Total cellular ribose nucleic acid analysis of multi-drug resistant cancer cells

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ABSTRACT

Objective: The present study is designed to elucidate the correlation between gene dosage and increased messenger ribose nucleic acid (RNA) level in multi-drug resistant cancer cells.

Methods: The human lymphoblasts CCRF-CEM (CEM) and CEM-vinblastine (VBL) 10, CEM-VBL 20, CEM-VBL 40, CEM-VBL 60, cell line CEM-VBL 80, and CEM-VBL 100 were derived from CEM VBL 10 by single step selection technique. For the analysis of total RNA and deoxyribonucleic acid (DNA), both Northern-Southern blot analysis were carried out on all sensitive and resistant cell lines. Part of the study was conducted at the Immunology Laboratory, Higher Institute of Health, Rome and the other part was conducted at the Middle Euphrates Center for Cancer Researches, Kufa University, Iraq, between 1990 and 2000.

Results: Total cellular RNA was analyzed to quantitate the levels of expression of multi-drug resistant-one gene with extent of its amplification in CEM-sensitive and resistant cell lines. Only the CEM-VBL resistant over–expresion the multi-drug resistant-one gene but in CEM-sensitive cells do not. The over-expresses appears to correlate proportionally with the level of drug resistance. No such correlation has been detected with regard to the genomic DNA.

Conclusion: It appears that the first step in the transition from drug sensitive to drug resistant is increasing levels of messenger RNA followed by gene amplification. Furthermore, once the gene is amplified, it remains at the same level of amplification regardless of the concentration of drug in which given cells have been selected.

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One of the most serious clinical obstacles which prevents successful cancer chemotherapy is the development of drug resistance by tumor cells. Ultimately, this resistance often displays a broad cross-resistance to a variety of other dissimilar drugs (for example Vinca alkaloids, anthracyclines, mitoxantrone, etoposide VP 16 and actinomycine-D) to which the cells have not been previously exposed. Such problems will lead undoubtedly to the failure of chemotherapy and patients subsequently relapse and eventually die. The phenomenon of cross-resistance has been referred to as multi-drug resistance (MDR). The human MDR gene family includes 2 members, MDR1 and MDR2. Nevertheless, it has been shown that increased expression of MDR1 was paralleled by a small increase in the MDR2 messenger ribose nucleic acid (mRNA) levels.[1,2] However, it is well established that both genes encode related proteins.[3] It is believed that MDR is due to the amplification of the MDR1 gene (when genes are amplified, more copies are produce in the cell nucleus), which encodes for p-glycoprotein.[4-6] This transmembranal glycoprotein has a molecular mass of approximately

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170,000 dalton protein which acts as an energy-dependent drug efflux system,[7,8] thus, they accumulate less drug than parental sensitive cells.[9,10] During deoxyribonucleic acid (DNA) amplification in vitro, rapid loss of gene copies in the absence of selective pressure is usual when the amplified DNA is located on an extra chromosome.[11] Little loss has been observed even after culture for more than a year without selective pressure, although it has been reported that chromosomal amplified genes can be lost rapidly from first and 2nd step mutants in the absence of the drugs. Despite a considerable volume of work which has been carried out to elucidate the nature of MDR little is known about its molecular mechanism in contrast to the clinical situations. In the present study, the correlation between mRNA level and gene dosage in multidrug resistant cancer cells was investigated.

Methods. ***Cell lines***. The human lymphoblasts CCRF-CEM (CEM) and CEM-vinblastine (VBL) 10 cell lines were kindly provided by W.T. Bcek (Memphis Tennessee, United States of America). CEM-VBL 40, CEM-VBL 60 and CEM-VBL 80 MDR cell lines were derived from CEM-VBL 10 by a single step selection technique. The number following the abbreviation of VBL indicates the selective drug concentration used (ng/ml) to isolate MDR cell variants as well as the drug concentration of the medium in which selected cell lines were routinely cultured.

Culture medium. The basic medium used for cell culture was a mixture of RPMI–1640 and Iscoves DMEM (4:1) supplemented with 10 mM L-Glutamine and 10% fetal calf serum (FCS). All lines herein described were incubated at 37ºC in a humid incubator in the presence of 5% CO_2 in air, using the standard conditions for cells growing in suspension.

Cytotoxic drugs. The following cytotoxic drugs were used: Vinblastine Sulfate (VBL, Lilly France, SA), Actinomycin D (ACT-D Merk Sharp and Dohm), Adriamycin (ADR, Farmitalia), Colchcine (Sigma). All the drugs used throughout were identical to those commercially available for clinical use .

Northern blot analysis. Total RNA was extracted from $5x10^7$ cells from both sensitive and MDR variants using the guanidinium isothiocyanate method.[12] Twenty µg of each preparation were loaded on a one percent agrose formaldehyde gel and transferred to a nylon membrane (hybond-N-one, Amersham, Aylesbury, United Kingdom) according to standard procedure. The MDR1 5A probe[13,14] was labeled at high specific activity with a random primer[15] A 2.2 kb B-actin probe was used to control the amount of RNA present on the filter.

Southern blot analysis. The DNA was digested to completion with *Escherichia coli* RY13 (*Eco*R1), separated on a 0.8% agarose gel and transferred to nitrocellulose by the method of Southern. Hybridization was performed at 42ºC in 40% formamide – 4 x SSC (1 x SSC is 0.15M NaC1 plus 15mM sodium citrate pH 7.0), 0.8 Denhardt solution (1X Denhardt solution is 0.02% BSA, 0.02% polyvinylpyrrolidone, and 0.02% Ficoll), 7mM Tris (PH 7.5), 20mg/m1 Salmon sperm DNA, 10% Dextran sulfate (Pharmacia) and $2x10^6$ cpm per ml of the MDR1 probe.[13,14] The washes were carried out in 0.1x SSC and 0.1% SDS at 50ºC. Washed filters were exposed to X-omat AR5 film (Eastman Kodak Co. Rochester, New York) at 70ºC with intensifying screen.

Growth assay to determine drug resistance. Parental cells were plated out in a 24-wells (Costar) plate at $5x10^4$ cell density in presence of various concentration of the drugs used. Within its inhibitory range, the drugs decreased growth of all cell lines in proportion to drug concentration. For all cell lines tested, a 3-day growth curve was determined. The relative growth was calculated using the expression: (En-Eo)/(chloroacetophenone [Cn] - cobalt [Co]) where Eo and En are the initial and the final cell concentration (24, 48, 72 hours) in the drugs containing culture. Cobalt and chloroacetophenone are the corresponding cell concentrations in untreated control culture. The inhibition concentration of a drug which lead to 50% cell death (IC-50) for all cell lines was determined from the plots of their growth for 48 hours versus drug concentration. The degree of the resistance of drug-resistant cell variants was determined by dividing their IC-50 by that of the parental line with the drug used in this study.

Results. To test for the cross-resistance of VBL-resistant mutants to various unrelated drugs, the drug concentration required for 50% inhibition of growth after 48 hours was compared for the mutants and for the parental cell lines. All the VBL mutants which have been selected exhibited cross-resistance to all the Cytotoxic drugs tested (Adriamycin, Actinomycin-D, Colchicine). The cells which show more cross resistant were the cells which were isolated at higher VBL concentration. However, all mutants which were tested for cross-resistance showed a variation in the degree of cross-resistance for the dissimilar drugs assayed, though they share the common character of being cross-resistance mutants. To exclude the possibility of mutant lines altering while in culture, all our mutants were thawed from frozen stocks a few days before conducting any experiment. Ribose nucleic acid and DNA analysis of CEM cell lines and their MDR variants. Total cellular RNA was analyzed to quantitate the level of expression of MDR1 gene with the extent of its amplification in CEM-sensitive and resistant cell lines. The data in **Figure 1** shows an autoradiograph of a northern blot of total RNA extracted from CEM (Lane l) CEM-VBL 10, CEM-VBL 20, CEM-VBL

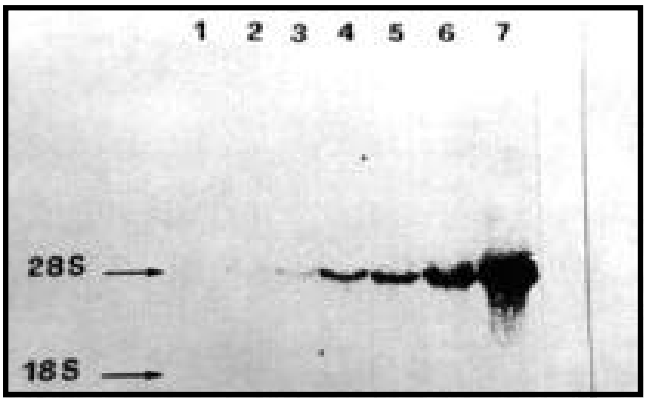

Figure 1 - Northern blot hybridization of total RNA from CEM cell lines and their drug variants (the size of MDR1 band is 4.7 kb). RNA - ribose nucleic acid, MDR - multi-drug resistant.

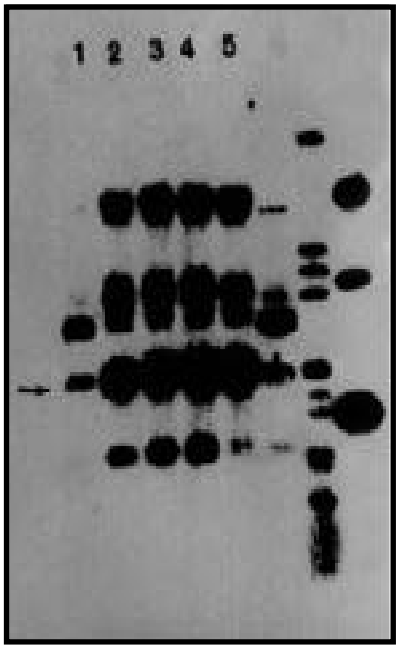

Figure 2 - Southern blot hybridization of *Eco*R1- digested genomic DNA from CEM cell lines and their resistant variants. *Eco*R1 - *Escherichia coli* RY13, DNA - deoxyribose nucleic acid.

40, CEM-VBL 60, CEM-VBL 80 and CEM-VBL 100 (Lanes 2-7). Cells hybridize with a probe for MDR1 gene (MDR 5A). It is clear from **Figure 1** that only the CEM-VBL resistant cells over-express the MDR1 gene. CEM – VBL sensitive cells do not. **Figure 2** shows an autoradiogragh of a southern blot of genomic DNA isolated from CEM-sensitive and their resistant variants as described in the materials and methods section, subjected to digestion with EcoR1 and hybridize to the MDR1 probe (MDR1 5A). **Figure 1** shows that there is no any sign of increase in the genomic sequence in the sensitive CEM cells (Lane1), while an obvious increase could be noticed in the CEM-VBL 10, CEM-VBL 20, CEM-VBL 40, VEM-VBL 60, CEM-VBL 80 (Lane 2, 3, 4, 5).

Discussion. It is clear from **Figure 1** that there is an obvious increase in the expression of MDR1 gene. The over-expression of MDR1 gene appears to correlate proportionally with the levels of drug resistance in multi-drug resistant cell lines. A good parallel between the extent of multi-drug resistance and levels of MDR1 mRNA have been reported previously.[1] The southern blot of genomic DNA has been conducted to compare the increasing levels of MDR1 mRNA with the corresponding genomic sequence. The result indicates that although there was an obvious parallel increase between the levels of MDR1 mRNA and the extent of multi-drug resistance for every step of selection, no such correlation has been detected with regard to the genomic DNA). Obviously the amplification of MDR1 had occurred in the CEM-VBL10 and the other higher resistant variants, but there is no apparent variation between them or parallel increase to correspond with the extent of drug resistance as has been noticed in MDR1 mRNA analysis. It appears that once the gene is amplified, it remains at the level of amplification regardless of the concentration of the drug in which given cells have been selected (namely the gradual increase in levels of drug resistant do not alter the level of amplification in the genomic DNA). This indicates that for some unknown reason the process of transcription is not related to gene dosage in a direct manner in MDR system. It appears that the first step in the transition from a drug-sensitive to drug-resistant cells is increasing level of mRNA followed by gene amplification. It has been reported that the increase in the expression of MDR1 in at least some cases occurs initially without gene amplification.[1,6] It is possible that the mechanism which leads to amplification starts either from blockages in the replication or from some problem connected with the transcription process. The results do not distinguish between the 2 possibilities.

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