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INCREASED SYNTHESIS OF A LOW MOLECULAR WEIGHT PROTEIN IN VINCRISTINE-RESISTANT CELLS

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SUMMARY: A 19,000-dalton peptide (pI = 5.7) that is synthesized in increased amounts in vincristine-resistant Chinese hamster cells (DC-3F/VCRd-5) has been identified by two-dimensional gel electrophoresis. Reduced amounts of the protein were present in a revertant line of DC-3F/VCRd-5, and only trace amounts were detected in control DC-3F cells. A similar protein (M_r = 19,000; pI = 5.7) was also found in a vincristine-resistant mouse line. Two vincristine-resistant human neuroblastoma cell lines likewise contained elevated levels of a low molecular weight acidic protein. Increased biosynthesis of the 19,000-dalton polypeptide in DC-3F/VCRd-5 cells coincides with the presence of a homogeneously staining region, HSR, on a metaphase chromosome.

INTRODUCTION

A metaphase chromosomal anomaly known as a homogeneously staining region, or HSR, has been found in several different antifolate-resistant Chinese hamster lung fibroblast sublines (1). The HSRs have been shown, in our laboratory, to be associated with DNA sequence amplification (2). These amplified genomic regions, appearing after prolonged exposure of hamster cells to antifolates, code for target enzyme dihydrofolate reductase. Cellular overproduction of that enzyme accounts at least partially for the high levels of resistance to antifolate (3).

A vincristine-resistant subline, derived from the same parental Chinese hamster line (DC-3F) as the antifolate-resistant cells, has been isolated.

Abbreviations used: HSR, homogeneously staining region, a nonbanded region of a metaphase chromosome observed after staining of chromosome spreads with trypsin-Giemsa; 19-KD, 19,000-dalton; 26-KD, 26,000-dalton.

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Vincristine is an antimitotic Vinca alkaloid included in a number of cancer chemotherapeutic protocols. All cells of the vincristine-resistant subline, DC-3F/VCRd, have an abnormal chromosome with a well-defined HSR (4,5). Its presence suggests that gene amplification, in response to vincristine exposure, might have occurred in DC-3F/VCRd in a manner analogous to that occurring in cells exposed to antifolates. If the analogy is correct, the vincristine-resistant cells could overproduce a specific protein species. Identification of such a protein was sought by two-dimensional gel electrophoretic analysis of protein content in a clone of DC-3F/VCRd, designated DC-3F/VCRd-5. The comparison control cells included parental DC-3F, DC-3F/AD X (an actinomycin D-resistant line), several antifolate-resistant sublines also derived from DC-3F, and a revertant of DC-3F/VCRd-5, designated VCRd-5-U. A mouse tumor cell line, MAZ, and its vincristine-resistant subline, MAZ/VCR, were also studied as were two independently-derived human neuroblastoma lines and their vincristine-resistant derivatives.

MATERIALS AND METHODS

Cells. All cells are grown in Falcon tissue culture flasks in Eagle's MEM and Ham's F-12 (1:1) supplemented with 5% fetal bovine serum (M. A. Bioproducts), nonessential amino acids (Eagle's formulation), 100 IU penicillin/ml and 100 µg streptomycin/ml. The origin of the near-diploid Chinese hamster lung fibroblast line, DC-3F, and the method of development of drug-resistant sublines have been described (6). DC-3F/VCRd, selected by stepwise increases in concentration of vincristine up to 10 µg/ml, was cloned in 5 µg/ml of drug. The clone, DC-3F/VCRd-5, is maintained at 5 µg/ml. The mouse cell line, MAZ, was originally explanted from a methylcholanthrene-induced tumor in a C57BL/6 mouse (5) and the vincristine-resistant subline, MAZ/VCR, is now maintained on 10 µg/ml of vincristine. The origins of the human neuroblastoma clonal lines, SH-SY5Y and MC-IXC, have been described (7), and their respective vincristine-resistant sublines are now maintained on 2 µg/ml (SH-SY5Y/VCR) or 0.1 µg/ml (MC-IXC/VCR) of drug. Vincristine was generously provided by Eli Lilly and Company. Drug-resistant cells were routinely grown in drug-free medium for 10-20 days before experimentation.

Cell fractionation. Substrate-attached cells in stationary growth phase were exposed to 10 µCi/ml [^{35}S]-methionine (New England Nuclear) in methionine-deficient growth medium for various periods of time up to 4 hours. Cells were removed from the substrate with 0.02% EDTA. After washing with phosphate-buffered saline, the cells were lysed by sonication in 20 mM Tris-HCl (pH 7.4) containing 5 mM $MgCl_2$ or by treatment with Nonidet P-40(8). The sonicated cells were centrifuged at 16,000 rpm (RC-5B

Sorvall) for 30 min and both supernatant and pelleted fractions were subjected to analysis by two-dimensional gel electrophoresis.

Two-dimensional gel electrophoresis. The pulse-labeled proteins from the drug resistant and control cells were analyzed by the two-dimensional electrophoretic technique described by O'Farrell et al. (9) with the use of a pH 3-10 ampholyte (Bio-Rad) gradient in the first dimension. A 5-13% acrylamide gradient SDS gel (13 x 22 cm) was used in the second dimension. The slab gels were run at 20 mA for approximately 5 hours. Gels were treated with sodium salicylate (10) before drying and autoradiography on Kodak XR-5 X-ray film. All chemicals for the gels were purchased from Bio-Rad.

RESULTS AND DISCUSSION

Table I lists the cell lines examined in this study and shows a specific result of the electrophoretic analysis of cytosol proteins. A 19,000-dalton acidic protein or a counterpart molecular weight species is synthesized in relatively large amounts by vincristine-resistant Chinese hamster, mouse, and human cells (Fig. 1). The parental lines synthesize substantially less

Table I. Response of Vincristine (VCR)-Resistant and Control Chinese Hamster (DC-3F), Mouse (MAZ) and Human Neuroblastoma Cells (SH-SY5Y and MC-IXC) to VCR: Drug Resistance, Chromosome Characteristics and Results of Two-Dimensional Gel Electrophoresis

Cell Line	Selective Agent	ED_{50} of VCR (ng/ml)	Increase in Resistance to VCR	Presence of HSR	Presence of 19,000- or 26,000-dalton polypeptide on gels*
DC-3F	None	0.02	1	-	-
DC-3F/VCRd	Vincristine	13.0	650	+	+
DC-3F/VCRd-5	Vincristine	10.0	500	+	+
VCRd-5-U**	None	0.63	32	-	±
DC-3F/AD X	Actinomycin D	28.0	1400	-	-
DC-3F/MQ19	Methasquin	ND		+	-
DC-3F/A3	Methotrexate	ND		+	-
MAZ	None	0.006	1	-	-
MAZ/VCR	Vincristine	2.0	308	-	+
SH-SY5Y	None	0.0039	1		±
SH-SY5Y/VCR	Vincristine	2.5	641		+
MC-IXC	None	0.00026	1		±
MC-IXC/VCR	Vincristine	0.32	1231		+

Methods for determining ED_{50} of vincristine for the cells (6) and methods of trypsin-Giemsa banding of metaphase chromosomes for HSR detection (3) have been described.

*Chinese hamster and mouse vincristine-resistant lines oversynthesize a 19,000-dalton protein; the human neuroblastoma vincristine-resistant lines oversynthesize a 26,000-dalton protein. A (±) sign indicates small amounts of the protein and a (-) sign indicates that the protein is visible only after prolonged exposure of the labeled gels.

**Data for VCRd-5-U were obtained after the line had been maintained in drug-free medium for about 2 years.

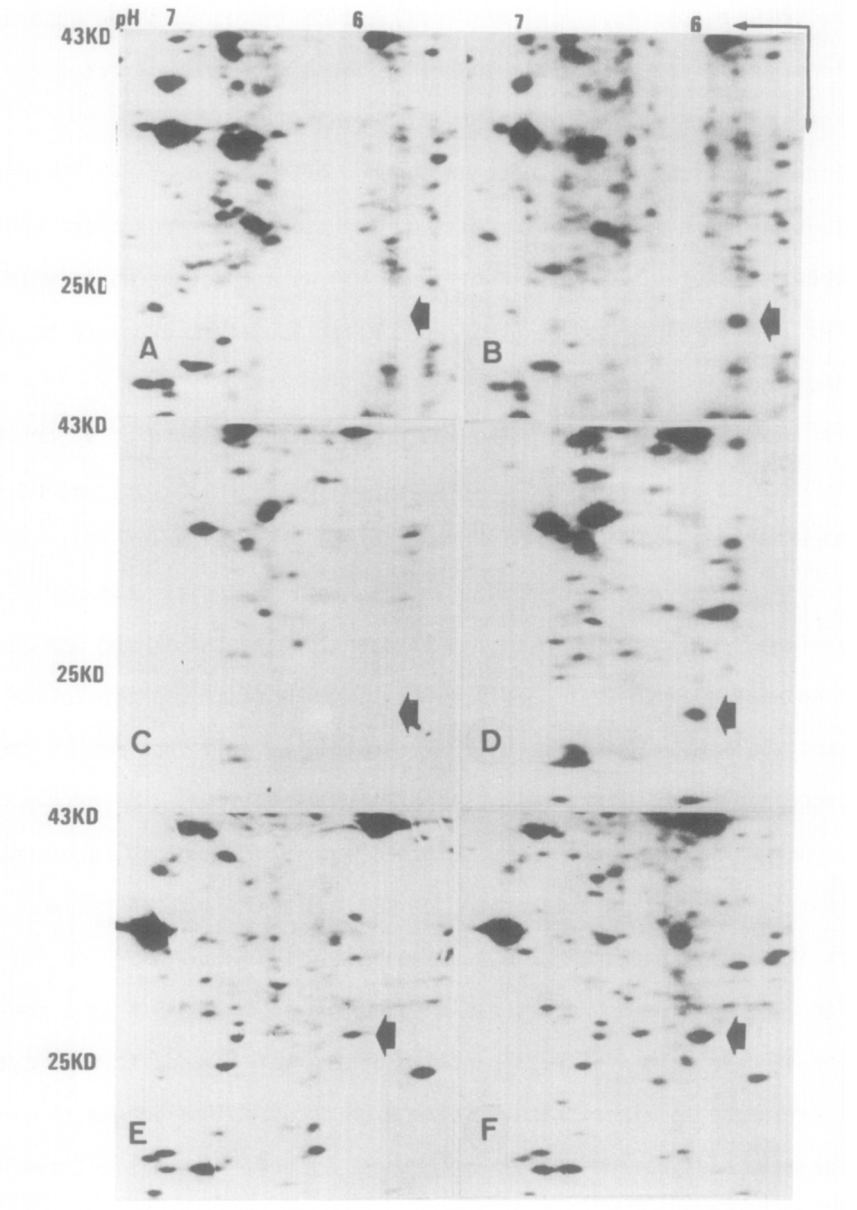

Figure 1. Two-dimensional gel electrophoretic analysis of [^{35}S]-methionine-labeled cytosol proteins from (A) DC-3F, (B) DC-3F/VCRd-5, (C) MAZ, (D) MAZ/VCR, (E) MC-IXC, and (F) MC-IXC/VCR cells. In each case a 4-hr pulse of [^{35}S]-methionine was given and cells were lysed by sonication as described in Materials and Methods. Acid-precipitable radioactivity was determined and 3×10^5 - 6×10^5 cpm (20-40 μg of protein) were loaded on each gel. See Materials and Methods for the two-dimensional gel electrophoresis and radioautography procedures. The arrows indicate the position of the 19,000-dalton protein in the case of (A), (B), (C), and (D) and a 26,000-dalton protein in (E) and (F). Cytosols from vincristine-resistant cells lysed by treatment with buffer containing Nonidet P-40, as described by Ben Ze'ev et al. (8), also contain the newly-identified protein.

of this protein species. The 19-KD polypeptide is found in the supernatant fractions and is not detectable in the pelleted membranous fractions. These findings have been partially reported in brief (11).

For comparison, the presence or absence of the chromosomal abnormality, HSR, is also shown in Table I. Cells of the vincristine-resistant subline of Chinese hamster DC-3F cells contain an HSR as a constant karyotypic feature. The HSR appeared in DC-3F/VCRd after long-term exposure to vincristine (5).

The revertant line, VCRd-5-U, was grown in the absence of vincristine for over two years. The level of resistance to the antimitotic agent decreased markedly but has not returned to the parental value (5); the 19-KD protein is synthesized in that line in amounts intermediate between DC-3F and DC-3F/VCRd-5. Cells of the VCRd-5-U line no longer exhibit the HSR (Table I), a finding analogous to that obtained for the HSR-containing antifolate-resistant sublines. When the latter were grown in drug-free medium there was concomitant loss of drug resistance and HSRs (3).

DC-3F/AD X cells, which were selected with actinomycin D, are cross-resistant to vincristine but contain no HSR and synthesize only minor amounts of the 19-KD protein. Other studies of this subline (5,12,13) indicate that cell membrane alteration occurred in DC-3F/AD X as a concomitant of development of resistance to actinomycin D and, to some extent, cross-resistance to vincristine. Cells with acquired resistance to vincristine also have altered plasma membranes (5) but, in addition, overproduce the 19-KD peptide. Both cellular changes are probably components of a complex mechanism of resistance to vincristine. However, vincristine apparently elicits a response, viz., increased synthesis of a 19-KD protein, not elicited by exposure to actinomycin D.

The antifolate-resistant sublines, DC-3F/MQ19 and DC-3F/A3, contain HSR-bearing chromosomes (1,3), but do not oversynthesize the 19-KD peptide. Therefore, synthesis of this protein is probably unrelated to HSR formation

per se. The HSR gene product in antifolate-resistant cells is dihydrofolate reductase which is overproduced in both lines (3) and not in the HSR-containing vincristine-resistant cells.

Unlike the near-diploid Chinese hamster cells, the murine MAZ cells are heteroploid. Vincristine-resistant MAZ/VCR cells have no well-defined HSR. This finding does not, of course, preclude the occurrence of gene amplification in that line (14). MAZ/VCR cells, like the drug resistant Chinese hamster cells, oversynthesize a 19-KD protein with an isoelectric point of 5.7.

Vincristine-resistant sublines of the human neuroblastoma lines, MX-IXC (Fig. 1) and SH-SY5Y (data not shown), also overproduce a low molecular weight polypeptide with the same pI (5.7) as the 19-KD species. However, the species in these human cells has an apparent molecular weight of 26,000. Chromosome analysis of the neuroblastoma lines is in progress.

Because vincristine is known to affect microtubule assembly in vivo (15), we anticipated that the 19-KD protein might be associated with microtubules or tubulin in the Chinese hamster cells. Microtubules from DC-3F/VCRd-5 cells were assembled in vitro according to the procedure of Shelanski et al. (16). The 19-KD species did not co-assemble with tubulin and other accessory proteins. Further, the molecular weight and isoelectric point values of the new protein do not coincide with those reported for known microtubule-associated proteins such as MAPS or the tau (τ) proteins (17,18). For these reasons we believe that, if the 19-KD polypeptide is involved in microtubule assembly, its role may be that of an assembly or disassembly control substance rather than that of a structural component. Our current speculation is that the protein may serve as a site of binding of vincristine and, thereby, as a microtubule inhibitory factor. A microtubule inhibitory protein (M_r = 15,000) has been isolated from Dictostelium discoideum and its function as a negative control factor suggested (19).

We plan to isolate and investigate the nature of the 19-KD polypeptide in terms of its function in mammalian cells, its role in the development of resistance to vincristine, and its association with the HSR in the vincristine resistant Chinese hamster cells. The presence of an HSR in these cells provides us with the possibility of defining a mechanism of resistance to vincristine that involves selective gene amplification. The 19-KD species of rodent cells and the 26-KD species of human cells will be compared in order to ascertain their relationship.

The finding of a low molecular weight acidic polypeptide preferentially synthesized in four independently-derived vincristine-resistant sublines of three different mammalian species suggests that the protein may be of general significance in development of resistance to vincristine.

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