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"DOUBLE MINUTE CHROMOSOMES IN A 6-MP RESISTANT MOUSE LEUKEMIA L1210 CELL LINE"

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ABSTRACT

6-Mercaptopurine (6-MP) resistant strains of L1210 v/c murine leukemic cells were obtained *in vivo* 6-MP treatment of a DBA/2 mouse inoculated with sensitive L1210 cells. In one of these 6-MP resistant strains, double minutes (DMs) were found in 12% of the metaphase cells, the number of DMs per cell ranging from 5 to 20. When these 6-MP resistant cells were subcultured in 6-MP free medium for 20 months, only 2% of the cells had DMs ranging from 1 to 5 per cell. The decrease of DMs in number and appearance rate was accompanied by a lowering of the maximum allowable concentration (MAC) of 6-MP necessary to induce resistance in the cells from 1 μg to 0.1 μg/ml. These observations suggest that 6-MP resistance in L1210 cells is the result of an unknown gene amplification.

INTRODUCTION

Gene amplification is a common mechanism for achieving drug resistance in mammalian cells[19]. Stepwise selection of cultured cells in increasing concentrations of methotrexate (MTX) results in cells with increased levels of the target enzyme for MTX inhibition, dihydrofolate reductase (DHFR), and an amplification in the DHFR gene[1]. In these MTX resistant cells, chromosome aberrations such as the homogeneously staining region (HSR) or double minutes (DMs) were observed, and demonstrated to be the sites of amplified DHFR genes by *in situ* hybridization[19].

HSR or DMs were also reported in human tumor cells, especially in neuroblastomas[6,16]. Molecular analysis of HSR and DMs in this tumor demonstrated

that these abnormalities are the cytological manifestation of gene amplification [11,17].

We have examined the mechanism whereby cultured murine leukemic cells (L1210) become resistant to the carcinostatic purine analogue, 6-mercaptopurine. This chemotherapeutic agent used in the treatment of human leukemia frequently induces resistance in tumor cells[7].

We observed DMs in 6-MP resistant L1210 cells, suggesting that the amplification of an unknown gene is involved in acquiring the resistance.

Materials and Methods

The L1210 v/c murine leukemia cell line (2n=39), kindly supplied by Dr. M. Shimoyama (National Cancer Research Institute, Tokyo), was usually maintained in RPMI 1640 medium supplemented with 50 U/ml penicillin, 50 μg/ml streptomycin and 20% fetal calf serum. For the in vivo experiment, 10^6 viable cells were injected intraperitoneally into male or female DBA/2 mice (5-6 weeks, 21±1 g). The mice survived for an average period of 7-8 days.

6-MP treatment was started 5 days after cell inoculation. To continue successive therapy by 6-MP, 10^4 of L1210 cells in ascites fluid were transplanted into a new mouse. The 6-MP dose was increased from 1 to 20 mg/kg when the L1210 cells were transferred to the new mouse. At every transplantation, the presence of 6-MP resistant cells was assayed on soft agar plates with 6-MP (5 μg/ml). After purification of cells resistant to 6-MP on an agar plate without 6-MP for 5 days (about 10 generations), the resistant level was measured by changing the concentration of 6-MP from 1 to 30 μg/ml. The resistant level was expressed by maximum allowable concentration (MAC) on colony formation and classified into three groups: high resistance strains (MAC$\geqq$30 μg/ml), low resistance strains (MAC=1.0-0.1 μg/ml) and sensitive strains (MAC$\leqq$0.01 μg/ml).

To obtain chromosome preparations, L1210 cells in ascites fluid were harvested and treated with 0.25 μg/ml colchicine for 15 min. Then the cells were spun down and resuspended in 0.075 M KCl at 37°C for 15 min. The cells were collected by centrifugation and fixed with methanol-acetic acid (3 : 1) for three changes. Metaphase chromosomes were differentially stained by G-banding[13] and by C-banding method[21].

Results

Twenty-four 6-MP resistant L1210 strains were isolated from ascites fluid and subcultured in RPMI liquid medium. After one month of subculture of the resistant cell line in the absence of 6-MP, 60% of the high resistance strains retained their resistance against 30 μg/ml of 6-MP, though only 14% of the low resistance strains maintained a resistance to 1.0-0.1 μg/ml of 6-MP. Although most of the low resistance strains became 6-MP sensitive, very few shifted to a high resistance strain.

Karyotypes were analysed in these resistant strains for the presence of HSR or DMs. No drastic karyotype changes were observed except for strain #16, in which DMs were

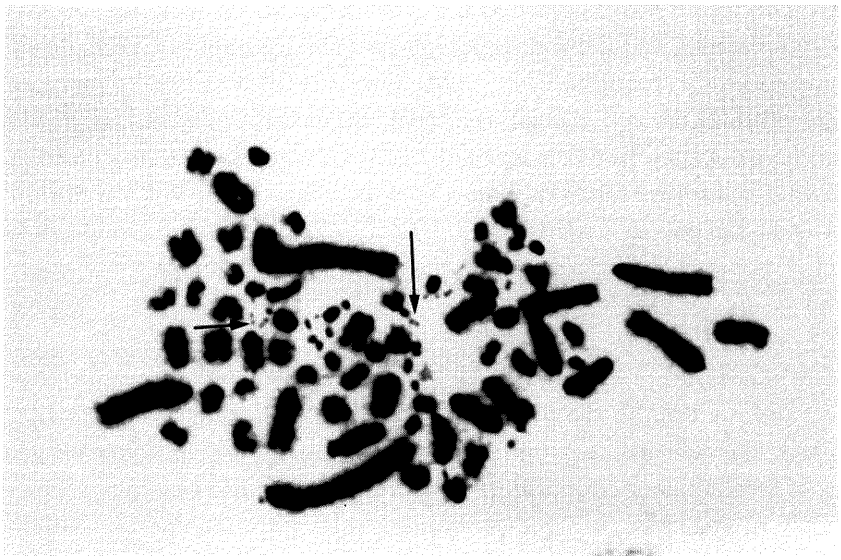

Fig. 1. G-banded metaphase of the strain #16 of L1210 v/c, showing the double minute chromosomes. (arrows)

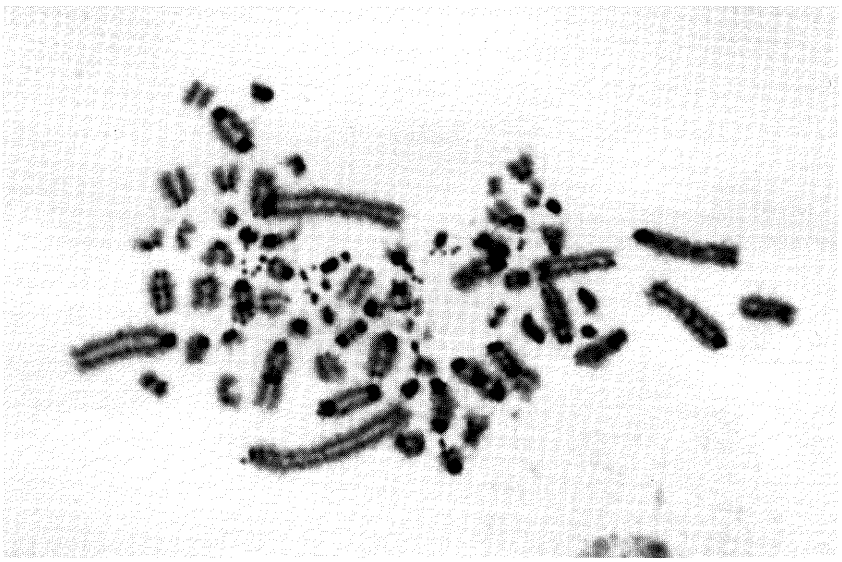

Fig. 2. C-banded metaphase of strain #16 indicating the absence of centromeric heterochromatin.

observed. This strain was classified as a low resistance strain (1 μg/ml of 6-MP). Figs. 1 & 2 show G-banded and C-banded metaphase cells of strain #16, revealing a lack of centromeric heterochromatin in DMs. DMs were identified in 12% of metaphase cells, ranging from 5 to 20 per cell, though the modal chromosome number was 2n=39 with no major karyotype change from the original L1210 cells (Table 1).

Even after subculture for twenty months in the absence of 6-MP, 2% of the cells still exhibited DMs ranging in number from 1 to 5. Consistent with the decrease in the number of DMs per cell after subculture, the resistant level against 6-MP dropped from 1 μg/ml to 0.1 μg/ml (Table 2).

Discussion

Appearance of DMs in the 6-MP resistant murine leukemia cell line L1210 (# 16) indicates that the cell acquired the resistance by amplifying an unknown gene. The resistance of human tumor cells against 6-MP *in vivo* may also be explained by the same amplification mechanisms.

It is now widely accepted that the observed DMs or HSR are the result of gene amplification in both drug resistant cells and tumor cells[10,20]. In MTX resistant cell lines, the presence of the amplified DHFR gene in the DMs or HSR has been demonstrated by *in situ* hybridization[18]. At present, more than 10 drugs are known to induce resistance in the mammalian cells by gene amplification, accompanied by DMs or HSR. For instance, the CAD (Carbamyl phosphate synthetase, Aspartate transcarbamylase, Dihydroorotase) gene in PALA (Phosphonacetyl-L-aspartate) resistance[22], methallothioneins gene in cadmium resistance have been studied in detail[4]. In colchicine or vincristine

Table 1. Distribution of chromosome numbers and appearance rate of DMs. #16 strain was subcultured in liquid medium without 6-MP every fourth day.

Time (month)	Cell line (L1210)	Range	Modal No. of chromosome	Rate of modal No. of chromosome (%)	No. of metaphase analyzed	Rate of DMs(%)	Range of DMs number in a cell
0	Control	38-99	39	72	100	0	
	#16	36-83	39	66	100	12	5-20
20	Control	37-99	39	68	100	0	
	#16	37-105	39	80	100	2	1-5

Table 2. Decrease of MAC level in #16 strain after 20 months subculture. Each figure indicates the number of the colonis. - ; not done

6-MP (μg/ml) / Time (month)	0	0.01	0.1	1	10
0	136±16	—	139±11	133±19	0
20	79±10	82±18	72±2	5±2	0

resistant cell lines, DMs or HSR were found in the metaphase cells, though the amplified gene has not been clarified[3,15].

Since the demonstration of amplified genes in DMs or HSR in MTX resistant cells[19], it has been suggested that the DMs or HSR observed in human tumor cells are cytological manifestations of gene amplification[5]. This supposition has been demonstrated by cloning the amplified genes from DMs or HSR and doing further analysis by *in situ* hybridization in human neuroblastoma [11,17] and in the mouse adenocarcinoma cell line Y -1[8]. These observations indicate that DMs or HSR result from chromosome aberration induced by gene amplification in general.

In the present study, DMs appeared only in strain # 16, and other 6-MP resistant strains lacked DMs or HSR. We speculate that these strains also become resistant by an unknown gene amplification. There may exist small DMs, which can not be identified by light microscopic analysis, or small HSR in the chromosomes, which are undetectable in the conventional G-banded chromosomes. Indeed, the presence of the small DMs, which are about 5,000 Kb in size and unidentifiable in cytological preparations for light microscopy, has been noted in the MTX resistant mouse 3T3 cells[9]. Furthermore, in one of the human neuroblastoma cell lines TNB-1, neither DMs nor HSR were distinguished in the metaphase cells[12], though N-myc[14], clone # 8[11] and pG 21[2] are coamplified about a hundred fold in the genome (Kanda, unpublished data). These observations suggest that the absence of DMs or HSR in the cytological preparations does not necessary mean the lack of amplified DNA.

In strain # 16, the number of DMs per cell decreased from 5-20/cell to 1-5/cell after a twenty-month subculture *in vitro*. This suggests that amplification is still unstable even in the resistant cells with DMs, consistent with the observations noted in the MTX resistant mouse cell [18].

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