Cancer Chemother Pharmacol (1984) 12: 146–150

Cancer Chemotherapy and Pharmacology

Cytotoxicity of mitomycin C on clonogenic human carcinoma cells is not enhanced by hypoxia

Christian U. Ludwig*, Yei Mei Peng, Jon N. Beaudry, and Sydney E. Salmon

University of Arizona, Department of Internal Medicine and the Cancer Center, Tucson, Arizona 85724, USA

Summary. *The bioreductive alkylating agent mitomycin C (mitomycin) has been shown to have greater activity under hypoxic than oxic conditions on murine cell lines such as the EMT-6 fibrosarcoma cell line. Solid tumors are known to contain hypoxic cells and are relatively resistant to ionizing radiation and some chemotherapeutic agents. We tested the cytotoxicity of mitomycin against fresh biopsies of human carcinomas under both hypoxic and oxic conditions in the human tumor clonogenic assay (HTCA). Additionally, we examined the metabolism of mitomycin by sonicates of the murine EMT-6 cells and the human WiDR colon carcinoma cells. We confirmed that under our clonogenic assay conditions the EMT-6 cell line was more sensitive to mitomycin under hypoxic than oxic conditions. Additionally, we established that EMT-6 cells also metabolize mitomycin at a more rapid rate under hypoxic than oxic conditions. However, these effects of hypoxia on mitomycin activity were not demonstrable for the human WiDR colon cancer cell line. In addition to these findings, the cytotoxicity of mitomycin was either unchanged or reduced under hypoxic conditions for ten fresh human tumors tested for mitomycin sensitivity in HTCA. Based on these observations, we conclude that the potentiating effect of hypoxia on mitomycin metabolism and biological activity may be peculiar to the murine EMT-6 and S-180 cell lines and that mitomycin C is not likely to have differential efficacy against hypoxic human carcinoma cells.*

Introduction

It has been proposed that hypoxic cells exist in solid tumors [19] and that these cells are relatively resistant to the cytotoxic effects of ionizing radiation [4, 5, 10, 13] and some chemotherapeutic agents [17]. Cytotoxic drugs with preferential toxic effects on hypoxic cells could therefore be of significant clinical interest. Lin et al. [15, 16] postulated that bioreductive drugs like mitomycin had such a particular toxicity for hypoxic tumors, and that the decreased oxygen tension in hypoxic tumor cells created conditions conductive to reductive processes hypothesized to be necessary for the activation of mitomycin C. The reduction of the benzoquinone ring of the mitomycin molecule to dihydrobenzoquinones has been proposed as an essential step for the biological activity of this drug [16], and it has been classed as a 'bioreductive alkylating agent'. Using the murine cell lines EMT-6 and S-180, Kennedy et al. have demonstrated that mitomycin is preferentially activated and metabolized by hypoxic murine tumor cells (EMT-6 and S-180) [11, 12].

In this study, we examined the cytotoxic effects of mitomycin on human carcinomas under oxic and hypoxic conditions in the human tumor clonogenic assay (HTCA). We also measured the rate of metabolism of mitomycin by EMT-6 cells and a human colon tumor cell line, WiDR, under both oxic and hypoxic conditions. Although we have been able to confirm the observations previously reported of hypoxia-induced enhancement of mitomycin metabolism and activity against EMT-6 cells in our system, these effects of hypoxia on mitomycin did not appear to pertain to either a human colon cancer cell line or the fresh human tumors we tested in HTCA.

Materials and methods

Fresh human tumor samples. The procedure for collecting, preparing, and culturing single-cell suspensions of human tumor cells has been described elsewhere [7, 8]. Tumor cells were obtained from solid tumor specimens, bone marrow, or ascitic or pleural fluid. An aliquot of the tumor cell suspension was cytologically examined to confirm the malignant histology.

Tumor cell lines. The human colon cancer cell line WiDR was obtained from the American Type Culture Collection, Rockville, Maryland. The human endometrial cell line HEC-1A was kindly provided by Dr J. Fogh, Memorial Sloan-Kettering Cancer Center, New York. The cells were cultured in RPMI 1640 medium with 10% fetal bovine serum (FBS).

The EMT-6 murine cell line was kindly provided by Dr E. Gerner, University of Arizona, Tucson. The cells were cultured in McCoy's medium 5A with 10% FBS. All cell lines were fed three times weekly and harvested with 0.25% Trypsin-EDTA buffer.

Hypoxic condition and drug exposure. Cells were rendered hypoxic by gassing the suspension containing medium and 10%

Offprint requests to: Sydney E. Salmon, M.D., University of Arizona, College of Medicine, Department of Internal Medicine, Section of Hematology/Oncology, Tucson, Arizona 85724, USA

* *Present address:* Christian U. Ludwig, M.D., Clinic for Oncology, Department of Internal Medicine, University Hospital Basel, Switzerland

FBS in completely gas-tight tubes designed for obtaining and maintaining extreme anaerobic culture conditions (Bellco Glass, New Jersey). Two 22-gauge needles were inserted through the rubber stopper, one as a gas inlet and the other as a gas vent. The tubes were flushed for 4 h with a humidified mixture of 5% CO_2 and 95% N_2 (certified, Liquid Air, Inc., Phoenix, Arizona). Control tubes were kept under normal atmospheric conditions. Cell viability examined by trypan blue exclusion did not change under the different incubation conditions. Mitomycin C was obtained as the clinical formulation from Bristol Laboratories (Syracuse, New York). The initial dilution was made in sterile distilled water (1 ng/ml), with subsequent dilutions in sterile saline (0.85%). Individual aliquots to be used one each were stored at −80° C in the dark. Mitomycin has been found to be stable under these storage conditions. Mitomycin at a final concentration of 0.01−0.1 μg/ml (equivalent to 10% and 100% of clinically achievable plasma concentrations) was injected through the rubber stopper at the end of the 4-h gassing period without altering hypoxic conditions, and the tubes were incubated for an additional 1 h at 37° C. To minimize adherence of cells to the glass the tubes were shaken every 30 min. The effectiveness of this system for rendering cells hypoxic was assessed by in vitro radiation survival studies on cell lines. We found that the oxygen-enhancing ratio (OER) was about 3, thereby confirming that the hypoxic conditions we had utilized reduced radiosensitivity to the range normally observed in the hypoxic state [10]. As controls, untreated cells kept under normal atmospheric conditions (in all oxic cultures) or cells gassed with 5% CO_2 and 95% N_2 (in all hypoxic cultures) were incubated along with the drug-treated cultures.

Human tumor clonogenic assay (HTCA). The HTCA has been described in detail elsewhere [7, 8]. Briefly, underlayers of augmented McCoy's medium 5A and 0.5% Difco agar were prepared in 35-mm petri dishes. For fresh human tumors, 5×10^5 cells from the tumor cell suspension were plated in 0.3% agar in enriched CMRL 1066 over a 0.5% agar underlayer. The WiDR and HEC-1A cell lines were plated at a cell concentration of 5×10^4/plate in RPMI 1640 with 10% FBS. The EMT-6 cell line was seeded at a cell concentration of 6×10^4/plate in McCoy's medium 5A with 10% FBS in RPMI 1640. Both the fresh tumors and the cell lines were plated in triplicate in the agar culture system. Dishes were incubated at 37° C in a humidified atmosphere of 7% CO_2. All plates were monitored for aggregation on the morning after plating and discarded if more than twenty aggregates were present. Cultures were subsequently reviewed by inverted microscopy every 3 days. The fresh human tumor samples were counted between days 14 and 17, using a Bausch and Lomb FAS II image analyzer equipped and programmed for tumor colony counting [14]. At the time of counting the control plates were also compared with additional control plated fixed in 3% glutaraldehyde kept at 4° C for the incubation period (to prevent proliferation). The human and murine tumor cell lines plated in HTCA were counted between days 7 and 10. If not otherwise stated, clonal growth units of 60 μm or larger were counted as colonies. The results reported are from tumors that gave rise to at least 30 colonies per control plate. The median number of colonies in HTCA on fresh tumors after plating 5×10^5 cells is 120, and the median number for the cell lines is significantly higher (so that these must be plated at lower concentrations). For example, with EMT-6 cells, plating of 6×10^4 cells gives rise to 400−650 colonies. Data are expressed in terms of the percentage change in survival of tumor colony-forming units from the control. Comparison of the triplicate results of oxic and hypoxic studies was done with the chi-square test.

Studies of mitomycin metabolism. Metabolism studies were carried out with minor modifications of the techniques used by Kennedy et al. [11]. EMT-6 or WiDR cells were resuspended in 0.2 ml phosphate-buffered saline (PBS); 0.2 ml distilled water was added and incubated on ice for 10 min to facilitate disruption of the cells. After addition of 1.8% NaCl solution to achieve isotonicity, the cell suspensions were subjected to three 10-s cycles of sonication at 0° C. The sonicated cell preparations were diluted with 0.05 *M* Tris-0.15 *M* KCl buffer (pH 7.4) to give a final concentration of 20×10^6/ml cells (or about 10 mg protein/ml).

Incubation mixtures for the sonicated cell preparations contained 1.3 moles NADP, 9.8 μmoles glucose-6-phosphate, 10 μmoles $MgCl_2$. 2 U gucose-6-phosphate dehydrogenase in 0.05 *M* Tris-0.15 *M* KCl buffer (pH 7.4), and about 3 mg cell sonicate protein. The total volume of the reaction mixture was 1.0 ml. Hypoxic conditions were achieved by stoppering tubes and pregassing reaction mixtures on ice for 15 min under a continuous flow of pure nitrogen. The incubation tubes were then warmed at 37° C for 2 min, and the reaction was initiated by the addition of mitomycin (5 μg/ml final concentration) under the continuous flow of nitrogen at 37° C. Control tubes were routinely incubated simultaneously under normal aerobic conditions.

The reaction was stopped at time intervals by adding 1 ml isopropanol : chloroform (1 : 1) mixture, and the samples were mixed rapidly for 60−90 s and centrifuged at 13,000 *g* for 3 min. Twenty-five microliters of the resulting upper solvent layer were analyzed for mitomycin content by HPLC.

HPLC analysis was performed with minor modifications of the method described by Den Hartig et al. [3], using two model M45 solvent delivery systems, a model 710B WISP autoinjector, a model 441 UV detector, a model 730 data module, and a model 720 system controller (Waters Associates, Milford, Massachusetts). A Waters Associates μ Bondapak C_{18} reversed-phase column was used for all analyses. The mobile phase consisted of 15% CH_3CN and 85% H_2O at a flow rate of 3.0 ml/min. Mitomycin was detected at 356 nm.

Results

Effects of hypoxia and mitomycin in HTCA

The cytotoxicity of mitomycin at 0.01 and 0.1 μg/ml under oxic and hypoxic conditions was tested against 10 fresh human tumor samples (6 ovarian, 1 thyroid, 1 renal, 1 pancreatic, and 1 breast) in the HTCA. None of these 10 samples showed a statistically significant reduction in survival after mitomycin exposure under hypoxic conditions ($P > 0.05$, chi-square test). In six of the 10 samples the activity of mitomycin was independent of oxygenation status, and in four it was diminished by hypoxia. As the difference in TCFU survival between oxic and hypoxic conditions was always in the same range, at 0.01 and 0.1 μg/ml of mitomycin, only survival data at 0.1 μg/ml are shown (Fig. 1).

In contrast to the results with fresh human tumors, the murine EMT-6 cell line manifested a significant increase in cytotoxicity to mitomycin under hypoxic conditions (Fig. 2). However, the human colon cancer cell line, WiDR, failed to show increased cytotoxicity to mitomycin under hypoxic

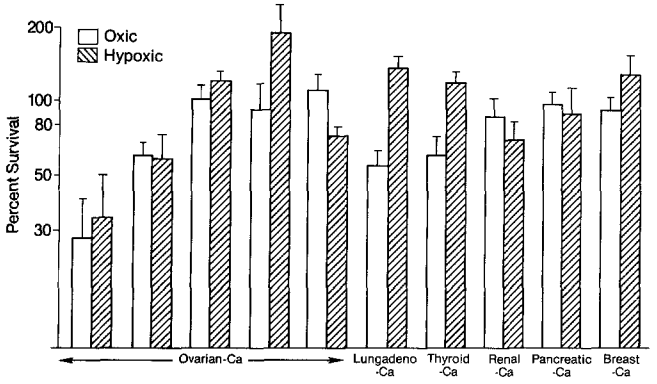

Fig. 1. Effect of mitomycin C (0.1 g/ml) under oxic and hypoxic conditions on clonogenicity of fresh human tumor samples

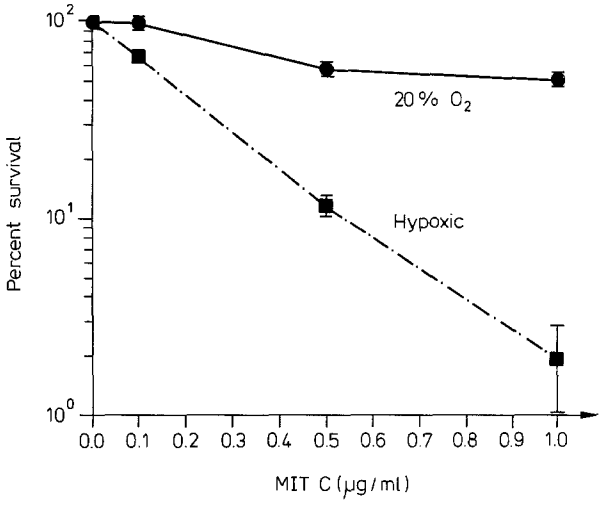

Fig. 2. Effect of mitomycin C under oxic and hypoxic conditions on the clonogenicity of the murine EMT-6 fibrosarcoma cell line. This is a representative example from three experiments and is shown as the mean and standard deviation from triplicate tubes

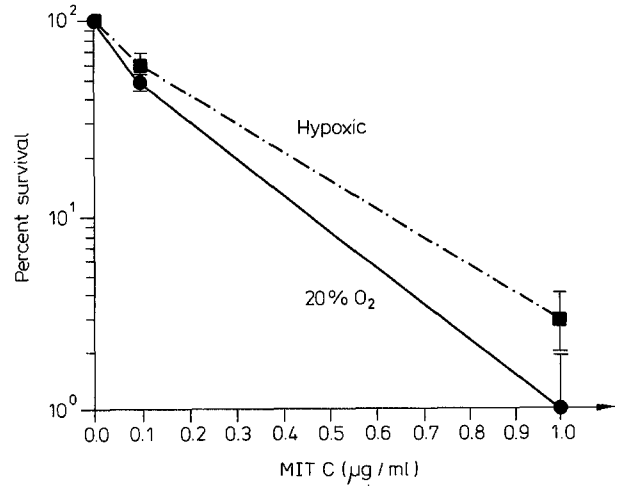

Fig. 3. Effect of mitomycin C under oxic and hypoxic conditions on the clonogenicity of the human WiDR colon cancer cell line. This is a representative example from three experiments and is shown as the mean and standard deviation from triplicate tubes

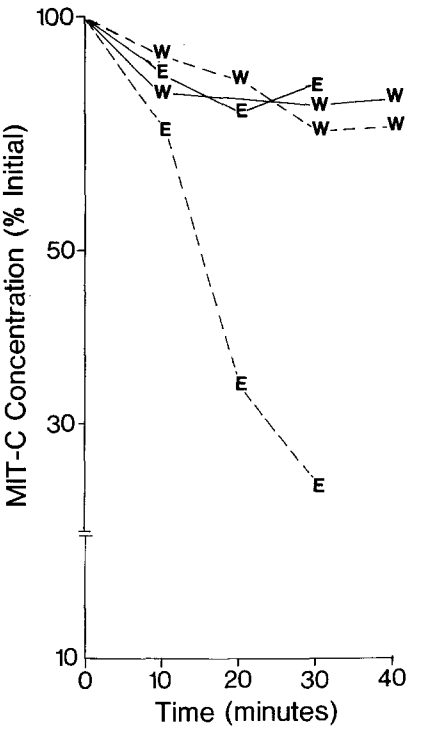

Fig. 4. Metabolism of mitomycin C by sonicates of EMT-6 cells (*E*) and of WiDR cells (*W*) under oxic (———) and hypoxic (- - - -) conditions. Disappearance of the parent compound was measured by HPLC as described in the *Materials and methods* section

conditions (Fig. 3) and was thus similar to the fresh human specimens. A second human tumor cell line, HEC-1A (endometrial), also failed to exhibit a difference in activity of mitomycin under oxic and hypoxic conditions (data not shown).

Effects of hypoxia on mitomycin metabolism

The disappearance of the parent compound, mitomycin, was measured by HPLC as a function of time under oxic and hypoxic conditions, sonicates of either EMT-6 cells or WiDR cells being used as the source for the microsomal enzyme system. Figure 4 depicts the disappearance of the parent compound of mitomycin over time. When sonicates from EMT-6 cells were used the parent compound, mitomycin, disappeared significantly faster under hypoxic than under oxic conditions. Under hypoxic conditions, EMT-6 sonicates metabolized 50% of the parent compound within 15 min, whereas there was virtually no metabolism within 30 min under oxic conditions. When WiDR sonicates were used under the same conditions very little mitomycin metabolism occurred during the first 40 min, and there was no significant difference between oxic and hypoxic conditions (Fig. 4).

Discussion

Hypoxic cells in solid tumors are considered to be relatively insensitive to radiotherapy and chemotherapy [4, 5, 10, 13, 18]. The concept that bioreductive drugs may have greater activity under hypoxic conditions and selectively kill hypoxic tumor cells [15, 16] is therefore very attractive. Based on this concept, it has been proposed that mitomycin at relatively low doses be

included in chemotherapeutic regimens to maximize the differential toxicity of this agent on hypoxic cells [11, 12].

Our data obtained with fresh human tumor samples and human tumor cell lines raise the question as to whether the data obtained with mitomycin under hypoxic conditions with murine sarcoma cell lines are representative or have significance for human cancers. None of 10 fresh human tumor samples showed a statistically significant increase in mitomycin cytotoxicity under hypoxic as opposed to oxic conditions. In six of the 10 samples the activity of mitomycin was independent of the oxygenation state and in four mitomycin activity was actually diminished under hypoxic conditions. Additionally, the human colon tumor line, WiDR, and the human endometrial cell line, HEC-1A, did not manifest increased sensitivity to mitomycin with hypoxia, despite the fact that both lines are quite sensitive to mitomycin. It is unlikely that we missed a relevant difference in the cytotoxicity of mitomycin C under oxic and hypoxic conditions by using 0.01 and 0.1 μg/ml of mitomycin C for the fresh human tumor samples. In the EMT-6 and WiDR cell lines a significant reduction in survival of colony-forming cells was demonstrated with mitomycin C under hypoxic conditions at 0.1 μg/ml. If mitomycin C did act in the same way on fresh human tumor samples as on the EMT-6 or WiDR cell lines, greater cytotoxic effects of mitomycin C under hypoxia would have been seen.

We still cannot exclude the possibility that mitomycin C might have shown more activity against the fresh human tumor samples under hypoxic or oxic conditions at higher concentrations. However, the goal of the present study was to look for potentiating effects at clinically relevant mitomycin concentrations. Our metabolic studies clearly show that very little mitomycin parent compound is metabolized by sonicates of the WiDR cell line during the first 40 min and further document that there is no significant difference in the rate of metabolism of mitomycin by this cell line under oxic and hypoxic conditions. In contrast, sonicates of the EMT-6 cells readily metabolized mitomycin C under the same hypoxic and enzymatic conditions.

One explanation for the discrepancy of our results and the reports of Kennedy et al. [11] is that an enzyme system for the bioreductive activation of mitomycin may be present only in murine tumor cell lines, such as EMT-6 and S-180. On the basis of the parallels we observed between the cloning results and the metabolic studies, it seems likely that most of the human tumors we tested lacked an oxygen-sensitive mitomycin-metabolizing enzyme system. The activity of mitomycin seen in some of our samples and in the WiDR cell line is probably due to other mechanisms. It has been postulated that quinone anticancer drugs such as mitomycin C may act through the formation of peroxides [9]. But cells have extensive and fully developed protective mechanisms against superoxides, which are a normal component of the cellular enzyme system [6]. Bachur has proposed that the cytotoxic quinone anticancer agents are converted intracellularly to site-specific free radicals [1, 2]. If these semiquinone free radical drug intermediates are sufficiently stable, they may enter the nucleus and bind to molecular DNA through intercalation. Lown has also demonstrated that mitomycin C will cross-link DNA *without* reduction activation [17].

We have only tested a limited number of fresh human tumor samples, and several of these tumors showed at least some degree of resistance to the drug. It would be of interest to test additional fresh human tumor samples which exhibit more marked sensitivity to mitomycin. However, the human colon cancer cell line, WiDR, did exhibit marked mitomycin sensitivity but no augmentation of lethality with hypoxia. Metabolic studies on fresh human tumor samples may prove necessary to answer the question about the presence of the enzyme system for the bioactivation of mitomycin in human tumors. There could be a difference in metabolism of mitomycin between human and rodent species. However, thus far our data do not support the concept that mitomycin will be particularly useful for the treatment of hypoxic cells in large human solid tumors.

Acknowledgements. These studies were supported by grants CA-17094, CA-21839, and CA-23074 from the U.S. Public Health Service, Bethesda, Maryland 20205. Christian U. Ludwig was the recipient of a traveling scholarship from the Swiss Cancer League.

References

1. Bachur N, Gordon S, Gee MV (1978) A general mechanism for microsomal activation of quinone anticancer agents to free radicals. Cancer Res 38: 1745–1750
2. Bachur N, Gordon S, Gee MV, Kon H (1979) NADPH cytochrome P-450 reductase activation of quinone anticancer agents to free radicals. Proc Natl Acad Sci USA 76: 954–957
3. Den Hartig J, van Oort WJ, McVie G, Bocher MCYM, Pinedo HM (1981) A sensitive method for analysis of mitomycin-C in clinical pharmacology studies. (Abstract) Proc Am Assoc Cancer Res, p 709
4. Dewey D (1960) Effects of oxygen and nitric oxide on the radiosensitivity of human cells in tissue culture. Nature 186: 780–782
5. Duncan W (1973) Exploitation of the oxygen enhancement ratio in clinical practive. Br Med Bull 29: 33
6. Fridovich I (1972) Superoxide radical and superoxide dismutase. Accounts of Chemical Research 5: 321–326
7. Hamburger AW, Salmon SE (1977) Primary bioassay of human tumor stem cells. Science 197: 461–463
8. Hamburger AW, Salmon SE, Alberts DS (1980) Development of a bioassay for ovarian carcinoma colony-forming cells. In: Salmon SE (ed) Cloning of human tumor stem cells. Alan R. Liss, New York, pp 63–73
9. Handa K, Sato S (1975) Generation of free radicals of quinone group containing anticancer chemicals in NADPH-microsome system as evidenced by initiation of sulfite oxidation. Gan 66: 43–47
10. Hewitt HB, Wilson CW (1961) Survival curves for tumor cells irradiated in vivo. Ann NY Acad Sci 95: 818–827
11. Kennedy KA, Rockwell S, Sartorelli AC (1980a) Preferential activation of mitomycin-C for cytotoxic metabolites by hypoxic tumor cells. Cancer Res 40: 2356–2360
12. Kennedy KA, Teicher BA, Rockwell S, Sartorelli AC (1980b) The hypoxic tumor cell: a target for selective cancer chemotherapy. Biochem Pharmacol 29: 1–8
13. Koch CJ, Kruuv J, Frey HE (1973) Variation in radiation response of mammalian cells as a function of oxygen tension. Radiat Res 53: 33–42
14. Kressner BE, Morton RRA, Martens AE, Salmon SE, Von Hoff DD, Soehnlen B (1980) Use of an image analysis system to count colonies in stem cell assays of human tumors. In: Salmon SE (ed) Cloning of human tumor stem cells. Alan R. Liss, New York, pp 179–193
15. Lin AJ, Pardini RS, Cosby LA, Lillis BJ, Shansky CW, Sartorelli AC (1973) Potential bioreductive alkylating agents. 2. Antitumor effect and biochemical studies of naphthoquinone derivatives. J Med Chem 16: 1268–1271
16. Lin AJ, Cosby LA, Sartorelli AC (1976) Potential bioreductive alkylating agents. In: Sartorelli AC (ed) Cancer chemotherapy. American Chemical Society, Washington, pp 71–86

17. Lown JW (1979) The molecular mechanism of antitumor action of mitomycins. In: Carter SK, Crooke ST (eds) Mitomycin C, current status and new developments. Academic Press, New York, pp 5–26
18. Teicher BA, Lazo JS, Sartorelli AC (1981) Classification on antineoplastic agents by their selective toxicities toward oxygenated and hypoxic tumor cells. Cancer Res 41: 73–81
19. Thomlinson RH, Gray LH (1955) The histological structure of some human lung cancers and the possible implication for radiotherapy. Br J Cancer 9: 539–549

Received June 16, 1983/Accepted November 16, 1983