Gene Amplification–associated Cytogenetic Aberrations and Protein Changes in Vincristine-resistant Chinese Hamster, Mouse, and Human Cells

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ABSTRACT We carried out cytogenetic studies of four Chinese hamster, mouse, and human cell lines selected for high levels of resistance (500- to 4,000-fold) to vincristine (VCR) by a multistep selection procedure. All cells examined contained gene amplification–associated metaphase chromosome abnormalities, either homogeneously staining regions (HSRs), abnormally banding regions (ABRs), or double-minute chromosomes (DMs); control actinomycin D– and daunorubicin-resistant hamster lines did not exhibit this type of chromosomal abnormality. VCR-resistant Chinese hamster sublines exhibited both increased synthesis of the protein V19 (M_r 19,000; pI = 5.7) and increased concentrations of V19 polysomal mRNA. When VCR-resistant cells were grown in drug-free medium, level of resistance, synthesis of V19, and amount of V19 mRNA declined in parallel with mean length of the HSR or mean number of DMs per cell. Cross-resistance studies indicate that VCR-resistant cells have increased resistance both to antimitotic agents and to a wide variety of agents unrelated to VCR in chemical structure and/or mechanism of action. Our studies of tubulin synthesis in Chinese hamster cells indicate no overproduction of tubulin or presence of a mutant tubulin species. Comparison with antifolate-resistant Chinese hamster cells known to contain amplified dihydrofolate reductase genes localized to HSRs or ABRs strongly suggests that the HSRs, ABRs, or DMs of the *Vinca* alkaloid-resistant sublines likewise represent cytological manifestations of specifically amplified genes, possibly encoding V19, involved in development of resistance to VCR.

Recognition of gene amplification as a mechanism whereby mammalian cells may develop resistance to cytotoxic agents began with the discovery of a novel chromosome abnormality, the homogeneously staining region (HSR)[1] (1). Appearing in trypsin-Giemsa banded cells as long chromosomal segments that stained with relative uniformity and intermediate intensity, HSRs were observed in Chinese hamster lung (CHL) cell lines with high levels of acquired resistance to antifolates and high levels of target enzyme dihydrofolate reductase (DHFR) (1, 2). It has been demonstrated that increased activity of DHFR in resistant sublines derived in cell culture by progressive increases in selective drug concentration is due to an increased rate of biosynthesis of the enzyme (3–5), accompanied by increase in cellular concentration of DHFR-specific mRNA (6–8). The basis of these overproduction phenomena was found to be selective amplification of DHFR-encoding genes (9, 10). Moreover, in antifolate-resistant, HSR-containing hamster and mouse cells, HSRs are sites of amplified DHFR genes (11–14).

Another distinctive chromosomal abnormality indicative of DNA sequence amplification is the double-minute chromosome (DM) (15–17). DMs are small, paired extrachromo-

[1] *Abbreviations used in this paper* ABR, abnormally banding region; CHL, Chinese hamster lung; DHFR, dihydrofolate reductase; DM, double-minute chromosome; ED_{50}, half-maximal effective dose; HSR, homogeneously staining region; 2D, two-dimensional; VCR, vincristine.

THE JOURNAL OF CELL BIOLOGY · VOLUME 100 FEBRUARY 1985 588-597

somal chromatin bodies that appear to lack a centromere. Variable in both number and size, DMs have been found in various methotrexate-resistant mouse (15, 18, 19) and human (20, 21) cell lines. Specific hybridization of cloned segments of the DHFR gene to DM-enriched DNA and quantitative correlations between DHFR level and number of DMs per cell (15, 16), as well as hybridization in situ of C_0t-purified amplified DNA to DMs of human neuroblastoma cells (17), have provided evidence that DMs carry amplified DNA sequences.

We report here that four Chinese hamster, mouse, and human cell lines with high levels of acquired resistance to vincristine (VCR) contain gene amplification–associated chromosomal abnormalities and that these structures are lost in revertants in parallel with loss of resistance. CHL cells selected for resistance to actinomycin D or daunorubicin do not contain these types of abnormalities. Cells of a VCR-resistant CHL line, DC-3F/VCRd, contain HSRs (22); cells of a second VCR-resistant CHL line described in this report, DC-3F/VCRm, contain abnormally banding regions (ABRs). ABRs have been shown to encompass low numbers of amplified genes in antifolate-resistant cells derived from DC-3F (23). Mouse tumor and human cells selected with the *Vinca* alkaloid contain DMs (19, and this report). The presence of HSRs, ABRs, or DMs in the resistant cells is suggestive of the possibility that at least one mechanism of resistance to VCR involves gene amplification.

A possible product of amplified genes is the previously described protein V19 (M_r 19,000; pI = 5.7) (24). V19 is overproduced in VCR-resistant CHL and mouse tumor cells. Increased synthesis of V19-specific mRNA and loss of the peptide during phenotypic reversion, as described in this report, further associate V19 and the drug-resistant phenotype in these cells. In contrast, we have observed no relationship between resistance, HSRs, or DMs, and overproduction of tubulin, to which VCR binds (25, 26). Cabral et al. (27, 28) Connolly et al. (29), and Keates et al. (30) have found mutant tubulin or altered microtubules in cells resistant to other antimitotics. VCR-resistant CHL cells, however, do not appear to synthesize a mutant tubulin species.

MATERIALS AND METHODS

Derivation of Cell Lines and Clones: Cell lines DC-3F/VCRd and DC-3F/VCRm are independently derived VCR-resistant sublines of the CHL cell line, DC-3F (31). Both were selected by exposure to stepwise (2–2.5-fold) increases in VCR concentration and are maintained in 10 μg/ml drug. Clonal sublines DC-3F/VCRd-1 and DC-3F/VCRd-5 were isolated from DC-3F/VCRd cells inoculated into Falcon Microtest II plates in 5 μg/ml VCR. After initial characterizations, both clones were transferred to drug-free medium, designated DC-3F/VCRd-1-U and DC-3F/VCRd-5-U, and grown in absence of drug for 2½ yr. Clone DC-3F/VCRd-5 was also further selected with higher concentrations of VCR; the resulting, highly resistant subline designated DC-3F/VCRd-5L grows in 50 μg/ml VCR. The actinomycin D– and daunorubicin-resistant CHL sublines DC-3F/AD X (2,450-fold resistant) and DC-3F/DM XX (883-fold resistant) have been described (31, 32). DC-3F/AD XC (50,000-fold resistant) was selected from DC-3F/AD X cells by multistep increases in concentration of actinomycin D from 10 to 100 μg/ml. The drug-sensitive mouse tumor line, MAZ, was clonally isolated from cells established in vitro from a hydrocarbon-induced tumor of a C57BL/6 mouse (22). Selection with VCR yielded the resistant cell line, MAZ/VCR, now maintained at 20 μg/ml drug. The VCR-resistant human neuroblastoma cell line SH-SY5Y/VCR was selected from SH-SY5Y, a clonal subline of the neuroblastoma line SK-N-SH (33, 34). Revertant sublines MAZ/VCR-U and SH-SY5Y/VCR-U were initiated from drug-treated sublines without prior cloning and grown in drug-free medium for 14 and 8 mo, respectively. VCR-resistant cell lines are listed in Table I.

Cell Culture Techniques: Chinese hamster and mouse cells are cultured either in Eagle's minimum essential medium or in a 1:1 mixture of that medium and Ham's F12 medium, supplemented with 5 or 10% fetal bovine serum, 100 IU/ml penicillin, and 100 μg/ml streptomycin. Drug-resistant sublines are routinely maintained in presence of drug but are grown in drug-free medium for 10–15 d (CHL cells) or 2–4 d (MAZ cells) before experimentation. Procedures for determination of drug sensitivity have been described (31). To obtain ED_{50} (half-maximal effective dose) values, we carried out two or three independent dose-response assays for each cell line. Modifications of experimental conditions for the more slowly growing human neuroblastoma cells include increase in serum concentration to 15% and a longer period of growth (6 d) in dose-response assays. VCR was generously provided by Eli Lilly and Co.

Chromosomal Analyses: Cells for karyotype studies were prepared and stained by standard procedures (35) modified from the method of Seabright (36), with or without pretreatment with 0.001–5 μg/ml Colcemid. Preparations were stained both by trypsin-Giemsa techniques for chromosome identification and by conventional methods to better assess DM size and number. HSR length was determined from photographs of 15–20 metaphase plates per sample, as described earlier (35). Chinese hamster chromosome nomenclature has been modified from earlier, published analyses (22) as suggested by Ray and Mohandas (37). Human chromosome nomenclature follows the conventions of ISCN (1978): An International System for Human Cytogenetic Nomenclature (1978) (38).

Chromosomal DNA replication patterns were determined as previously described (1, 2).

Protein Analyses: Cells were grown in methionine-deficient growth medium containing 10 μCi/ml [^{35}S]methionine (New England Nuclear, Boston, MA) for 4 h, except where noted, then harvested and lysed by sonication as previously described (24) unless otherwise stated. Two-dimensional (2D) gel electrophoresis was carried out according to the methods of O'Farrell et al. (39). The completed 5–13% acrylamide gradient slab gels (0.75 x 14 x 35 cm) were treated with sodium salicylate (Aldrich Chemical Co., Inc., Milwaukee, WI) (40), dried, and exposed at −70°C with X-OMAT AR film (Eastman Kodak Co., Rochester, NY). The 2D gels are oriented with basic proteins at left and acidic proteins at right, in each panel. The acid side of the 2D gels in this report is on the right.

Microtubules from radiolabeled DC-3F and DC-3F/VCRd-5 cells were assembled through one cycle of polymerization as described by Shelanski et al. (41). The microtubule pellets were solubilized in O'Farrell buffer (39) before 2D gel analysis. In some experiments, cells were treated with 5 μg/ml colchicine to stop *de novo* synthesis of tubulin, then lysed with detergent (42). Absence of

TABLE I

Drug Resistance Levels and Presence of Resistance-related Cytogenetic Abnormalities in Vincristine-resistant and Control Cell Lines

Cell line	Selective concentration	Increase in resistance*	Chromosomal abnormality
	μg/ml		
Chinese hamster			
DC-3F	0	1	—
DC-3F/VCRd	10	650‡	HSR
DC-3F/VCRd-1	5	650	HSR
DC-3F/VCRd-5	5	500‡	HSR
DC-3F/VCRd-5L	50	2,750‡	HSR
DC-3F/VCRd-1-U	0	1	—
DC-3F/VCRd-5-U	0	33	—
DC-3F/VCRm	10	550	ABR
Mouse			
MAZ	0	1	—§
MAZ/VCR	20	3,939	DMs
MAZ/VCR-U	0	4	—
Human			
SH-SY5Y	0	1	—
SH-SY5Y/VCR	10	1,333	DMs
SH-SY5Y/VCR-U	0	8	—

* Degree of resistance was determined in dose-response assays as described previously (31).

‡ Previously published values (19, 47, 48).

§ Control MAZ cells contain a low number of DMs; however, both the number of DMs and the frequency of DM-containing cells are greatly increased in the resistant subline. See text.

spots representing tubulin on 2D gels of proteins from the treated cells assisted in identification of those peptides. Identity of α- and β-tubulin spots on the 2D gels was confirmed by co-electrophoresis of pig brain tubulin, prepared according to Shelanski et al. (41), with labeled CHL cell protein.

In Vitro Translation: Procedures for polysome isolation, mRNA purification, and cell-free protein synthesis were carried out as described by Melera et al. (8).

RESULTS

Resistance and Cross-resistance of Cell Lines Selected with VCR

As part of an ongoing investigation to uncover genetic mechanisms of resistance to a variety of cancer chemotherapeutic agents, we independently selected two VCR-resistant sublines, DC-3F/VCRd and DC-3F/VCRm, from DC-3F, one VCR-resistant line from the mouse tumor line, MAZ, and a fourth subline from the cloned human neuroblastoma line, SH-SY5Y. Levels of resistance of drug-treated cells range from 500- to 4,000-fold (Table I).

To assess the presence and extent of the multidrug-resistant phenotype in cell lines selected with the *Vinca* alkaloid, we carried out cross-resistance assays on the 650-fold VCR-resistant DC-3F/VCRd CHL cells and, to a more limited extent, on VCR-resistant mouse and human cells. In general, degree of cross-resistance of DC-3F/VCRd cells is greatest for drugs known to affect microtubule polymerization; indeed, the VCR-resistant cells show reduced sensitivity to all antimitotic drugs and experimental analogs tested (Table II). DC-3F/VCRd cells are cross-resistant, to a lesser extent, to a variety of other agents. The low levels of cross-resistance to 6-thioguanine, arabinosylcytosine, methotrexate, and 1,3-bis(2-chloroethyl)-1-nitrosourea are probably not significant (31). The mouse and human VCR-resistant cell lines, like CHL cell sublines, are cross-resistant to actinomycin D, exhibiting increases of 571- and 200-fold, respectively, as compared with controls.

Chromosomal Abnormalities Characterizing VCR-resistant Cell Lines

CHINESE HAMSTER SUBLINES: Routine karyotypic analysis of the 650-fold resistant subline, DC-3F/VCRd, revealed the presence of an HSR (Fig. 1). HSRs were subsequently observed in cells of all DC-3F/VCRd sublines (DC-3F/VCRd-1, DC-3F/VCRd-5, DC-3F/VCRd-5L). The HSR is located interstitially on a marker chromosome and comprises, on average, 2.7% of the total chromosome length in a cell. It stains uniformly although somewhat more intensely than a typical HSR in antifolate-resistant CHL cells (1, 2, 35). The HSR of DC-3F/VCRd cells commences and completes DNA replication early in S-phase as shown by [^{3}H]thymidine-labeling studies (Fig. 2, *a–c*).

The labeling studies also demonstrated that the segment of the marker chromosome arm distal to and including an apparent secondary constriction region (Fig. 1) is clearly late-replicating (Fig. 2*c*). This segment thus most probably comprises the distal half of the long arm of an X chromosome (bands Xq21-Xqter) that, in normal cells, stains similarly to an HSR. The origins of the grey band proximal to Xq21 (the secondary constriction region) and of a wide, palely staining band between the HSR and a seemingly normal entire chromosome 8 are ambiguous. The marker chromosome thus comprises a t(X;8;HSR;?)(8qter→8p19::?::HSR::?::Xq21→Xqter).

Chromosomal analysis of the second, independently-derived VCR-resistant CHL cell line, DC-3F/VCRm, revealed the presence of an ABR, a long, obscurely banded region comprising 3.8% of the total chromosome length, on a pre-

TABLE II

Resistance of DC-3F/VCRd Cells to Chemotherapeutic and Cytotoxic Agents

Agent	Molecular weight	ED_{50}* for DC-3F/VCRd		ED_{50} for DC-3F		Increase in resistance‡
		$\mu g/ml$	10^{-6} M	$\mu g/ml$	10^{-6} M	
Antimitotic agents						
Vincristine sulfate	922	13	14	0.020	0.022	650
Vindesine sulfate	851	7.8	9.2	0.014	0.017	557
Vinblastine sulfate	980	2.2	2.4	0.0043	0.0047	512
Colchicine	399	7.0	18	0.020	0.050	350
LY 108555§	1,820	19	10	0.065	0.036	292
VP-16-213	588	7.9	13	0.057	0.097	139
LY 119863§	906	11	12	0.15	0.17	73
Demecolcine	371	0.36	0.97	0.013	0.034	28
Other agents						
Mithramycin	1,089‖	30	28	0.068	0.062	441
Actinomycin D	1,256	0.30	0.24	0.0024	0.0019	125
Puromycin	471	160	330	1.5	3.1	107
Daunorubicin	528	2.1	4.0	0.023¶	0.044	91¶
Ethidium bromide	394	20	51	0.30	0.76	67
Hydroxyurea	76	12	160	2.0	26	5.9
6-Thioguanine	167	0.091	0.54	0.044	0.26	2.1
Arabinosylcytosine	243	0.076	0.31	0.040	0.16	1.9
Methotrexate	454	0.012	0.026	0.0067	0.015	1.8
1,3-Bis(2-chloroethyl)-1-nitrosourea	214	2.4	11	1.9	8.9	1.3

* ED_{50}: concentration effective in reducing cell growth to 50% of that of drug-free controls, as measured in a 3-day assay.
‡ ED_{50} of DC-3F/VCRd::ED_{50} of DC-3F.
§ Experimental *Vinca* alkaloid derivatives provided by Dr. George Boder of Eli Lilly and Co.
‖ Tentative molecular weight.
¶ Revised from earlier publication (31).

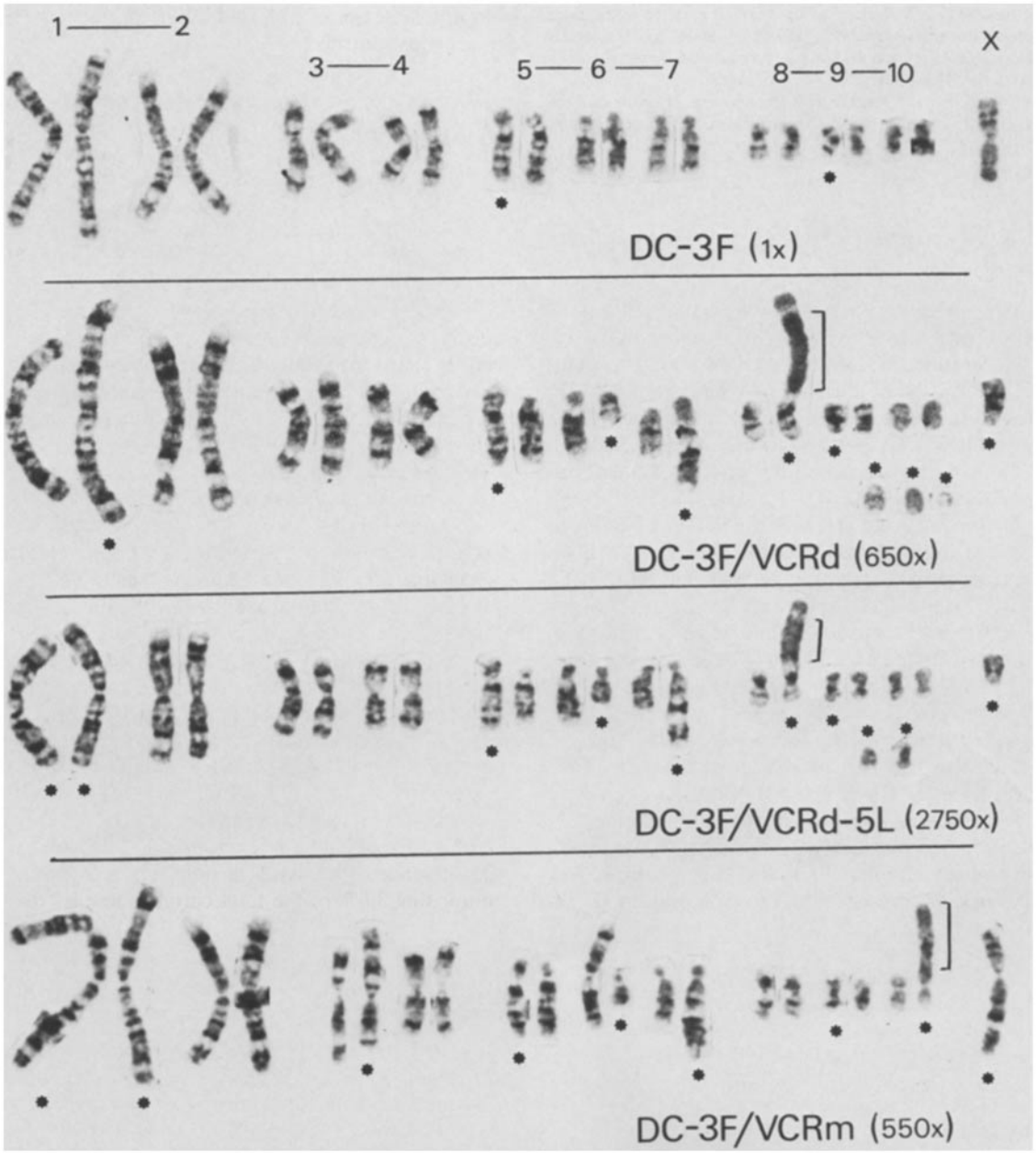

FIGURE 1 Trypsin-Giemsa banded karyotypes of the drug-sensitive CHL cell line DC-3F and vincristine-resistant sublines. Asterisks indicate consistent, structurally altered chromosomes; brackets delineate the HSR (DC-3F/VCRd and DC-3F/VCRd-5L) or ABR (DC-3F/VCRm). The number in parentheses denotes degree of resistance to vincristine. The translocation involving the first chromosome 6 in the DC-3F/VCRm cell shown is unique to this cell. × 1,500.

sumptive chromosome 10p21 (Fig. 1). In these cells, both chromosome 8 homologues are normal. Moreover, although the single X chromosome has been rearranged distal to the secondary constriction region of the long arm, the region between the centromere and band Xq22 is normal. Thus, there appears to be no correspondence between the locations of the HSR in DC-3F/VCRd cells and the ABR in DC-3F/VCRm cells. Control DC-3F cells and the actinomycin D- and daunorubicin-resistant lines DC-3F/AD X and DC-3F/DM XX, also used as controls in these studies, contain no anomalous regions (nor DMs). Even the 50,000-fold resistant DC-3F/AD XC cells have no chromosomal regions with abnormal band patterns corresponding to the ABR of DC-3F/VCRm.

Both of the VCR-resistant CHL sublines contain numerous structurally altered chromosomes, including two that are common to all DC-3F sublines (Fig. 1). DC-3F/VCRd sublines also contain 2–3 small supernumerary chromosomes. Whether these small, abnormal chromosomes or unidentifiable segments present on rearranged chromosomes represent ABRs is an open question.

MOUSE TUMOR LINES: Drug-sensitive MAZ cells have a modal chromosome number of 67–70 and contain an average of 24 DMs per cell in 65% of cells. DM number and frequency increased markedly after exposure to VCR; 100% of MAZ/VCR cells, selected at 20 μg/ml VCR and ~4,000-fold resistant to drug (Table I), are characterized by an average of 183 very small DMs near the resolving limit of the light microscope (Fig. 2*d*). Of 100 cells examined, no cell contained <50 DMs, and two-thirds of the cells had >100 DMs

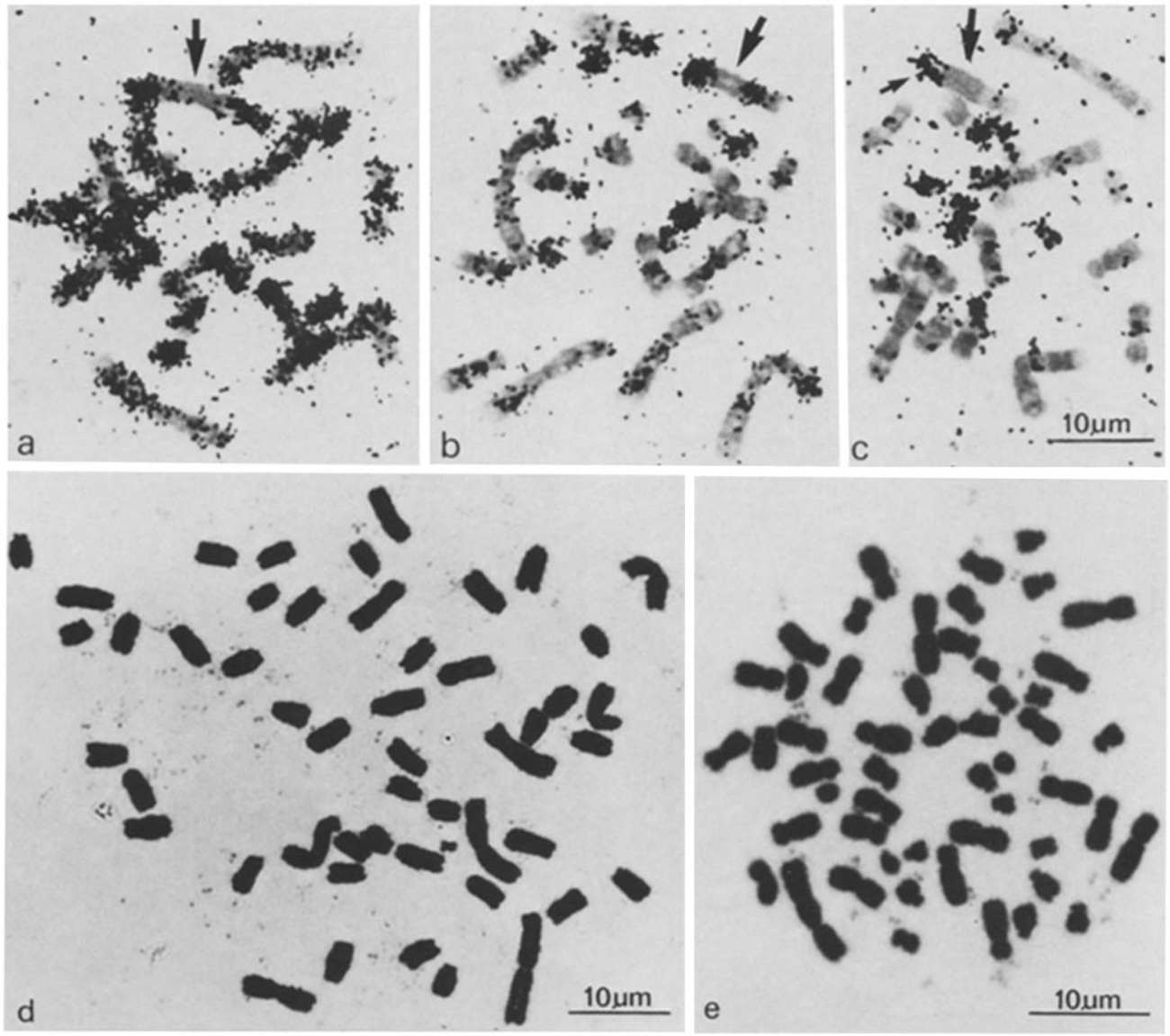

FIGURE 2 Tritiated thymidine labeling of DC-3F/VCRd chromosomes (*a–c*) and DMs in VCR-resistant mouse (*d*) and human (*e*) tumor cells. Cells in *a*, *b*, and *c* were labeled for 10 min with 1 μCi/ml [^{3}H]thymidine, swelled and fixed after 3–15 h as described previously (1, 2). Chromosome regions replicating (*a*) early, (*b*) midway, and (*c*) late in S-phase. The HSR is indicated by a large arrow; the late replicating Xq chromosome segment in *c* is indicated by a small arrow. (*d*) A conventionally stained cell of the mouse MAZ/VCR cell line with more than 250 barely visible DMs. (*e*) A conventionally stained cell of the DM-containing human SH-SY5Y/VCR subline. Bar, 10 μm.

per cell. Resistant cells do not exhibit long HSRs or obvious ABRs; short HSRs or ABRs, however, could be present but not as yet identified owing to the type and extent of rearrangement characteristic of MAZ cells.

HUMAN NEUROBLASTOMA CELL LINES: A VCR-resistant subline was also selected over a 7-yr period from the human neuroblastoma line, SH-SY5Y. Unlike most human neuroblastoma cell lines described so far (43), hyperdiploid SH-SY5Y cells and the SK-N-SH line from which the thrice-cloned subline was isolated have never been observed to contain HSRs or DMs (34). The 1,300-fold resistant cells, however, are characterized by an average of 40 DMs per cell in 100% of cells (Fig. 2*e*); no cell contains <10 DMs, 30% of cells have >50, and 6% have >100 DMs.

Protein Changes in VCR-resistant CHL Cells

A possible protein product of amplified genes in VCR-resistant cells is a protein we have designated V19 (24). V19 is seen on 2D gels of cytosol proteins of DC-3F/VCRd-5 cells (Fig. 3) and of the more highly resistant DC-3F/VCRd-5L cells. Further, DC-3F/VCRm cells also synthesize V19, although in lesser amounts (data not shown). The relative abundance of V19 seen on these gels reflects an increased abundance of V19 mRNA in the VCR-resistant cells. 2D gels of translation products of polysomal mRNA from DC-3F/VCRd-5L cells show a prominent V19 spot (Fig. 4*b*) compared with control (Fig. 4*a*), whereas DC-3F/VCRm cell material had lower levels of V19-specific mRNA (data not shown). Two other proteins are also overproduced in DC-3F/VCRd-5 cells: V61 (M_r 61,000; pI = 7.2) (Fig. 3) and V92 (M_r 92,000; pI = 7.1) (not shown). V61, however, is not seen on gels from DC-3F/VCRm, and neither V61 nor V92 is identifiable on gels of translation products of DC-3F/VCRd-5L polysomal mRNA (Fig. 4). V19 is also readily identified on 2D gels of MAZ/VCR cell proteins (Fig. 5); V61 and V92 are not seen on MAZ/VCR gels. In earlier studies, cytosol

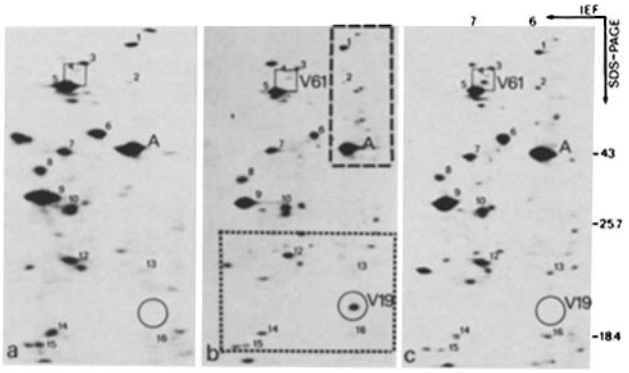

FIGURE 3 Two-dimensional gel electrophoretic separation of [^{35}S]-methionine-labeled soluble proteins from (*a*) DC-3F, (*b*) DC-3F/VCRd-5, and (*c*) DC-3F/VCRd-5-U cells lysed by sonication. Aliquots of supernatant material obtained after centrifugation of cell homogenates, containing ~5 × 10^5 cpm (30–50 μg of protein), were applied to the first dimension gels. Completed gels were exposed to x-ray film for four d. Complete procedures are described in Materials and Methods. The numbers on the photographs are for orientation purposes only. Spot 11, designated *A*, is known to be actin. V61 and V19 positions are enclosed. Dotted lines enclose areas of gel depicted in Fig. 5 (V19 area) and dashed lines delineate areas shown in Figs. 6 and 7 (tubulin area). Acid side of the 2D gels is on the right.

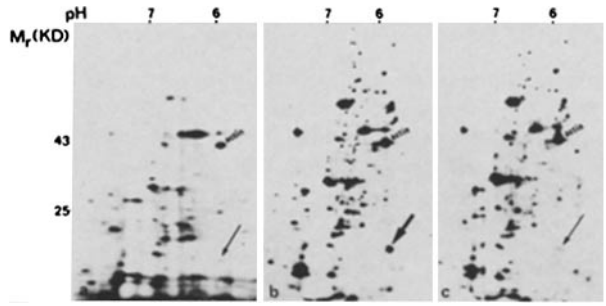

FIGURE 4 Fluorograms of 2D gels of protein products of in vitro translations directed by poly(A$^+$) RNA from (*a*) DC-3F, (*b*) DC-3F/VCRd-5L, and (*c*) DC-3F/VCRd-5-U cells. Equal amounts of RNA were used for each reaction (~2 μg) and equal amounts of trichloroacetic acid-precipitable radioactivity in translated products (2–3 × 10^5 cpm) were subjected to electrophoresis. Arrows point to V19. Additional procedural information is in Materials and Methods.

proteins of the VCR-sensitive and -resistant hamster and mouse cell lines were examined by sodium dodecyl PAGE. In addition to V19, two other protein species (M_r 43,000 and 32,000–35,000) were present in increased amounts in resistant cells (data not shown). These two proteins are not consistently identifiable on 2D gels but are of potential future interest.

The high degree of cross-resistance of DC-3F/VCRd cells to various antimitotic agents (Table II) and studies in other laboratories (27–30) suggested the possibility that tubulin protein was overproduced or altered in the resistant cells. Analysis by 2D gel electrophoresis of polymerization-competent tubulin (Fig. 6) or of unpolymerized tubulin in absence and presence of colchicine (Fig. 7) showed no evidence for either overproduction or mutation of α- or β-tubulin in VCR-resistant CHL cells. V19 does not copolymerize with tubulin; it remains in the supernatant material above the in vitro polymerized microtubule pellet, and V19 synthesis is not altered by colchicine treatment (data not shown).

Reversion of Resistance and Loss of HSR, DMs, and V19 Expression

To assess the stability of the VCR-resistant phenotype in absence of drug selection pressure, we cloned the DC-3F/VCRd subline in the presence of 5 μg/ml VCR. We then transferred two clonally derived sublines, DC-3F/VCRd-1 and DC-3F/VCRd-5, to drug-free medium and redesignated them DC-3F/VCRd-1-U and DC-3F/VCRd-5-U (Table I). Levels of resistance declined between the first two assays done after 3 and 9 wk in the absence of VCR (Table III). During the first 4 mo of the 2½-yr experimental period, the rate of decrease was more rapid for DC-3F/VCRd-1-U than for DC-3F/VCRd-5-U cells ($t_{1/2}$ ≃ 60 and 120 d, respectively). After an initial decline, resistance of DC-3F/VCRd-1-U cells stabilized for 8 mo at an intermediate level, and again declined rapidly until reaching control level (Table III). In contrast, the resistance level of DC-3F/VCRd-5-U cells decreased pro-

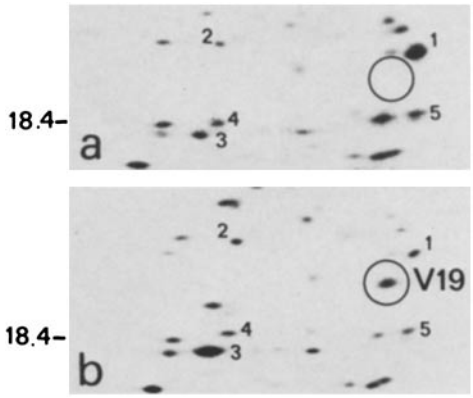

FIGURE 5 Details of fluorograms of [^{35}S]methionine-labeled soluble proteins from (*a*) MAZ and (*b*) MAZ/VCR separated by 2D gel electrophoresis. Experimental details are as described in Fig. 3. The numbers on the photographs are for orientation purposes. V19 and its expected location in MAZ are circled. Area of this detail is enclosed by dotted lines in Fig. 3.

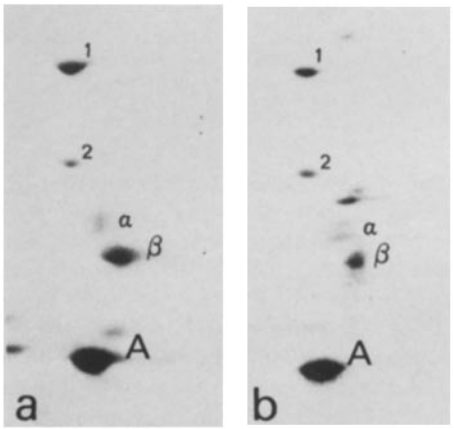

FIGURE 6 Details of fluorograms of [^{35}S]methionine-labeled proteins in microtubule pellets obtained by one cycle of in vitro assembly and separated by 2D gel electrophoresis. Equal amounts of trichloroacetic acid-precipitable radioactive proteins (about 1 × 10^5 cpm/sample) from (*a*) DC-3F and (*b*) DC-3F/VCRd-5L cells were analyzed. Complete procedures are given in Materials and Methods.

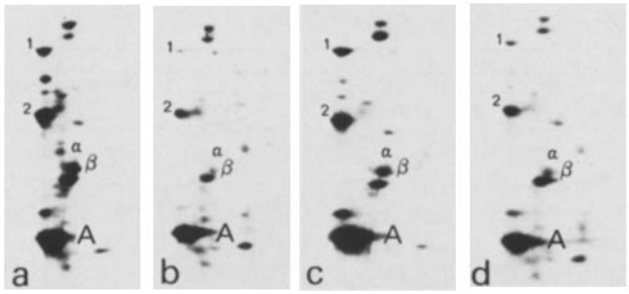

FIGURE 7 Details of fluorograms of [^{35}S]methionine-labeled soluble proteins separated by 2D gel electrophoresis from (*a* and *b*) DC-3F and (*c* and *d*) DC-3F/VCRd-5 cells either treated (*b* and *d*) or untreated (*a* and *c*) with 5 μg/ml of colchicine for 5.5 h before a 30-min exposure to radioactive methionine. The cells were lysed by treatment with Nonidet P-40 (30) and soluble material containing 5–6 × 10^5 cpm per aliquot was applied to first dimension gels. Procedures for these experiments were described by Ben-Ze'ev et al. (42) as stated in Materials and Methods. Numbers are used for orientation purposes. Spot *A* is actin, α is α-tubulin and β is β-tubulin. Two forms of α-tubulin (α_1 and α_2) are evident in DC-3F untreated gels and are not observed on the gels of colchicine-treated cell samples. α-Tubulin was frequently not observed or was seen as an indistinct spot on DC-3F/VCRd-5L gels.

TABLE III

Concomitant Decrease in Resistance to Vincristine and in HSR Length in Two Clones Isolated from the DC-3F/VCRd Cell Line

	DC-3F/VCRd-1-U		DC-3F/VCRd-5-U	
Time off drug	Degree of resistance*	HSR length‡ ± SD	Degree of resistance*	HSR length‡ ± SD
weeks				
0–3	650	0.52 ± 0.057	500	0.68 ± 0.11
9	345		425	
12–13	290	0.45 ± 0.12	385	0.62 ± 0.20
18–24	175		325	
28–30	150	0.33 ± 0.099	150	0.37 ± 0.13
35–39	150		100	
49–55	180		55	0.043 ± 0.064§
67–78	110		32	
119	1.2	0.073 ± 0.0032	33	

* ED_{50} of resistant cells::ED_{50} of parental DC-3F cells.
‡ Length of the HSR::average length of chromosome 2p.
§ At this time point, only 40% of cells contained HSRs.

gressively for ~1½ yr, ultimately stabilizing at a level 33-fold higher than that of control DC-3F cells (Table III).

Decrease in resistance was paralleled by a decrease in the mean length of the HSR (Table III). At any one time point during the reversion process, there was considerable heterogeneity among cells with respect to HSR length (Fig. 8). However, diminution in length of the HSR was not associated with obvious chromosomal breakage or rearrangement. In nearly all cells examined, the composition of the marker chromosome, i.e., 8-HSR-Xq, remained constant; only the length of the interstitial HSR was altered. After 28 mo, HSRs had diminished in size to the approximate width of a normal chromosome band, situated between the secondary constriction (band Xq21) of the X chromosome and the elongated, palely staining 8p+ region. Whether the remaining band represents residual HSR material or a normal Xq18 chromosome band cannot be determined at this time. The revertant cells, however, retained the structurally rearranged chromosomes 1, 6, and 7 that are characteristic of the drug-resistant progenitors but not seen in drug-sensitive cells. The presence of these marker chromosomes and of the 8-Xq chromosome indicated unequivocally that the revertant cells, even those that had completely lost resistance, were derived from the resistant cells and were not cell contaminants.

Decrease in resistance and in HSR length in DC-3F/VCRd-5-U cells was also accompanied by a decrease in V19 synthesis (Fig. 3*c*) and a decreased concentration of V19 mRNA (Fig. 4*c*). The amount of the other two proteins, V61 (Fig. 3*c*) and V92, remained elevated, even in cells with only a 33-fold residual resistance level.

Both DM-containing VCR-resistant sublines, mouse MAZ/VCR and human SH-SY5Y/VCR, also underwent phenotypic reversion when grown in drug-free medium. Decline in resistance was rapid for both lines, with $t_{1/2} \simeq 10$ d for MAZ/VCR-U and 30 d for the more slowly growing human SH-SY5Y/VCR-U cells. Resistance of the MAZ/VCR-U subline stabilized at a level fourfold higher than that of control MAZ cells (Table IV) after ~5 mo and remained at that level for another 9 mo, at which time the experiment was terminated. Decrease in resistance was paralleled by a decrease in DM number and frequency (Table IV; Fig. 9) and by a decrease in synthesis of V19 (Fig. 9). The V19 spot, as seen in Fig. 5*b*, is absent in MAZ/VCR-U protein gels (not shown).

DISCUSSION

Resistance and cross-resistance patterns observed for DC-3F/VCRd cells, in the present study, differ from those previously reported for actinomycin D- and daunorubicin-resistant CHL cells (22, 44). The levels of resistance or cross-resistance of DC-3F/VCRd cells to actinomycin D and daunorubicin are low compared with VCR itself and other antimitotics such as vindesine and colchicine, suggesting that there is a resistance mechanism in these cells involving microtubule function alteration. Actinomycin D–resistant (DC-3F/AD X) and daunorubicin-resistant (DC-3F/DM XX) cells, on the other hand, show relatively high levels of cross-resistance to vincristine and other alkaloids. This suggests that drug exclusion plays a more important role in expression of resistance in the antibiotic-resistant cells. In general, all of the cross-resistance

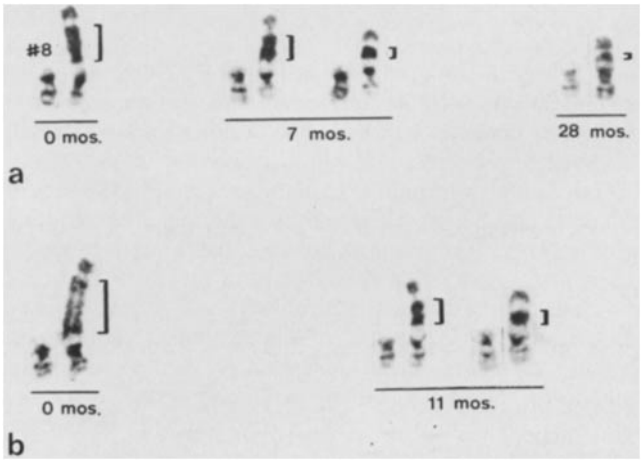

FIGURE 8 Decrease in HSR length in revertant (a) DC-3F/VCRd-1-U and (*b*) DC-3F/VCRd-5-U cell lines during growth in vincristine-free medium. Brackets denote HSRs.

TABLE IV

Decrease in Resistance and DM Number in Murine MAZ/VCR Cells in Absence of Drug

Time off drug	Degree of resistance*	Frequency (%) of cells with: 0 DMs	1–10 DMs	11–50 DMs	51–100 DMs	101–200 DMs	>200 DMs	Mean number of DMs
weeks								
0	4,545	0	0	0	34	42	24	183
0.5	3,333	0	6	20	30	26	18	139
1.5	2,515	0	0	17	33	23	27	171
2.0	1,576							
3.0–3.5	1,242	0	4	44	34	16	2	59
4.0	939	2	20	58	16	4	0	34
7.5–8.0	333	4	18	44	26	8	0	43
12.0	76							
19.0–57.0	4							
Control‡	1	35	24	30	5	5	1	24

* ED_{50} of resistant cells::ED_{50} of drug-sensitive parent cells.
‡ Drug-sensitive MAZ cells.

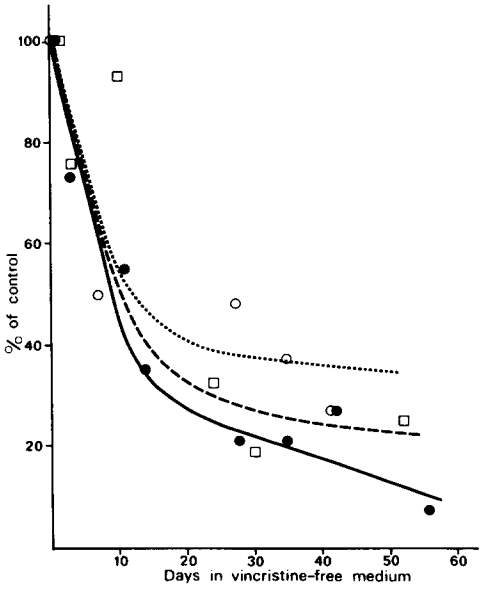

FIGURE 9 Decrease in level of resistance (●), in numbers of DMs (□), and in synthesis of V19 (○) in MAZ/VCR cells grown in the absence of drug. For determination of amounts of V19, cells were metabolically labeled with [^{35}S]methionine; aliquots of soluble radiolabeled proteins, containing 5 × 10^5 cpm, were subjected to two-dimensional or one-dimensional gel electrophoresis. Two-dimensional gel spots corresponding to V19 were cut out and counted (values plotted here), or bands from single dimension gels were first visualized by Coomassie Blue stain and then cut out and counted. The intensity of stain in the V19 band decreased with time as did the amount of radioactivity (data not shown). Data are reported as a percent of values obtained for MAZ/VCR cells grown continuously in the presence of drug.

results are consistent with the idea that there are multiple pathways for the development of resistance in multidrug resistant cells.

The cytogenetic and reversion data strongly implicate a gene amplification mechanism in development of resistance to VCR. Four independently derived, VCR-resistant cell lines selected from CHL, mouse tumor, or human neuroblastoma parental populations are characterized by gene amplification–associated chromosomal abnormalities that disappear in absence of selection pressure. Preliminary evidence from in situ hybridization experiments with the use of C_0t-purified amplified DNA from DC-3F/VCRd-5L cells as probe confirms the presence of amplified DNA sequences in the HSRs of VCR-resistant CHL cells (Scotto, K. W., and P. W. Melera, unpublished observations).

VCR-resistant CHL cells share many of the characteristics of HSR- and ABR-containing antifolate-resistant sublines also derived from control DC-3F cells: (*a*) as in highly resistant methotrexate- and methasquin-resistant DC-3F cells (1, 35) and in numerous other cell lines selected with methotrexate (11–13, 45, 46) and known to contain amplified genes coding for DHFR, DC-3F/VCRd and DC-3F/VCRd-5L cells with the highest level of VCR resistance contain an HSR on a single chromosome; (*b*) the HSRs of the VCR-resistant cells stain homogeneously but somewhat more darkly than those typifying antifolate-resistant cells; they comprise ~3% of the total chromosome length (22) compared with 4–5% for the antifolate-resistant cells (35); (*c*) DNA of the HSR characterizing both methotrexate- and VCR-resistant cells is replicated synchronously and starts replication early during the S-period (1) (Fig. 2), consistent with the possibility that it is a transcriptionally active region; (*d*) like antifolate-resistant CHL sublines with low levels of resistance (23, 25), a second, independently derived VCR-resistant subline, DC-3F/VCRm, with a slightly lower level of resistance, has an ABR; (*e*) when clonally derived sublines of DC-3F/VCRd were grown in drug-free medium, there was a decline in resistance and mean length of the HSR (Table III), as previously demonstrated for antifolate-resistant DC-3F sublines (35).

The VCR-resistant CHL cell system differs from the antifolate-resistant system in the chromosomal location of the HSR or ABR. In the latter, the regions are preferentially (but not always) located on a particular arm of chromosome 2 (1, 11, 35). In the VCR-resistant cells, chromosome 2 is not involved. However, we have only two independent VCR-resistant sublines and thus cannot infer the permanent location of a putatively amplified gene.

There are also clear similarities between the VCR-resistant mouse MAZ/VCR subline and the previously described antifolate-resistant subline MAZ/A/MQ (19), both derived from the same control cell line MAZ. The 4,000-fold resistant MAZ/VCR cells contain high and variable numbers of DMs (an average of 183 per cell). The 50,000-fold resistant MAZ/A/MQ cells average >300 DMs per cell (19); the association between the presence of DMs and amplification of the DHFR-encoding gene is well-established (15, 18, 20, 21). When each

of the lines was maintained in the absence of selective agent, there was a rapidly initiated and rapidly progressive loss of resistance and mean number of DMs per cell (Table IV) (19).

One important attribute that may distinguish gene amplification–associated drug resistance from resistance developing as a consequence of other genotypic alterations, e.g., base substitution or deletion, is instability of the resistant phenotype in the absence of drug selection pressure (19). In all four cell lines tested for stability of resistance, two of the lines derived from mass cell populations and two isolated as clonal subpopulations, resistance decreased markedly during growth in drug-free medium. For two cell lines, sensitivity to VCR returned to near-control levels, suggesting that in these lines, DC-3F/VCRd-1-U and SH-SY5Y/VCR-U, amplification of one or several genes may be the major mechanism of resistance. In DC-3F/VCRd-5-U cells, however, resistance stabilized at a level 30-fold higher than that of the controls. Whether this residual resistance indicates the operation of a second resistance mechanism not associated with gene amplification or presence of a small number of amplified genes integrated into one or more chromosomally undetectable sites, an apparently stable arrangement in antifolate resistant cells (19), is unknown.

One candidate protein product of the amplified genes is V19. This previously reported (24) cytosolic protein is overproduced in VCR-resistant CHL and mouse tumor cells. Its synthesis reflects increased abundance of V19 mRNA in those cells. Although levels of V19 synthesis are closely associated with levels of resistance to VCR and with number of DMs in the mouse tumor cell system, as indicated by the reversion study, the role this peptide plays in expression of resistance, if any, is not yet known. However, the consistent finding of increased synthesis of V19 in VCR-resistant cells warrants continued investigation of this protein species.

In contrast to studies in other laboratories of cells resistant to other antimitotics (27–30), our studies have not demonstrated any apparent mutant tubulin species in the VCR-resistant CHL cells, nor do they indicate overproduction of tubulin. Thus a major mechanism conferring resistance on DC-3F/VCRd-5 cells may be associated with microtubules, but is probably not directly involved with tubulin proteins.

A second protein, somewhat less compelling as a candidate product of amplified genes, is the high molecular weight glycoprotein species, gp150, characterizing drug-resistant CHL cells selected with actinomycin D or daunorubicin as well as with VCR (22, 47). Chromosomal structures indicative of gene amplification have not been seen in the CHL cells resistant to either daunorubicin or actinomycin D, even in cells raised to an extraordinarily high level of resistance (50,000-fold) to the latter drug. Nevertheless, the phenotypic similarities between resistant CHL cells selected with each of the three drugs (19, 22, 44, 48), the instability of gp150 expression in revertant CHL cells grown in the absence of either actinomycin D or VCR (19, 22, 44, 47), and the finding of an HSR in drug-resistant Djungarian hamster cells selected with actinomycin D (49) all raise the possibility that gp150 could be the product of amplified genes that are not detected by current cytogenetic procedures in the actinomycin D- or daunorubicin-resistant CHL cells.

Taking into account all the above considerations, our overall results strongly support the view that the VCR-resistant cells have undergone more than one genetic alteration (49). One alteration is related to drug uptake differences and cross-resistance phenomena and affects most or all multidrug-resistant cells. A second one involves gene amplification associated with overproduction of V19 or some other as yet unidentified protein. This second change contributes to the phenotype of the VCR-resistant cells under study in our laboratory and of certain other multidrug-resistant cells as well (44, 50, 51).

This work was supported in part by National Institutes of Health grants CA-08748 and CA-28595, and the Kleberg Foundation.

Received for publication 10 July 1984, and in revised form 22 October 1984.

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