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A Cell Line with Unusual Characteristics from an Ovarian Carcinoma Patient: Modulation of Sensitivity to Antitumour Drugs

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A cell line (GZL-8) was established by cloning from ascitic fluid of an untreated ovarian carcinoma patient. The cells grew rapidly, accumulated lipids and showed chromosomal alterations. One of the marker chromosomes showed characteristics of a Y-like chromosome. This unusual finding was confirmed by DNA hybridisation using specific probes to the Y chromosome. The cells stained with fluorescent antibodies to desmoplakin and cytokeratins 8, 18, 19, and weakly with vimentin but not with desmin. The presence of epithelial membrane antigen, human milk fat globulin, α-lactalbumin, α-fetoprotein, placental alkaline phosphatase and oestrogen receptor-related antigen was demonstrated by indirect immunoperoxidase staining, but no CA-125 antigen could be detected. The cells showed positive reaction with antibodies to P-glycoprotein. The function of the P-glycoprotein transport system was demonstrated by the rhodamine-123 release test. The cells were initially responsive to doxorubicin, and to high concentrations of cisplatin. Growth inhibition by doxorubicin, especially at low doses was enhanced by the addition of verapamil or tamoxifen. This was shown by the soft agar clonogenic assay, by direct cell counting and by the MTT reducing test. Our results show that combination between drug and sensitivity modulators may be of potential clinical value in ovarian cancer.
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INTRODUCTION

MANY STUDIES on the chemosensitivity of tumour cells *in vitro* were designed to predict the *in vivo* response. For this purpose clonogenic assays, short-term cultures as well as cell lines of neoplastic origin were used [1]. During our study on the sensitivity of ovarian cancer cells to antitumour drugs, a cell line was generated from an untreated patient's ascitic fluid. This line showed features typical for both epithelial and ovarian carcinoma cells, as well as an unusual karyotype including a Y-like chromosome.

The sensitivity of this cell line (which was not previously exposed to antitumour drugs) to doxorubicin and cisplatin was investigated. These drugs are widely used antitumour agents in ovarian cancer therapy. However, drug toxicity, such as the cumulative-dose dependent cardiotoxicity of doxorubicin and the development of multidrug resistance, are the main obstacles to successful chemotherapy [2–5]. Circumvention of drug resistance once it emerged and potentiation of drug activity before development of resistance are of major importance. Several classes of membrane active agents have been shown to enhance drug sensitivity of resistant cells [2, 6, 7]. However, the effect of modulators on drug activity in non-resistant cells and the question of whether they act through the P-glycoprotein transport system responsible for multidrug resistance (MDR) have been less studied and the reports are controversial [2, 6–8]. We report here the effect of modulators on drug activity in a drug-sensitive ovarian cancer cell line with unusual characteristics.

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MATERIALS AND METHODS

Cell culture

A cell line (GZL-8) from ascitic fluid of a previously untreated patient with poorly differentiated adenocarcinoma of the ovary was initiated on January 1988. The cells were pelleted by centrifugation, resuspended in phosphate buffered saline (PBS) and separated on Ficoll–Hypaque density gradient. The cells were washed with PBS and cultured at 37°C in a mixture of DMEM (4.5 mg glucose/ml) and Ham's F12 (1:1) medium supplemented with 10% fetal calf serum and antibiotics (Biological Industries, Israel). After several passages with trypsin-EDTA a monolayer of tumour cells free from fibroblasts was obtained.

A single cell preparation was obtained by trypsinisation and the cells were maintained in suspension culture in the above medium in bacteriological plastic dishes. Floating colonies were formed and single colonies were cloned separately. All the subsequent experiments were performed with one of the clones. Population doubling time was determined from cell counts at daily intervals. Cells (10^4/500 μl) were seeded in 24-well plates, and quadruplicate samples were detached daily with EDTA (1 mmol/l) in PBS and counted in a Coulter counter for 4 consecutive days.

Cell cycle distribution

Cells were detached with trypsin, washed and adjusted to the concentration of 10^6/ml PBS. Staining solution containing 50 μg/ml propidium iodide (Sigma) and Triton X-100 (0.1%) was added to the cells and the sample was filtered through 40 μ nominal pore size nylon gauze. Cytofluorometric analysis was performed with a FACS 440 flow cytometer (Becton Dickinson) with a Consort 40 data analyser.

Karyotype analysis

Tumour cells were grown on glass coverslips and chromosomal analysis was performed using the *in situ* processing method. G-banding [9] C-banding [10] and Q-banding [11] were performed according to routine cytogenetic methods.

DNA analysis for the presence of Y chromosome material

DNA was prepared from cultured cells and from control blood leucocytes of male and female by standard methods [12]. 5 μg DNA of each sample were digested by *Taq* I, separated by electrophoresis of 0.8% agarose gel and transferred to nylon membranes.

Two probes from the short arm of Y chromosome were kindly supplied by D.C. Page: pDP61 (DXYS8) is a genomic 1.0 kb *Eco* RI-*Taq* I fragment cloned into pUC8. On *Taq* I blots it detects an Y-specific fragment of either 2.1 or 2.6 kb and an X-specific fragment of 2.8 kb; pDP34 (DXYSI) is a genomic 2.2 kb fragment cloned into pBR322. On *Taq* I blots it detects a Y-specific fragment of 15 kb and an X-specific fragment of 12 kb [13]. Inserts excised from a low melting point gel were labelled by the random priming method (Multiprime, Amersham). Hybridisation was performed at 42°C and blots were washed with 0.1% SDS/0.1 × SSC at 58°C. Blots were exposed to CURIX RP-2 film for 5 days.

Staining for lipids

Cell cultures were washed with PBS fixed with formol-calcium and stained with oil red O [14] and haematoxylin.

Indirect immunofluorescence

Intermediate filament distribution in the cells was investigated. Cells were grown on glass coverslips. The cultures were washed with PBS, fixed for 10 min with a mixture of 50% ethanol 50% acetone at −20°C, and air dried. Monoclonal antibodies to intermediate filaments and goat antimouse serum fluorescently labeled with Texas red were applied. The monoclonal antibodies to vimentin, desmin and cytokeratin (CK) 18 were obtained from Biomakor. Desmoplakin, CK 8 and CK 19 were from Progen and CK AE1/AE3 was from Biogenex. CK 7 and CK 17 were provided by B. Czernobilsky and M. Fogel.

Immunohistochemical staining

Various tumour-associated antigens were studied with a panel of specific monoclonal or polyclonal primary antibodies by the indirect immunoperoxidase method. Secondary anti-mouse or anti-rabbit antibodies were from the streptavidin–biotin universal kit (Immustain, DPC) and were used according to the manufacturer's instructions. Cell cultures were washed *in situ* with PBS, fixed with ethanol and treated with the primary antibodies followed by the above detection system. Rabbit antibodies to carcinoembryonic antigen (CEA), α-fetoprotein, calcitonin, thyroglobulin lysozyme and mouse antibodies to epithelial membrane antigen (EMA), S-100 protein, prostate specific antigen, glial fibrillary acidic protein (all from DPC) were used. Polyclonal antibodies to human α-lactalbumin (ALA) and human placental alkaline phosphatase (PLAP) were obtained from DAKO. Rabbit antibodies to human milk-fat globulin (HMFG) (Serotek) and monoclonal antibodies to oestrogen receptor-related antigen (ER-D5, Amersham) were also used. The presence of CA-125 antigen was investigated with the Histo-CA kit (Cis, France, kindly donated by Pharmatope, Israel). Multidrug resistance (MDR)-associated P-glycoprotein was detected using the C219 (Centrocor Diagnostics) monoclonal antibodies.

Accumulation of rhodamine-123

The function of the MDR transport system (P-glycoprotein) was investigated using the fluorescent dye rhodamine-123 (Sigma) as molecular probe [8]. Cells were seeded into 50 mm culture dishes at the concentration of 7×10^5 cells/3 ml medium. After 24 h the medium was replaced by rhodamine-123 solution (5 μg/ml medium) with or without verapamil (10 μg/ml) or tamoxifen (10^{-5} mol/l) and the cells were incubated at 37°C for 1 h. The cells were rinsed in PBS and further incubated at 37°C in 3 ml of the corresponding dye-free medium. At different time intervals (0, 1, 2 and 24 h) duplicate plates were removed, rinsed in PBS and scraped in PBS with a rubber policeman. A sample was taken for protein analysis [15]. After centrifugation, the pellet was extracted with 1 ml 1-butanol and the fluorescent cell-associated dye was determined (λ_{ex}=480 nm, λ_{emm}=532 nm). The results are expressed as ng rhodamine-123 per mg cell protein.

Drug sensitivity

Drug sensitivity was investigated by the clonogenic assay and by using monolayer cell cultures. The number of surviving cells was estimated either by direct Coulter counting or by the tetrazolium (MTT) dye reducing assay.

Clonogenic assay. The assay was performed as described previously [16] based on the method of Hamburger and Salmon [17]. 5×10^3 cells/ml were seeded in 0.3% agar in medium containing 10% fetal calf serum and in the presence or absence of different concentrations of doxorubicin (donated by Abic, Israel) (0.05 and 0.1 μg/ml), and either verapamil (3 μg/ml) or tamoxifen (10^{-5} mol/l). The colonies formed were counted after 2 weeks of incubation.

Monolayer cultures. For Coulter counting, cells (10^5/500 μl) were incubated for 3 days in 24-well plates in the presence of different concentrations of doxorubicin applied alone or in combination with verapamil (3 μg/ml) or tamoxifen (10^{-6} mol/l). Cells were detached with EDTA (1 mmol/l in PBS) and counted in a Coulter counter. For MTT testing, cells at the concentration of 1.2×10^4/100 μl were seeded into 96-well plates. After 24 h, fresh medium containing different concentrations of doxorubicin alone or in combination with various amounts of verapamil or tamoxifen was added. Following 3 days of incubation the number of viable cells was determined by the tetrazolium dye (MTT) reducing assay [1]. Briefly, the medium was discarded and 50 μl of 2 mg/ml MTT (Sigma) in HBSS without phenol red was added and the plates were incubated at 37°C for 3 h. The plates were centrifuged, the free dye was aspirated and the formazan crystals were dissolved in 100 μl dimethylsulfoxide. Optical density was read using Dynatech microplate reader MR 600 at 570 nm. The sensitivity of the cells to different concentrations of cisplatin (abiplatin solution, cisplatin 0.5

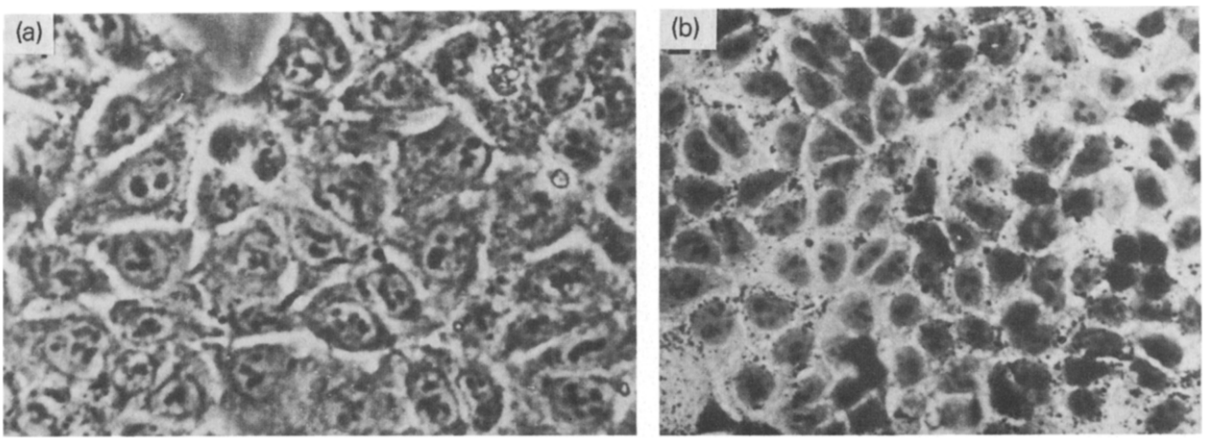

Fig. 1(a) Phase contrast micrograph of GZL-8 cell line derived from ascitic fluid of untreated ovarian carcinoma patient (×200). (b) Photomicrograph of GZL-8 cells stained for lipids by the oil red 0 method (×200).

mg/ml prepared and donated by Abic, Israel) was also investigated by the MTT method. All assays were performed twice in quadruplicates. Statistical significance was calculated by the unpaired *t* test.

RESULTS

Cell line characteristics

Monolayer growth pattern of one clone of the new human cell line GZL-8 isolated from ascitic fluid of an untreated patient with ovarian carcinoma is shown in Fig. 1. The cells are small, have a large nucleus and form a compactly organised monolayer with a population doubling time of 15 h. Cell cycle analysis showed the following distribution: 44.2% of cells were in the G_0/G_1 phase, 41.2% were in S phase and 14.6% in G_2/M phase. The cells also contained lipid droplets that stained with oil red O (Fig. 1b). The profile of alkaline phosphatase activity was previously shown to correspond with the placental Regan I type [18].

Karyotype analysis

An abnormal karyotype of the cells is shown in Fig. 2. The short arm of chromosome 1 is broken and an additional particle can be seen on chromosome 8. One of chromosomes 11, 17 and X is missing and three additional marker chromosomes could be found. One of the markers (M1) showed morphology resembling that of Y chromosome and was the only chromosome stained by C-banding (Fig. 2b) and Q-banding (not shown). Karyotype of

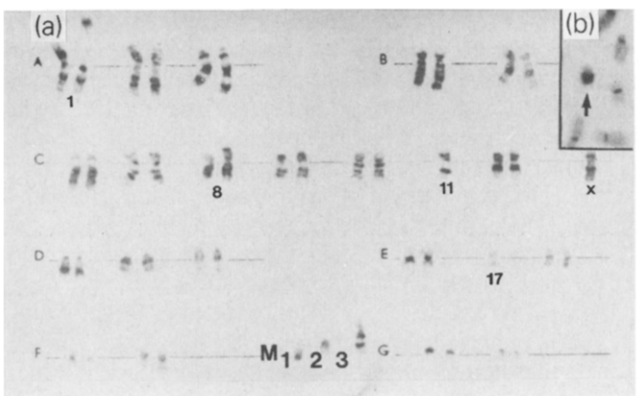

Fig. 2. Karyotype of GZL-8 cells. (a) G-banding, MI–M3 indicate marker chromosomes; (b) partial C-banded karyotype showing M1 marker chromosome staining.

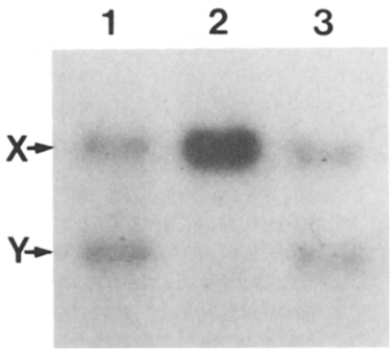

Fig. 3. Hybridisation of DNA from GZL-8 with a probe from the Y chromosome. DNA was prepared from (1) GZL-8 cell line, (2) leucocytes from a woman, (3) leucocytes from a man. Arrows show Y and X-specific fragments.

the cell line was reexamined after more than a year in culture (130 passages) and proved to remain stable. The presence of a Y-like chromosome was further confirmed by hybridisation of the GZL-8 DNA with two probes from the short arm of the Y chromosome: pDP61 (Fig. 3) and pDP34 (not shown) which gave similar results.

Immunohistochemical staining

Intermediate filament distribution was investigated by specific fluorescent antibodies. The cells showed positive staining for cytokeratin AE1/AE3 and desmoplakin, a weak reaction for vimentin and negative for desmin. The presence of individual cytokeratins 8, 18 and 19 is shown in Fig. 4. Cytokeratins 7 and 17 were not detected. The presence of tumour-associated antigens was determined by the indirect immunoperoxidase method. The results are summarised in Table 1. It has to be mentioned that the cells did not show the presence of CA-125 antigen, and with the ER-D5 antibodies the staining was cytoplasmic [19]. Positive staining was obtained for the P-glycoprotein associated with multidrug resistance.

Intracellular rhodamine-123 content

The function of the P-glycoprotein transport system was further investigated using rhodamine-123 as a substrate. Influx of rhodamine as well as dye release were determined in the presence or absence of verapamil and tamoxifen. These modulators are known MDR inhibitors and the results are presented

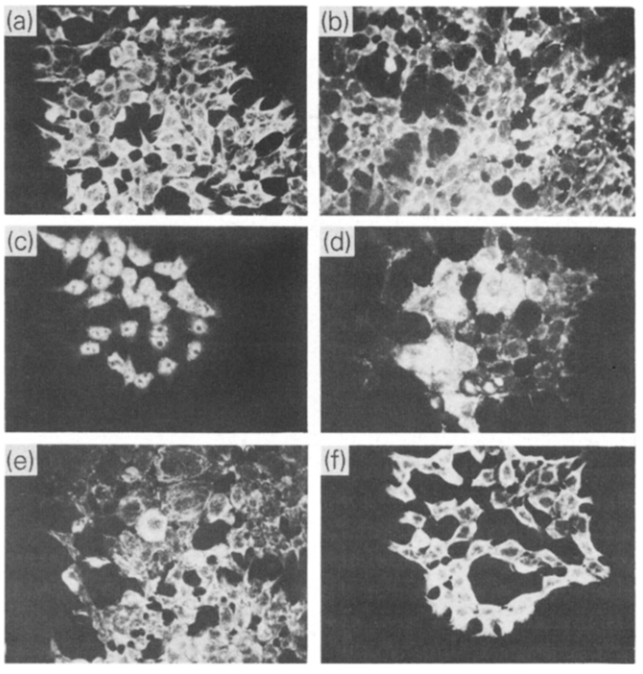

Fig. 4. Immunofluorescent micrographs of cultured GZL-8 cells incubated with antibodies against (a) cytokeratin AEI/AE3, (b) desmoplakin, (c) vimentin, (d) cytokeratin 8, (e) cytokeratin 18, (f) cytokeratin 19 (×200).

Table 1. Immunoperoxidase staining of GZL-8 cells with various antibodies against tumour-associated antigens

Antibodies against	Reaction
CA 125	–
P-glycoprotein	+
Epithelial membrane antigen (EMA)	+
Human milk fat globulin (HMFG)	+
α-lactalbumin (ALA)	+
α-fetoprotein (AFP)	+
Carcinoembryonic antigen (CEA)	–
Glial fibrillary acidic protein (GFAP)	–
S-100 protein	–
Prostate specific antigen (PSA)	–
Thyroglobulin	–
Calcitonin	–
Lysozyme	+
Placental alkaline phosphastase (PLAP)	+
Oestrogen receptor-related antigen (ER-D5)	+

in Table 2. Untreated cells released rhodamine slowly while dye release was significantly retarded by the presence of verapamil and tamoxifen.

Table 2. Intracellular rhodamine-123 retention at various time intervals

	Incubation in dye-free medium			
Additives	0	1h	2h	24h
None	195	189	118	32
Verapamil	207	185	194	166
Tamoxifen	217	172	171	113

ng rhodamine per mg cell protein.
Rhodamine-123 content was estimated following 1h incubation with dye solution and further incubations for 1, 2 and 24h in dye-free medium. The assay was performed in the presence or absence of verapamil or tamoxifen.

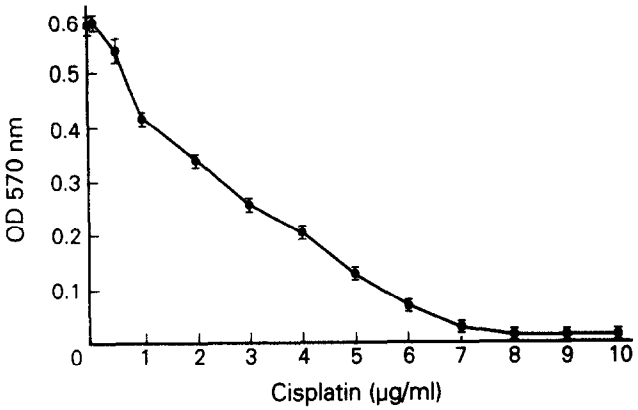

Fig. 5. The effect of cisplatin on cell viability, expressed as OD at 570 nm (MTT test).

Sensitivity of GZL-8 cells to chemotherapeutic drugs

The sensitivity of GZL-8 cells to cisplatin and to doxorubicin in the absence and presence of verapamil and tamoxifen was investigated. The sensitivity of the cells to various concentrations of cisplatin was determined using the MTT method in which the amount of formazan formed was proportional to the number of viable cells that survived drug action. The results in Fig. 5 demonstrate a dose-dependent response of the cells to cisplatin; the cells were sensitive to high concentrations of cisplatin (above 1 μg/ml).

The sensitivity to doxorubicin was investigated with or without the addition of verapamil or tamoxifen. The number of colonies formed following 2 weeks of incubation is shown in Table 3. The cells were sensitive to doxorubicin alone but the addition of verapamil or tamoxifen markedly decreased the number of colonies. Verapamil or tamoxifen alone had only a minor effect.

Sensitivity was also determined at different concentrations of doxorubicin with and without verapamil (3 μg/ml) and tamoxifen (1 μmol/l), using monolayer cell cultures by Coulter counting. Both modulators were applied within the *in vivo* achievable range of concentrations. The cells showed a dose dependent decrease in cell number following 3 days of incubation with doxorubicin. The addition of verapamil enhanced the growth inhibitory effect of doxorubicin which was particularly pronounced at low concentrations of the drug (Fig. 6a). It should be mentioned that the range of 0.01–0.05 μg/ml doxorubicin is achievable *in vivo*. Similar results were obtained when tamoxifen was added instead of verapamil (Fig. 6b). Tamoxifen as well was particularly effective with low doses of doxorubicin. Tamoxifen and verapamil alone did not affect cell number significantly. Response of the cells to different concentrations of doxorubicin alone and to the combinations consisting of doxorubicin with various amounts of verapamil (1.5, 3.0, 4.5 and 6.0 μg/ml) or tamoxifen (0.1, 1, 5 or 10 μmol/l) was investigated by the MTT

Table 3. Colonies formed after 2 weeks in the presence of doxorubicin with or without verapamil or tamoxifen

	Doxorubicin (μg/ml)		
Additives	0	0.05	0.1
None	609	265	154
Verapamil (3μg/ml)	500	96	29
Tamoxifen (10^{-5} mol/l)	581	160	29

No. of colonies.

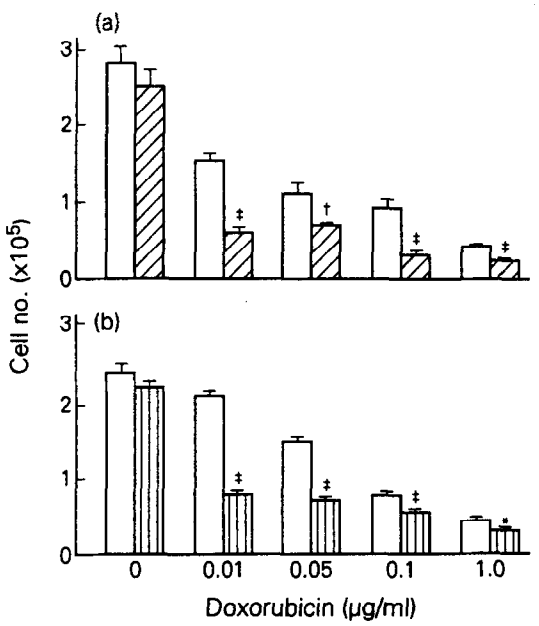

Fig. 6. The effect of various concentrations of doxorubicin without or with 3μg/ml verapamil (a)(▨) or 1 μmol/l tamoxifen (b)(□) on GZL-8 cell number following 3 days incubation. Vertical lines represent S.E. of eight replicates done in two different experiments. *P* values were calculated for cytotoxic drug + verapamil or tamoxifen versus cytotoxic drug alone. **P*=0.05, †*P*=0.01, ‡*P*=0.001.

methods. As seen in Fig. 7, mainly the combination between either 0.05 or 0.1 μg/ml doxorubicin with all the range of verapmil concentrations tested resulted in decreased cell viability. Parallel results were also obtained with the different concentrations of tamoxifen. The highest significance ($P<0.001$) was found with 100 mmol/l tamoxifen.

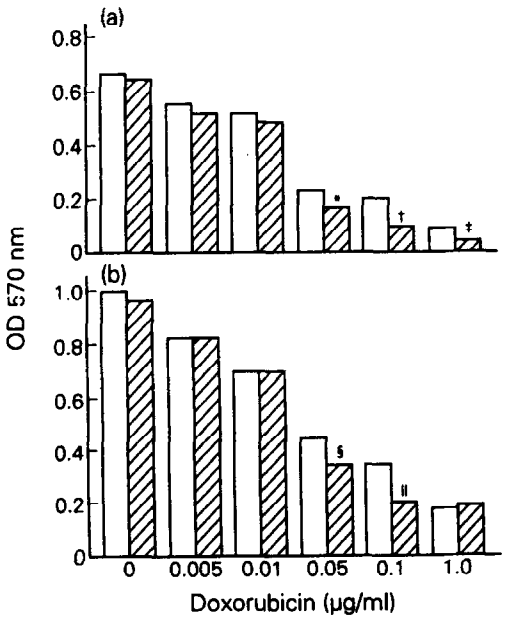

Fig. 7. The effect of doxorubicin □ or the combination of doxorubicin with 3 μg/ml verapamil (a) or with 5 μmol/l tamoxifen (b) □ on cell viability (MTT test) expressed as OD at 570 nm. *P* values for doxorubicin plus verapamil or tamoxifen versus doxorubicin alone: **P*=0.004, †*P*=0.001, ‡*P*=0.002, §*P*=0.05, ‖*P*=0.03.

DISCUSSION

Cell line GZL-8 was established from ascitic fluid of a patient with poorly differentiated ovarian carcinoma prior to the initiation of chemotherapy. The cells grew fast in monolayers with no fibroblastic contamination. The cells also formed colonies in suspension culture and in soft agar, suggesting the tumorigenic features of the cells. The epithelial character of the cells was confirmed by a positive immunofluorescent reaction with antibodies against CKs, especially 8, 18 and 19, against desmoplakin and a limited reaction with vimentin but not with desmin. This pattern of intermediate filaments was found in simple epithelia [20] and the absence of CK 7 was observed in some adenocarcinomas of the ovary [21]. Coexpression of CK and vimentin was found in human carcinoma cells derived from ascitic fluid [21].

Positive reactions with various antibodies directed against tumour associated antigens further support the epithelial origin of the cells. The cells showed a positive immunoperoxidase reaction with anti-EMA, which detects poorly differentiated neoplasms of epithelial origin [22]. The cells also reacted with a panel of antibodies against markers often associated with ovarian neoplasia such as PLAP [23, 24], HMFG [23] oestrogen receptor [21, 25] and α-lactalbumin [26]. However, the cells were negative for CA-125 and CEA. A relatively low rate of CEA positivity was reported in poorly differentiated or undifferentiated tumours [22]. In addition CEA was found mainly in mucinous rather than in serous tumours of the ovary [21]. The presence of intracellular lipid droplets was also associated with ovarian carcinoma cells [21, 23].

Karyotype analysis showed aneuploidy with loss of chromosomes and alterations in others, changes which remained stable over a year in culture. Numerical and structural abnormalities in karyotypes of ovarian carcinomas were frequently observed [27]. Of special interest is the appearance of a marker chromosome with morphology, C-banding and Q-banding patterns resembling that of Y chromosome. The presence of Y-like chromosome material was further verified by hybridisation of the DNA of GZL-8 cells with specific probes. The presence of Y-like chromosome material in ovarian cells derived from a fertile female is an unusual finding. The possibility of contamination and overgrowth by another established cell line present temporarily in our laboratory was considered. However, this seemed unlikely since all the cell lines in question were known to be of female origin. It has to be mentioned that a high incidence of mosaicism for Y chromosome has been found in tumours from gonadal dysgenesis patients. It was suggested that a certain gene on the Y chromosome was a contributory factor to tumour formation [28, 29]. The cells exhibited alkaline phosphatase activity with characteristics of placental Regan I type [18]. Special reference to the presence of placental type isoenzyme has been suggested for ovarian cacinoma [23, 24].

Taken together it appears that GZL-8 cells might represent a poorly differentiated epithelial cell line with an unusual Y-like chromosome and which demonstrated some features suggestive for ovarian tumour cell origin.

Further investigation showed that the cells were initially sensitive to doxorubicin, and to high doses of cisplatin. Combined treatment with doxorubicin and verapamil or tamoxifen increased markedly the growth-inhibiting effect of doxorubicin, whereas tamoxifen or verapamil alone had no or minor effect. Growth inhibition could be achieved at significantly lower concentrations of doxorubicin when verapamil or tamoxifen were added. This effect was shown by the soft-agar clonogenic

assay and using the monolayer cell cultures. Verapamil, tamoxifen and other modulators were shown to reverse multidrug resistance [2, 6, 7]. However, where drug-sensitive cells are concerned the reports on the contribution of these agents to chemotherapeutics are contradictory [2, 6–8]. MDR was often accompanied by decreased drug accumulation as well as by overexpression of P-glycoprotein [3–5]. The modulators were suggested to reverse resistance by interfering with the transport system, resulting in enhanced drug retention [4]. Using specific antibodies we showed that the non-resistant GZL-8 cells possessed P-glycoprotein antigen. Rhodamine-123 release testing further confirmed that the MDR-associated transport system was operating in the cells and could be inhibited by verapamil and tamoxifen.

Combinations of doxorubicin and verapamil [2] or tamoxifen [30, 31] have been assayed in clinical trials. The present studies suggest that the addition of modulators to chemotherapeutics might also be worthwhile where initially non-resistant tumour cells are concerned.

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