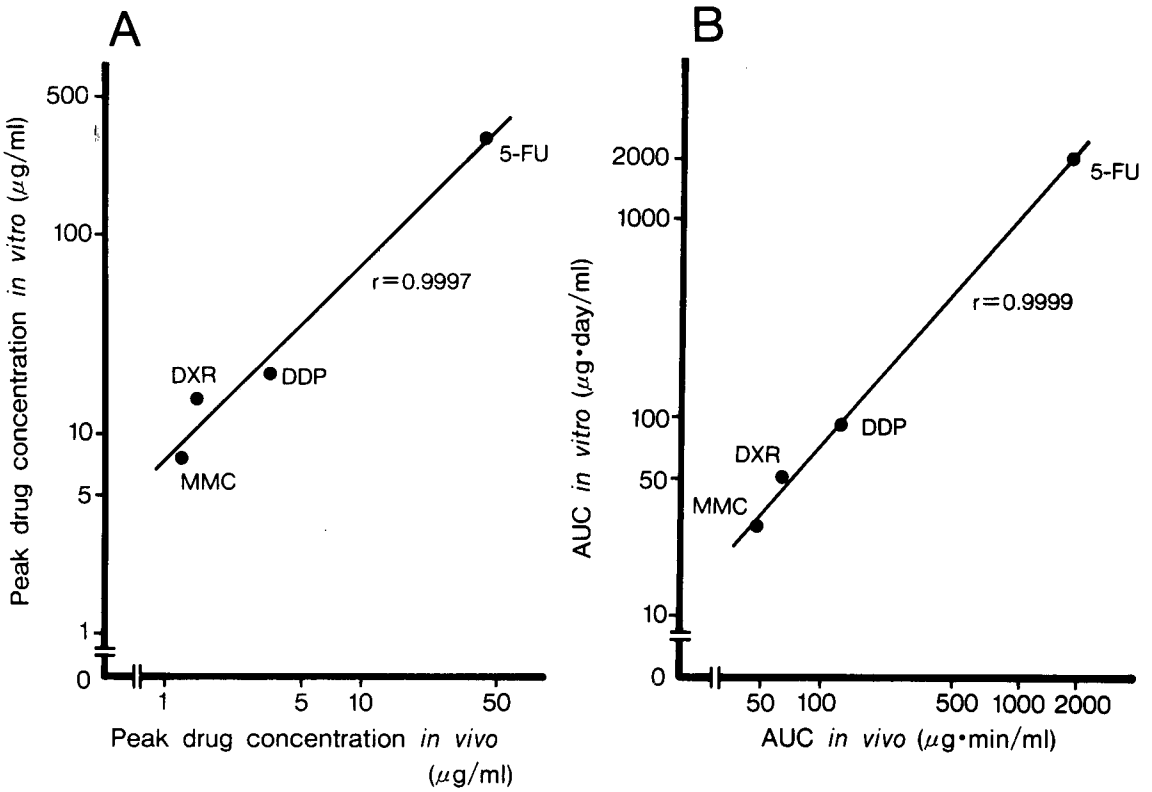

FIGURE 6 – *In vitro-in vivo* correlations of *(a)* peak drug concentrations (C_{maxs}) and *(b)* areas under the curves (AUCs) in mouse sera at the maximum tolerated doses and in media of the histoculture at the cutoff drug concentrations. *In vitro* AUCs were calculated from the drug concentrations measured at intervals for 7 days of the assay period. *In vivo* AUCs were calculated from the drug concentrations measured at intervals for 4 hr after drug administration.

the relatively high cutoff drug concentrations noted in this study with the MTT end point could be due to the fact that succinate dehydrogenase survives even after considerable cell damage (Maehara *et al.*, 1987) that is not registered by other end points which therefore have relatively lower cutoff drug concentrations. Therefore, the supra-pharmacological cutoff drug concentrations seemed to be optimal for the histoculture assay with the MTT end point for the best correlation with *in vivo* chemoresponsiveness at MTDs.

In the St-15 human gastric-cancer line and Lu-24 human small-cell lung-cancer lines, 2 false-positive and 2 false-negative correlations were observed respectively. As the chemosensitivities of these 2 tumors were correctly correlated in the cell-suspension assay with the MTT end point (Shimoyama *et al.*, 1989), the lower correlation rate in the histoculture assay may be due to histological peculiarities of these 2 lines. In future investigations, some factors which make certain tumors unsuitable for chemosensitivity testing in histoculture may be identified, and higher correlation rates may be achieved by testing these tumors in cell-suspension culture.

In conclusion, the Hoffman histoculture assay, with the MTT end point, correlates well with *in vivo* drug response. This safe, simple and convenient chemosensitivity test is therefore expected to be clinically useful.

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