Increased dihydrofolate reductase, double minutes and increased nucleolar activation in methotrexate-resistant HeLa cells

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Summary. HeLa cells resistant to methotrexate exhibit increased production of dihydrofolate reductase, double minutes and increased activity of the nucleolar organizing regions.

There is evidence that the synthesis of excess dihydrofolate reductase (DHFR) in various types of methotrexate (MTX) - resistant cells is due to amplification of the DHFR gene[2-6]. At the cytological level, the amplified genes have been localized either to expanded chromosome regions on single chromosomes, called 'homogeneously staining regions, HSRs'[7], or to small, paired acentric chromosomal elements, called 'double minutes, DMs'[3-8]. HSRs and DMs may represent two alternative aspects of the same phenomenon, since they can be converted into each other[9-13]. In this report we present evidence that production of DMs as well as increased nucleolar activation can be induced in HeLa cells when resistance to methotrexate is acquired.

Materials and methods. Both sensitive and resistant HeLa cells were grown in Mc Coy's 5a medium, supplemented with 10% fetal calf serum, penicillin 10^5 IU/l, streptomycin 10^5 µg/l, and amphotericin B 2 mg/l. All cells were free of mycoplasma and other contaminations as shown by ^{3}H-thymidine labeling, autoradiography, and microscopy[14]. Methotrexate (Lederle, USA) was added to the medium in the form of a concentrated solution to give the desired concentration prior to use. Concentrated MTX solutions were stored at −20 °C. Chromosome preparations of cells grown in MTX-free medium for two cell generations were stained for G-banding[15] or for nucleolar organizing regions (NOR)[16]. G-banded metaphases were examined for the presence of HSRs and DMs. Ag-stained metaphases were examined for the presence and intensity of NOR staining. The latter was estimated visually as absent (0), small (1), medium (2), and large or extra large (3)[17]. As silver staining is positive only for NORs that were functionally active during the preceding interphase[18], the amount of silver deposited on NORs can be considered as a measure of the intensity of nucleolar activation. Dihydrofolate reductase (DHFR) was assayed in cell lysates after electrophoresis on polyacrylamide gels. Cell lysates were prepared by sonication of cell suspensions (10^8 cells/ml in distilled water) and centrifuged at 2500 rpm for 15 min. The supernatant was diluted 2:1 with 40% sucrose to give a dense sample. Gels were loaded with samples containing 50-200 µg of protein as estimated by the Lowry assay[19]. Electrophoresis was carried out using the procedure of Davis[20], omitting the spacer and sample gels. The gels were stained for DHFR according to the procedure described by Huennekens et al.[21] and examined for band color intensity and R_f.

Results. HeLa cell lines resistant to doses of methotrexate ranging from 0.5 to 100 µg/ml were developed by stepwise increasing of the concentration of MTX starting from 0.1 µg/ml (LD_{50}). Three lines with acquired resistance to MTX were used in the present study: HeLa-MTX_1, HeLa-MTX_{10}, and HeLa-MTX_{100}; resistant to 1, 10, and 100 µg/ml respectively. The DHFR activity for these lines on polyacrylamide gels appeared as a band of significantly increasing color intensity with an increasing level of resistance. The parental HeLa-s showed no obvious DHFR activity.

Examination of metaphase preparations showed that DMs were present in all the 3 cell lines, in numbers increasing with higher levels of resistance: HeLa-MTX_1 cells contained DMs in 61% of the metaphases examined in num-

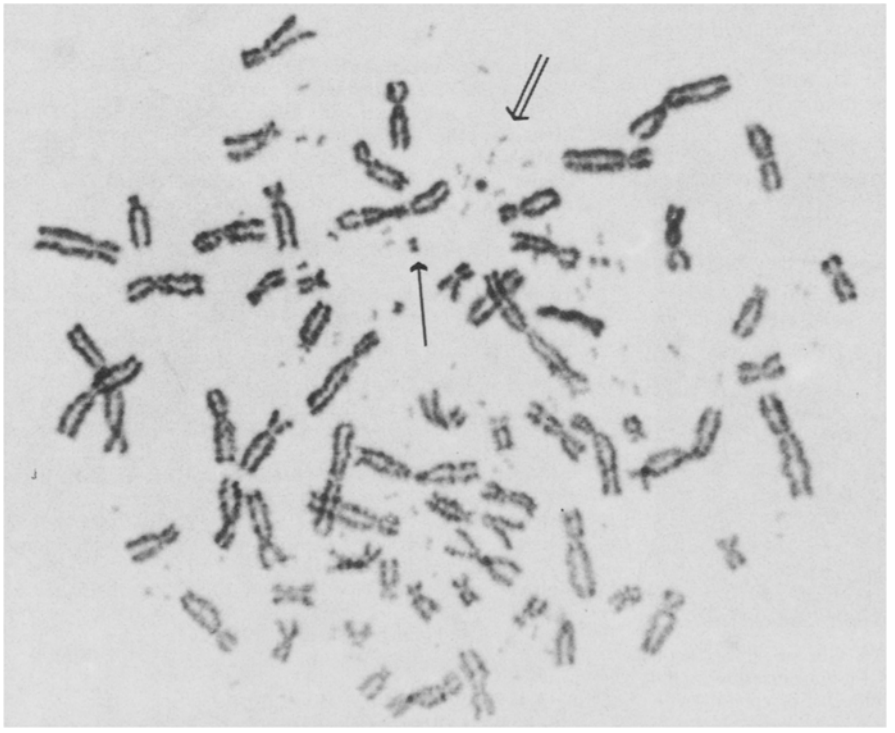

Figure 1. Metaphase of a HeLa-MTX_{100} cell containing numerous DMs. Note the presence of large (single arrow) and small DMs (double arrow). Giemsa staining.

bers varying from 2 to 32 DMs/cell (mean: 11); HeLa-MTX_{10} contained DMs in 90% of the metaphases examined in numbers varying from 6 to 220 DMs/cell (mean: 51); HeLa-MTX_{100} contained DMs in 100% of the metaphases examined in numbers varying from 4 to 260 DMs/cell (mean: 85) (80 cells were counted in each case) (fig. 1). These numbers did not change significantly after 5 months in culture. The parental HeLa-s cells exhibited neither DMs nor HSRs.

The mean number of Ag-stained acrocentric chromosomes per cell was 3.2 for MTX-sensitive HeLa, 3.4 for HeLa-MTX_1, 3.3 for HeLa-MTX_{10}, and 3.2 for HeLa-MTX_{100} (80 cells were counted in each case). The amount of Ag-stain scored according to Markovic et al.[14] was 1.2 for HeLa-s, 2.4 for HeLa-MTX_1, 2.8 for HeLa-MTX10, and 2.2 for HeLa-MTX_{100}. Figure 2 shows the remarkable amount and intensity of Ag-stain in a HeLa-MTX_{100} metaphase. In most metaphases the amount of Ag-stain deposited on the NORs was not equally distributed on the two short arms of the acrocentric chromosomes (Fig. 2, insert a). In some metaphases the Ag-stained regions of two different chromosomes or both arms of one chromosome were associated, forming a large mass (fig. 2, insert b). The NOR activity was most frequently distributed in 1-3 group D chromosomes and in 1-2 group G chromosomes in both MTX-resistant and sensitive cells.

Discussion. The interest in double minutes has been enhanced since they have been found in several types of human tumors[8,13] and have been determined as products of a complex process of gene amplification[2-5,22,23]. The fact that DMs have been found in various short- or long-term cultures of tumor cells[8,24,25] and also in stromal cells of tumors[26] encourages the belief that gene amplification may occur in these cells as a primary or secondary effect of the malignant process.

The HeLa-MTX cell lines described in this study are shown to contain DMs consistently and numerously. Therefore, they could be useful in further studies on the mechanism of origination, properties, and function of DMs.

The profound increase of the nucleolar activation in HeLa-MTX cells could be attributed to the need for synthesis of large amounts of rRNA. As the number of chromosomes carrying active NORs was not significantly different for MTX-resistant and sensitive HeLa cells, it can be postulated that the existing nucleolar activity in sensitive HeLa has been increased in the resistant cells. Variations in the activity of NORs have also been shown to occur in various tissues[27,28]. Since acrocentric chromosome associations have been correlated with nucleolus organization and NOR activity[18], and non-randomness of associations may indicate a genetic heterogeneity among different satellites[29], the HeLa-MTX lines produced may also be useful in finding out relationships between NOR activity level and chromosome a-sociations.

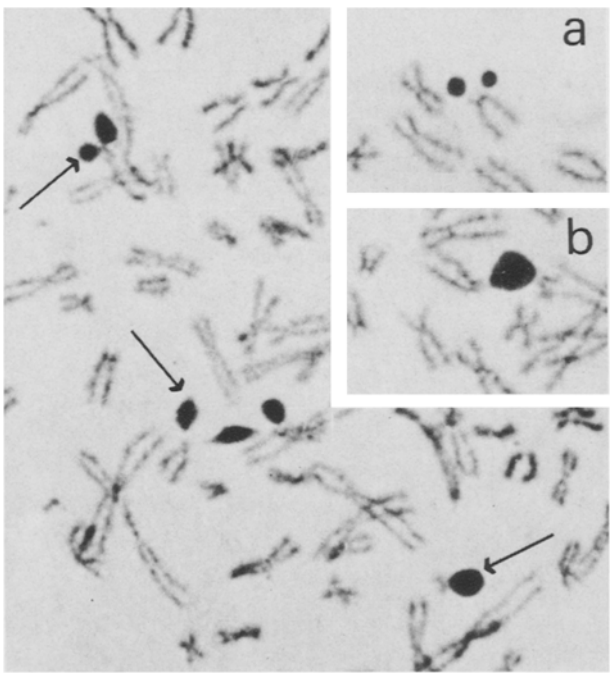

Figure 2. Ag-stained partial metaphase of a HeLa-MTX_{100} cell exhibiting remarkable amount and intensity of Ag-stain (arrows). Insert *a*: Partial metaphase of a HeLa-MTX_{10} cell showing unequal distribution of the Ag-stain on the two short arms of a D chromosome. Insert *b*: Partial metaphase of a HeLa-MTX_1 cell showing association of the Ag-stained regions of one G and one D chromosome.

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