MECHANISMS AFFECTING PEPLOMYCIN SENSITIVITY OF CHINESE HAMSTER CELL LINES

SHOGO OZAWA, AKITERU TAMURA, HIDEO SUZUKI, TOSHIO NISHIMURA and NOBUO TANAKA

Institute of Applied Microbiology, University of Tokyo, Tokyo 113, Japan

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Chinese hamster lung cell line V79 was *ca.* 13 times more resistant to peplomycin (PEP), and 6 times more resistant to bleomycin (BLM)-A_2 than Chinese hamster ovary (CHO) cell line. The natural resistance of V79 cells to PEP or BLM was attributed to higher levels of BLM hydrolase activity and lower cellular uptake of the antibiotic. The sensitivity to PEP of a mutant clone CHO/O-2 T-1 was similar to that of CHO. A hybrid clone of CHO/O-2 T-1×V79 showed an intermediate sensitivity to PEP between those of both parental cell lines, suggesting that the gene responsible for the natural resistance to PEP appears codominantly in the hybrid. The BLM hydrolase activity of the hybrid was also found intermediate between those of both parental cells.

Mutant clones CHO/O-2 T-5 and CHO/O-2 T-6 were 8.3~9.0 times more sensitive to PEP than CHO cells. Hybrid clones CHO/O-2 T-5×V79 and CHO/O-2 T-6×V79 displayed PEP sensitivity similar to that of V79, suggesting that the gene responsible for the PEP supersensitivity (PEP^{ss}) behaves recessively in the hybrids. Both PEP^{ss} clones showed levels of BLM hydrolase and cellular uptake of [^{3}H]PEP similar to the parental CHO cells, suggesting that the PEP^{ss} is due to neither BLM hydrolase nor cellular uptake of the antibiotic. Increased PEP-induced DNA cleavage and decreased DNA repair in the PEP^{ss} clones were demonstrated by alkaline sucrose density gradient sedimentation method. The results suggest that the PEP^{ss} of these mutant clones is attributed to decreased DNA-repairing activity and/or increased DNA-breaking activity.

Bleomycin (BLM) is widely used in the treatment of human squamous cell carcinomas, testicular carcinoma, and malignant lymphoma. The antibiotic is less effective for adenocarcinomas and sarcomas. The diverse sensitivity to BLM of various tumors is attributed to the drug uptake and BLM hydrolase activity (*cf.* a monograph[1]). However, the detailed mechanism of BLM sensitivity of various tumors still remains open to discussion.

MIYAKI *et al.*[2] reported that BLM-resistant rat ascites hepatoma cells show higher BLM hydrolase activity than BLM-sensitive hepatoma cells. AKIYAMA and KUWANO[4] isolated BLM-resistant mutants of Chinese hamster ovary (CHO) cells, due to high BLM hydrolase activity. We obtained BLM-resistant cell lines of murine lymphoma L5178Y, in which the resistance is attributed to alteration of the plasma membrane transport system[3].

We have studied the mechanism of action of BLM[5,6], and are interested in the mechanism of BLM resistance of tumor cells[3]. In the current studies, we mainly used peplomycin (PEP), an antibiotic of the BLM group, which contain [3-((*S*)-1-phenylethyl)aminopropyl]amino group[12]. We have found that Chinese hamster lung fibroblast-like cell line V79 is more resistant to PEP than CHO, and have also isolated PEP-supersensitive (PEP^{ss}) mutants of CHO cells. We have further studied these mechanisms as models of a variety of PEP or BLM sensitivity of human neoplasms[1]. The results are presented in this publication.

Materials and Methods

PEP and [^{3}H]PEP (phenyl-*m*-^{3}H, 250 μCi/mg)[7] were kindly provided by Nippon Kayaku Co., Ltd., Tokyo, Japan.

Cells and Culture Conditions

CHO and V79 cell lines were generously given by Prof. S. OKADA, School of Medicine, University of Tokyo. The cells were cultured in McCoy 5A medium supplemented with 10% calf serum and 5% fetal calf serum at 37°C in atmosphere of 5% CO_2 and 95% air.

Drug Sensitivity

The drug sensitivity was expressed by IC_{90}: *i.e.* drug concentrations reducing relative plating efficiency of a cell line to 10% of that obtained in the absence of drugs. Plating efficiency was determined by plating 200 cells in a well of 6-well plastic plates. After 6 to 8 days of incubation at 37°C, the plates were stained by 0.1% crystal violet, and colonies were scored. The relative plating efficiency was calculated as the ratio of the colony number at a given drug concentration to that obtained in the absence of drugs.

Bleomycin Hydrolase

Bleomycin hydrolase activity was determined, using HPLC[8] and bioassay, using *Bacillus subtilis* ATCC 6633 as a test organism[4]. Cells were homogenized with 1/15 M phosphate buffer (pH 7.2) in a Teflon homogenizer, and centrifuged at 105,000×*g* for 60 minutes. The supernatant was used as an enzyme solution, which (0.45 ml) was added to 0.05 ml of 8 mg PEP/ml solution, and incubated for 2 hours at 37°C. The reaction was terminated by addition of 0.5 ml of MeOH. The reaction mixture was centrifuged at 3,000 rpm for 10 minutes, and the supernatant was bioassayed by a paper disc method. For HPLC, 2 mg of cupric carbonate was added to the supernatant, and 20 μl of each sample was injected into HPLC column. HPLC conditions; Column, 4.6×250 mm, Senshu pak Aquasil SS-352N, particle size 5 μm (Senshu Scientific Co., Ltd., Tokyo). Mobile phase, MeOH - acetonitrile - 20% ammonium acetate - AcOH, 620: 380: 100: 0.5.

Cellular Uptake of [^{3}H]PEP[9]

Cells, harvested by trypsinization (10^7/ml), were incubated in the culture medium with [^{3}H]PEP (1.02 μg/ml) at 37°C for various periods. The uptake was terminated by rapid sedimentation, using an Eppendorf centrifuge 5412. 1.0 ml of cell suspension was added on 200 μl of an oil mixture, consisting of 80 parts of silicone oil SH500 and 20 parts of liquid paraffin. The mixture was sedimented at 15,000 rpm for 30 seconds. The cell pellet was solubilized with 0.5 ml of Protosol (New England Nuclear) by incubation overnight at 37°C. The radioactivity was determined in a liquid scintillation spectrometer, using scintillation fluid Dimilume-30 (United Technologies Packard).

Efflux of [^{3}H]PEP

The efflux of [^{3}H]PEP from cells was examined, following the method previously described[10]. Cells (10^6/ml) were incubated with [^{3}H]PEP (0.24 μg/ml) for 30 minutes at 37°C in the culture medium. Then a part of the cells was sedimented for determining the radioactivity incorporated as described above. The residual cells were washed 3 times by centrifugation, resuspended in the fresh medium (10^6/ml), and incubated for 30 minutes at 37°C. The radioactivity remaining in the cells, before and after incubation, was analyzed as described above.

Isolation of Ouabain- and 6-Thioguanine-resistant Clones

The parental CHO cells were treated with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) (0.5 μg/ml) for 3 hours at 37°C in the culture medium, and further incubated in MNNG-free medium for 3 days at 37°C. Then several ouabain-resistant clones were selected by 1 mM ouabain, and then treated with MNNG in a similar way to obtain ouabain- and 6-thioguanine-resistant clones.

Cell Hybridization

Hybrid cells of V79 and CHO were constructed as described previously[11]. V79 cells of 3×10^6 were mixed with the same number of CHO cells, and treated with 50% polyethylene glycol 4000 in

Table 1. Sensitivity of CHO and V79 cell lines to various antitumor agents.

Agent	IC_{90} (μg/ml) of		Ratio of IC_{90}
	V79	CHO	V79/CHO
Peplomycin	1.9	0.15	13
Bleomycin A_2	2.7	0.48	5.5
Aclarubicin	0.030	0.029	1.0
Doxorubicin	0.080	0.13	0.62
Mitomycin C	0.014	0.016	0.85
Actinomycin D	0.0013	0.0023	0.57
Neothramycin	0.063	0.13	0.50
Arabinosylcytosine	0.033	0.11	0.31

Table 2. BLM hydrolase activity of V79 and CHO cell lines.

Cell line	Cell extract	BLM hydrolase
V79	0.5*	4.1**
	1.0	3.6
CHO	0.5	1.8
	1.0	1.7

* mg protein/ml.

** Specific activity (μg PEP hydrolyzed/mg protein/minute).

BLM hydrolase was assayed by HPLC method.

serum-free McCoy 5A medium for 1 minute at 37°C. The cells were then washed and incubated in the same medium supplemented with 10% calf serum, 5% fetal calf serum, 100 μM hypoxanthine, 0.4 μM aminopterin, 16 μM thymidine and 1 mM ouabain (selection medium). Usually several hybrid cells were obtained out of 10^5 parental cells.

Chromosome Number

The chromosome number was determined in 100 individual metaphase spreads by the ordinary method. The cells ($1 \sim 2 \times 10^6$) were cultured in 10 ml of the culture medium with 0.2 μg Colcemid/ml for 2 hours at 37°C in a dish of 100 mm diameter. The cells were trypsinized, lysed in a hypotonic KCl, fixed in acetic methanol, and stained with Giemsa solution.

Alkaline Sucrose Gradient Sedimentation Analysis of DNA Strand Scission *In Vivo*

DNA breakage in the intact cells treated with PEP was examined, following the procedure described previously[13]. In each plate, the cells (*ca.* 10^6) were incubated with 0.5 μCi of [^{3}H]thymidine for 16 hours, washed, and then incubated with various concentrations of PEP for an hour at 37°C in the culture medium described above. The cells were harvested by trypsinization (*ca.* 10^5/0.2 ml) and gently applied on the top of 5~20% (w/v) alkaline sucrose gradient in 4.2 ml of 0.3 M NaOH, 0.7 M NaCl and 0.01 M EDTA, on which 0.2 ml of lysis solution (0.5 M NaOH, 0.02 M EDTA and 0.1% Triton X) had been layered. After standing at room temperature for 10 hours, centrifugation was carried out in a Beckman SW 50L rotor at 30,000 rpm for 90 minutes at 20°C. Samples were fractionated in 0.3 ml from the top of the gradient with an ISCO fractionator, Model 640. Each fraction was precipitated, after addition of 100 μg of bovine serum albumin as a carrier, by cold 5% TCA and the radioactivity was determined in a liquid scintillation counter.

Alkaline Sucrose Gradient Centrifugation Analysis of Repair of DNA Damage

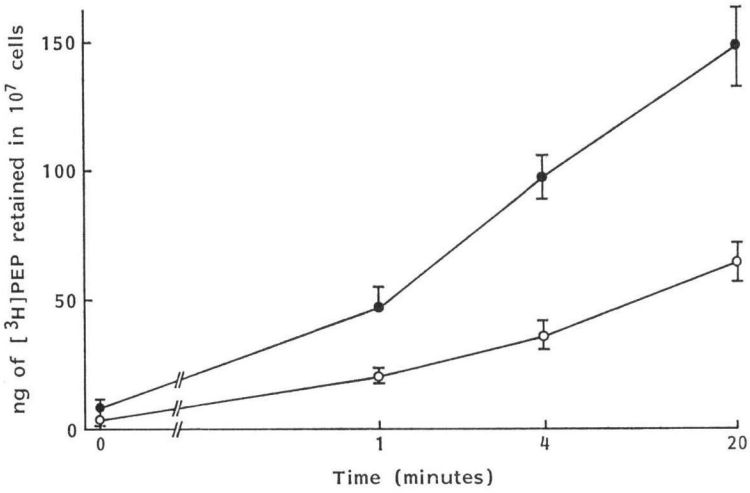
The cells, labeled with [^{3}H]thymidine as described above, were incubated in the culture medium with PEP (100 μg/ml) for 2.5 hours, and then in the PEP-free medium for various periods at 37°C. The size of DNA was analyzed by alkaline sucrose density gradient sedimentation as described above, except that fractions in 0.2 ml were collected.

Results

Drug Sensitivity of V79 and CHO Cell Lines

The drug sensitivity of V79 cells to various antitumor drugs was compared with that of CHO cells. The IC_{90} values, which reduce relative plating efficiency of the cells to 10% of control, are presented in Table 1. V79 cells were approximately 13 times more resistant to PEP and 6 times to BLM-A_2 than CHO cells. On the contrary, both cell lines showed equal sensitivity to aclarubicin, and V79 cells were a little more sensitive to doxorubicin, mitomycin C, actinomycin D, neothramycin

Fig. 1. Cellular uptake of [^{3}H]PEP by CHO (●) and V79 (○) cells.



or arabinosylcytosine than CHO cells. The results suggest that the natural resistance of V79 cells is limited to the bleomycin group of antibiotics.

BLM Hydrolase Activity of V79 and CHO Cells

For the purpose of elucidating the mechanism of BLM resistance of V79 cells, BLM hydrolase activity of cell extracts (0.5 or 1.0 mg protein/ml) was studied by HPLC and bioassay, using PEP as a substrate. The results obtained by HPLC method are presented in Table 2, and are in accordance with those by bioassay (Table 4). V79 cells displayed 2.1~2.3 times higher BLM hydrolase activity than CHO cells. The results suggest that BLM resistance of V79 cells is partially due to higher BLM hydrolase activity.

Cellular Uptake and Efflux of [^{3}H]PEP

The time course of [^{3}H]PEP uptake by V79 or CHO cells was studied in connection with the mechanism of BLM resistance of V79 cells, and the results are illustrated in Fig. 1. Cellular uptake of [^{3}H]PEP for 20 minutes was 148 ng/10^7 CHO cells, and 64.8 ng/10^7 V79 cells. The former was 2.3 times higher than the latter.

Efflux of [^{3}H]PEP from V79 or CHO cells occurred at the same rate (Fig. 2). [^{3}H]PEP retained in 10^6 CHO cells after 30 minutes reincubation in the fresh medium was 11.8 ng, and that in 10^6 V79 cells 5.28 ng. The retention of PEP in CHO cells was 2.2 times higher than that in V79 cells.

The results suggest that BLM resistance of V79 cells may also be attributed to decreased uptake and retention of PEP.

Sensitivity to PEP of Ouabain-Thioguanine-Resistant CHO Sublines and their Hybrid Cells with V79

CHO cell sublines resistant to ouabain and 6-thioguanine were established, for the purpose of constructing CHO×V79 hybrid clones. As presented in Table 3, the IC_{90} values of PEP were 0.14 μg/ml for parental CHO cells and 0.21 μg/ml for CHO/O-2 T-1 cells. Both cell lines showed a similar sensitivity to PEP. V79 cells were more resistant to PEP; the IC_{90} was 1.2 μg/ml.

Table 3. Sensitivity to PEP of ouabain-thioguanine-resistant CHO sublines and their hybrids with V79 cells.

Cell line	Chromosome number	Sensitivity to PEP IC_{90} (μg/ml)	Gene
CHO	21	0.14 (S)*	
CHO/O-2 T-1	21	0.21 (S)	
V79	22	1.2 (R)	
Hybrid (O-2 T-1 × V79)	43	0.81	Codominant
CHO/O-2 T-5	21	0.017 (SS)	
CHO/O-2 T-6	21	0.015 (SS)	
Hybrid (O-2 T-5 × V79)	46	1.1 (R)	Recessive
Hybrid (O-2 T-6 × V79)	44	0.81 (R)	Recessive

* Sensitivity to PEP: S; PEP-sensitive, R; PEP-resistant, SS; PEP-supersensitive.

Fig. 2. Efflux of [^{3}H]PEP from CHO (●) and V79 (○) cells.

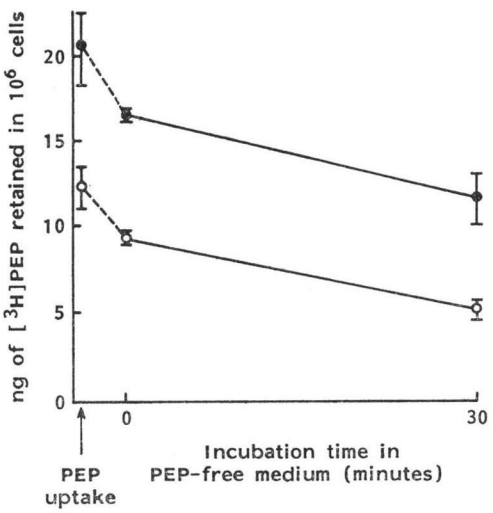


Table 4. BLM hydrolase activity of the hybrid of CHO/O-2 T-1 and V79 cells.

Cell line	BLM hydrolase
V79	3.7*
CHO/O-2 T-1	1.7
Hybrid (V79 × CHO/O-2 T-1)	2.3

* Specific activity (μg PEP hydrolyzed/mg protein/minute).
BLM hydrolase was determined by bioassay.
The cell extract used was 1.0 mg protein/ml.

The IC_{90} value of PEP for CHO/O-2 T-1 × V79 hybrid clone was 0.81 μg/ml, which was intermediate between those for CHO/O-2 T-1 and V79 cells (Table 3). The dose-response curve of O-2 T-1 × V79 hybrid clone for PEP was located between those of CHO/O-2 T-1 and V79 cells (data are not shown). The results suggest that the gene, responsible for natural resistance to PEP, appears codominantly in the hybrid cells.

The BLM hydrolase activity of the hybrid was found intermediate between those of parental cell lines (Table 4). The result is in accord with the above assumption.

The CHO/O-2 T-5 and CHO/O-2 T-6 sublines were 8.3~9.0 times more sensitive to PEP than the parental cells: *i.e.* IC_{90} were 0.017~0.015 μg/ml (Table 3).

The CHO/O-2 T-5 × V79 and CHO/O-2 T-6 × V79 hybrid clones displayed IC_{90} of 1.1~0.81 μg/ml, which were similar to IC_{90} for V79 cells, suggesting that the gene responsible for PEP^{SS} phenotype behaves recessively in the hybrid cells.

All the hybrid clones, described above, showed chromosome numbers nearly additive for the parental cell lines before cell-to-cell fusion (Table 3).

Sensitivity of PEP^{SS} Cells to Various Drugs

The sensitivity of CHO/O-2 T-6 (PEP^{SS} cells) to various agents was compared with that of the parental CHO cells, in order to examined whether CHO/O-2 T-6 cells exhibited supersensitivity to

Table 5. Comparison of drug sensitivity of CHO and its mutant O-2 T-6. IC_{90} (μg/ml) values.

Agent	Cell line	
	CHO	CHO/O-2 T-6
Peplomycin	0.14	0.015
Actinomycin D	0.0040	0.0040
Mitomycin C	0.020	0.020
Aclarubicin	0.060	0.050
Doxorubicin	0.096	0.094
Neothramycin	0.20	0.20
Arabinosylcytosine	0.15	0.15
4-Nitroquinoline oxide	1.8	0.96

Table 6. BLM hydrolase activity of cell extracts of parental and PEP-supersensitive cell lines of CHO.

Cell line	BLM hydrolase
CHO	0.61*
CHO/O-2 T-5	0.60
CHO/O-2 T-6	0.81

* Specific activity (μg PEP hydrolyzed/mg protein/minute).
The cell extract used was 3.0 mg protein/ml.

Fig. 3. Cellular uptake of [^{3}H]PEP by CHO (●) and CHO/O-2 T-6 (○) cells.

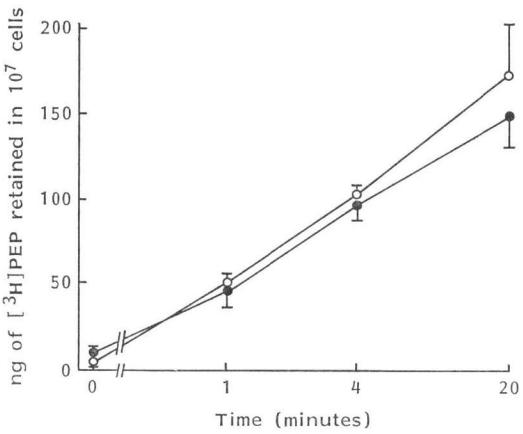
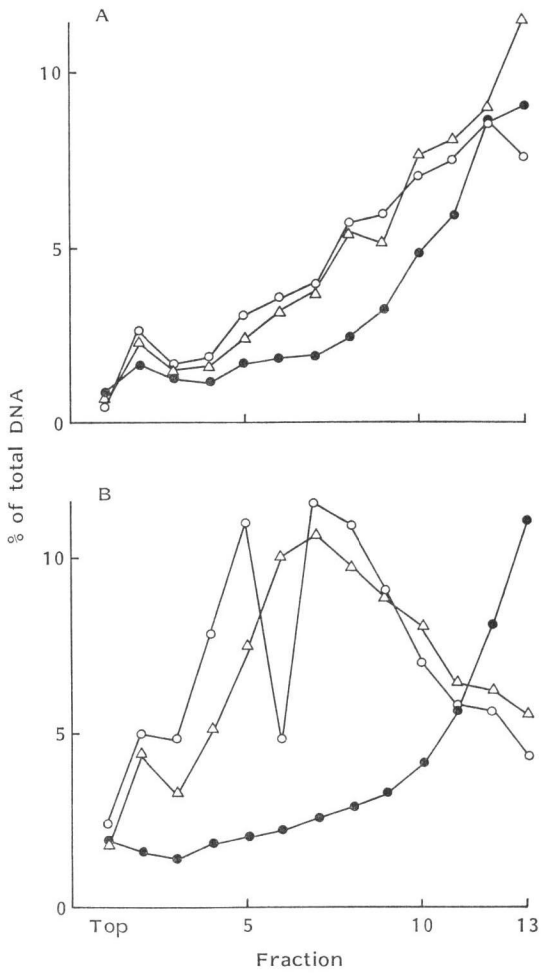


Fig. 4. DNA strand scission induced by PEP in CHO (A) and CHO/O-2 T-6 (B) cells: Sedimentation profiles.
○ 50 μg PEP/ml, △ 25 μg PEP/ml, ● 0.



other drugs. The results are summarized in Table 5. Both cell lines displayed similar sensitivity to actinomycin D, mitomycin C, aclarubicin, doxorubicin, neothramycin, and arabinosylcytosine. CHO/O-2 T-6 cells were 1.9 times more sensitive to 4-nitroquinoline oxide than the parental cells, but the difference of sensitivity was not so remarkable as that to PEP. The results indicate that the drug hypersensitivity of CHO/O-2 T-6 cells is limited to PEP and BLM.

BLM Hydrolase Activity of PEP^{ss} Clones

For the purpose of elucidating the mechanism of PEP^{ss} of CHO/O-2 T-5 and CHO/O-2 T-6 cells, the BLM hydrolase activity was determined by HPLC method in comparison with that of the parental cells (Table 6). Both PEP^{ss} clones showed similar levels of the enzyme activity as the parental

Fig. 5. Repair of PEP-induced DNA cleavage in CHO (A) and CHO/O-2 T-6 (B) cells: Centrifugation profiles of DNA.

The time represents incubation period in PEP-free medium.

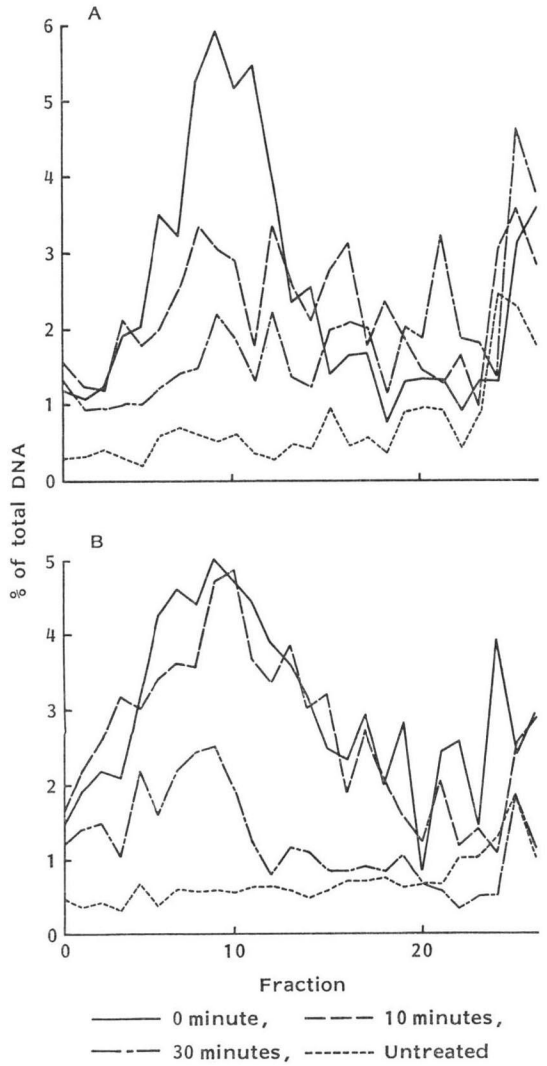


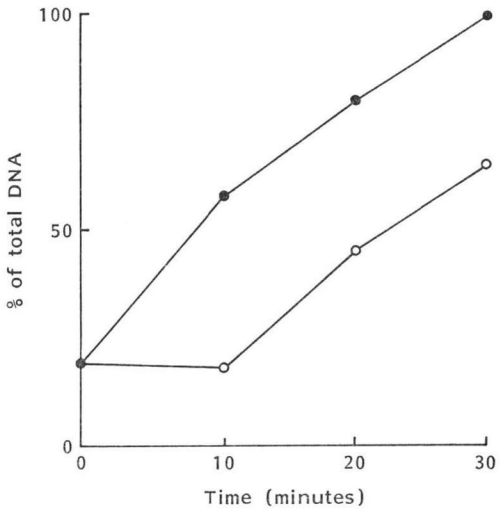
Table 7. DNA cleavage induced by PEP in PEP^{SS} cell line in comparison with that in the parental cell line: Large size DNA content sedimented to the bottom of centrifugation tubes (fractions 13~16).

PEP (μg/ml)	Cell line	
	CHO	CHO/O-2 T-6
0	67*(1.00)**	62*(1.00)**
25	50 (0.74)	18 (0.29)
50	49 (0.73)	13 (0.21)

* % of total DNA.

** The number in the parenthesis represents ratio of large size DNA content in PEP-treated cells to that in controls. The results were obtained in the experiments presented in Fig. 4.

Fig. 6. Repair of PEP-induced DNA breakage in CHO (●) and CHO/O-2 T-6 (○) cells: Large size DNA sedimented to the bottom of tubes.



cells, suggesting that the PEP^{SS} is not due to alteration of BLM hydrolase activity.

[^{3}H]PEP Uptake into PEP^{SS} Cells

The uptake of [^{3}H]PEP into CHO/O-2 T-6 (PEP^{SS} cells) was compared with that into the parental cells. The time course of PEP uptake is illustrated in Fig. 3. [^{3}H]PEP was incorporated into both cell lines in a similar rate, suggesting that the PEP^{SS} is not attributed to increased uptake of PEP into CHO/O-2 T-6 cells.

DNA Strand Scission Induced by PEP in PEP^{SS} Cells

The fragmentation of cellular DNA by PEP in CHO/O-2 T-6 (PEP^{SS} cells) was comparatively

studied with that in the parental CHO cells by alkaline sucrose density gradient centrifugation analysis. The sedimentation is recorded from left to right in Fig. 4. The damaged DNA of small molecular size was found more in CHO/O-2 T-6 cells than in CHO cells. The content of large size DNA, which was sedimented to the bottom of tubes (fractions 13~16), is presented in Table 7. The reduction of large size DNA by the treatment with PEP of 25 or 50 μg/ml, was 2.6 or 3.5 fold higher in CHO/O-2 T-6 cells than in CHO cells. The results suggest that cellular DNA is cleaved more markedly in CHO/O-2 T-6 cells than in the parental cells.

Repair of DNA Damage induced by PEP in PEP^{SS} Cells

Repair of DNA strand scission caused by PEP in CHO/O-2 T-6 cells was compared with that in the parental cells by alkaline sucrose density gradient centrifugation analysis. The sedimentation profiles, representing time course of DNA repair, are illustrated in Figs. 5 and 6. The PEP-induced DNA cleavage was repaired progressively after PEP was washed out and the cells were incubated in the PEP-free medium: *i.e.* the DNA content of small size decreased (Fig. 5), and that of large size increased (Fig. 6). The initial rate of DNA repair of CHO/O-2 T-6 cells was significantly slower than that of the parental cells, suggesting that the former shows lower ability of repairing DNA damage than the latter.

Discussion

The current studies revealed that V79 cells are more resistant to PEP than CHO cells, and this natural resistance is due to both higher BLM hydrolase levels and lower uptake of PEP. The results are of interest, because in BLM-resistant cells, reported up to present, the resistance is caused by either mechanism, but not by both[2~4]. However, the relationship of higher BLM hydrolase *activity to* lower uptake of PEP remains open to discussion.

The PEP^{SS} of CHO/O-2 T-6 clone is attributed to increased PEP-induced DNA cleavage and decreased repair of the DNA damage. The former may be derived from the latter. However, the detailed mechanism remains to be determined. We are now studying various enzymes involved in breakdown and repair of DNA. The ouabain- and thioguanine-resistance may be not directly linked to PEP^{SS}, because CHO/O-2 T-1 clone, another ouabain- and thioguanine-resistant mutant, is not PEP^{SS}.

The gene responsible for the natural resistance to PEP appears codominantly in the hybrid, which displayed intermediate BLM hydrolase activity between those of the parental cell lines. The results suggest that the expression of BLM hydrolase gene may not be disturbed in the hybrid as in the parental cell lines. The uptake of PEP by the hybrid seemed to be intermediate between those of the parental cell lines. However, the interpretation of the experimental results was difficult (data are not shown). In this connection, the results are in accord with the report[11] that macromomycin resistance, which is due to change of membrane transport, is a codominant trait in the hybrids of macromomycin-sensitive and -resistant L5178Y cells.

The PEP^{SS} phenotype is a recessive trait in the hybrids of PEP^{SS} and PEP^{R} cell lines. The mechanism remains to be determined. However, a plausible mechansim of PEP^{SS} is attributed to deficiency of a certain repair enzyme(s). PEP^{SS} clones CHO/O-2 T-5 and T-6 showed similar levels of UV sensitivity to CHO cells (data are not shown). The results suggest that repair mechanism of PEP-induced DNA cleavage or enzyme involved may differ from that of UV-induced DNA damage.

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