

ISOLATION, DRUG SENSITIVITY, AND SOME BIOCHEMICAL
AND GENETICAL PROPERTIES OF MACROMOMYCIN-RESISTANT
MOUSE LYMPHOBLASTOMA L5178Y CELLS

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(Received for publication December 9, 1983)

A macromomycin (MCR)-resistant subline of mouse lymphoblastoma L5178Y cells was isolated after successive treatment of tumor-bearing mice with the antibiotic for 7 transplant generations, followed by cloning in culture in MCR-containing soft agar medium. The resistant cell line was about 17 times more resistant to MCR than was the parental cell line and exhibited cross-resistance to neocarzinostatin, mitomycin C and adriamycin in a similar degree to MCR. No significant cross-resistance was observed with aclarubicin, bleomycin and neothramycin. Alkaline phosphodiesterase activity in the plasma membrane of resistant cells was higher than that of parental cells. Uptake and efflux studies with [³H]adriamycin suggested that the resistance is due to decreased uptake and increased efflux of the antibiotic in resistant cells. Hybridization studies with MCR-sensitive and -resistant cells showed that the MCR resistance is a codominant trait in somatic cell hybrids.

One of the most important problems in cancer chemotherapy is that cancer cells tend to acquire resistance to antitumor drugs during treatment which restricts the clinical use of drugs. To elucidate the mechanism of drug resistance in cancer cells, we have already isolated ADM^R, ACR^R and BLM^R sublines of mouse lymphoblastoma L5178Y cells by mutagenesis *in vitro* or repeated treatment of tumor-bearing mice with these drugs¹⁻³) and showed that the resistance to ADM and ACR is due to a change in the plasma membranes resulting in lowered retention of these drugs by the resistant cells^{4,5}).

MCR⁶), an antitumor antibiotic, isolated from the culture filtrate of *Streptomyces macromomyceticus*, is an acidic polypeptide with a molecular weight of 12,500⁷) and recently its amino acid sequence has been elucidated⁸). Like NCS⁹⁻¹²), MCR contains a low molecular weight chromophore, which is responsible for biological activity^{13,14}). MCR causes DNA strand scission *in vivo*¹⁵) and *in vitro*¹⁶) by generating a free radical in the presence of a reducing agent¹⁷). MCR exhibited a marked antitumor activity against various murine tumor systems^{8,15,18}).

We obtained a MCR-resistant subline of L5178Y cells by repeated injections of tumor-bearing mice with MCR. In this paper, we describe isolation, drug sensitivity and some biological and genetical properties of the resistant cell line.

Materials and Methods

Chemicals

MCR was generously supplied by Dr. K. WATANABE, Kanegafuchi Chemical Industry Co., Taka-

The abbreviations used are: ACR, aclarubicin; ADM, adriamycin; BLM, bleomycin; MCR, macromomycin; MMC, mitomycin C; NTM, neothramycin; ACR^R, ADM^R, BLM^R and MCR^R, resistant cell lines of L5178Y cells to ACR, ADM, BLM and MCR, respectively; AO, 8-azaguanine- and ouabain-resistant L5178Y cell line; HAT medium, hypoxanthine 10⁻⁴ M, aminopterin 4 × 10⁻⁷ M and thymidine 1.6 × 10⁻⁸ M containing medium; PBS, phosphate-buffered saline; IC₅₀, 50% inhibitory concentration.

sago, Japan and ACR, BLM A₂ (copper free) and NTM were from Dr. T. TAKEUCHI, Institute of Microbial Chemistry, Tokyo. MMC and ADM were supplied by Kyowa Hakko Kogyo Co., Tokyo and NCS was a product of Kayaku Antibiotics Research Co., Tokyo. Chromophores of MCR and NCS were extracted with methanol as described previously⁹⁾. Thymidine-5'-monophosphate-*p*-nitrophenyl ester was purchased from Sigma Chemical Co., St. Louis, Missouri and [2-³H]adenosine monophosphate (15.8 Ci/mmol) from Amersham, England. [³H]ADM (45.1 μCi/mg), a product of Farmitalia, was a kind gift of Kyowa Hakko Kogyo Co.

Cell Lines

Mouse lymphoblastoma L5178Y cells were generously given by Prof. S. OKADA, Faculty of Medicine, University of Tokyo in 1974 and were maintained in Fischer's medium supplemented with 10% horse serum (regular medium) in suspension. The AO cell line, which is derived from L5178Y cells and is resistant to 8-azaguanine and ouabain, was cloned in soft agar (0.15%) Fischer's medium with 15% horse serum (soft agar medium) containing 8-azaguanine (10 μg/ml) and ouabain (1 mM), after mutagenesis with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. The AO cells were sensitive to MCR, ADM, ACR and BLM. The parental and MCR^R cells were confirmed to be free of *Mycoplasma*. All of the cell lines were cultured in a regular medium in tightly capped culture tubes. They were, in some cases, cultured in RPMI1640 medium supplemented with 10% fetal calf serum in plastic dishes in a CO₂ incubator. Hybridomas were cultured in HAT medium containing 1 mM ouabain. Growth of cells was determined by measuring cell number in a Coulter counter or by counting viable trypan blue-excluding cells with a hemocytometer (trypan blue method).

Determination of Chromosome Number

Culture of logarithmically growing cells were treated with Colcemid (0.2 μg/ml) for 2~4 hours and karyotypes were prepared by standard procedures. After Giemsa staining, chromosome numbers were obtained from 100 individual metaphase spreads for parental, MCR^R and AO cells and 20 for hybridomas.

Enzyme Activities of Plasma Membrane Fraction

Crude plasma membranes were prepared by the method of KOIZUMI *et al.*²⁰⁾. Activities of alkaline phosphodiesterase and 5'-nucleotidase were determined as described previously²¹⁾, using thymidine-5'-monophosphate-*p*-nitrophenyl ester and [³H]adenosine monophosphate as substrates, respectively.

Uptake and Efflux Studies with [³H]ADM

A cell suspension (0.5 ml) containing 10⁷ cells/ml was incubated at 37°C with 4 μg/ml of [³H]ADM in regular medium on oil mixture (0.3 ml) for an indicated period and the transport was terminated by rapid sedimentation of cells using Eppendorf 5412 centrifuge, as described previously⁹⁾. The radioactivity in the cells was determined in 10 ml of Dimilume (Packard) in a liquid scintillation spectrometer, after solubilizing the cells by addition of 1 ml of Protosol (New England Nuclear). To determine efflux, parental cells (10⁷/ml) were preincubated with 4 μg/ml of [³H]ADM and MCR^R cells (10⁷/ml) with 8 μg/ml of [³H]ADM at 37°C for 20 minutes in regular medium. After brief centrifugation, the pellet was washed once with cold PBS and suspended in ADM-free medium. An aliquot (0.5 ml, 5 × 10⁶ cells) was incubated on oil mixture at 37°C and the radioactivity remained in the cells was determined as mentioned above. The average of triplicate samples was calculated.

Cell-to-cell Hybridization

The MCR^R or parental cells were hybridized with AO cells; actively growing cells of both cell lines (4.5 × 10⁶ cells each) were mixed and centrifuged at 1,200 rpm for 3 minutes and washed three times with PBS. After removing supernatant, 1 ml of 50% Polyethylene glycol 4000 (Merck) in serum-free Fischer's medium was slowly added to the cell pellet under gentle shaking at room temperature. One minute later, 9 ml of Fischer's medium was added to the cell suspension and centrifuged. The cells were washed twice with the same medium, suspended in regular medium and incubated for a further 24 hours at 37°C. The cell suspension was then centrifuged, suspended in HAT medium supplemented with ouabain (1 mM), divided into 15 tubes, and cultured. After 2 weeks, 10 viable cells from each tube were inoculated into 5 ml of soft agar medium containing HAT and ouabain. Individual hybri-

doma cell lines were established by picking up one colony from one tube and were maintained in HAT plus ouabain medium.

Results

Isolation of a MCR-resistant Subline of L5178Y Cells

L5178Y cells of 2×10^9 were intraperitoneally inoculated into CDF₁ (BALB/c \times DBA/2) mice, 7~8 weeks of age, which were then intraperitoneally injected everyday with MCR solution in PBS ($300 \mu\text{g}/\text{kg}/\text{day}$) for 7~8 days, starting 24 hours after tumor transplantation. The cells harvested from mice were transplanted into other CDF₁ mice, which were again treated with the antibiotic in the same way as above. After 7 successive transplant generations, the mean lifespan on MCR-treated group came near to that of untreated control group (Fig. 1). The ascitic cells at 7th transplant generation were inoculated into soft agar medium (10^5 cells/ml) containing MCR ($0.4 \mu\text{g}/\text{ml}$). After 2 weeks, the colonies formed were independently transferred into regular medium. Among several cell lines established, a clone No. 4 showed the highest resistance to MCR and was used for further experiments as a MCR^R cell line. The MCR^R cells were morphologically indistinguishable from the parental cells. The transplant experiment was continued up to the 15th generation, but it failed to obtain a subline showing higher resistance to MCR than the one obtained at the 7th generation.

Growth of the MCR^R cells was slower and its saturation level was lower than that of the parental cells. Doubling time of the former was approximately 18 hours and that of the latter 11 hours (see controls in Fig. 2). The MCR^R cells even grew at a concentration of $0.2 \mu\text{g}/\text{ml}$ of MCR, whereas the number of the parental cells was below 10^4 cells/ml on day 2 at $0.04 \mu\text{g}/\text{ml}$ of MCR and the cells eventually died (Fig. 2).

The dose response for growth of the parental and MCR^R cell lines in the presence of MCR was determined by viable trypan blue-excluding cell count after 3 days' treatment and is presented in Fig. 3. In this experiment, IC_{50} for MCR was *ca.* $0.35 \mu\text{g}/\text{ml}$ for the resistant cells and $0.021 \mu\text{g}/\text{ml}$ for the parental cells thus the MCR^R cells were *ca.* 17 times more resistant.

Cross-resistance of the MCR^R Cell Line to Other Antitumor Antibiotics

Cross-resistance of the MCR^R cells to other DNA-interacting antitumor antibiotics was examined, because MCR causes DNA strand scission and its chemoreceptor is thought to be DNA. As shown in Table 1, it showed cross-resistance to NCS, MMC and ADM. No significant cross-resistance to ACR, BLM and NTM was observed. The pattern of cross-resistance of MCR^R cells differed from those of ADM^R, ACR^R and BLM^R sublines of L5178Y cells which we had previously isolated¹⁻³⁾. The biologically active principle of MCR and NCS is a low molecular weight chromophore and not the protein moieties⁹⁻¹⁴⁾. MCR^R cells showed cross-resistance to chromophores extracted by methanol from these

Fig. 1. Change of lifespan of CDF₁ mice bearing L5178Y cells treated with MCR during successive transplants.

Shaded area means range of lifespan of untreated control mice and points show a mean lifespan of 4 MCR-treated mice.

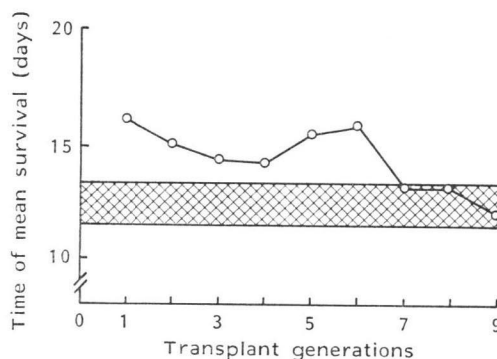


Fig. 2. Effect of MCR on growth of parental and MCR^R L5178Y cells. Viable cell number was measured by trypan blue method.

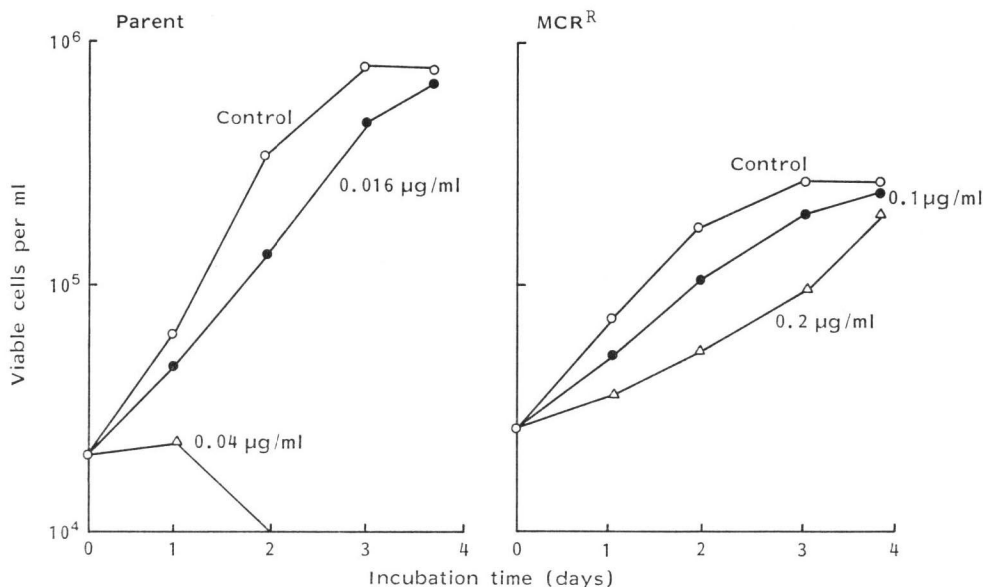


Fig. 3. Comparison of sensitivity to MCR of MCR^R cells with that of parental cells.

Viable cell number was determined by trypan blue method after 3 days treatment with MCR and is presented as % of control.

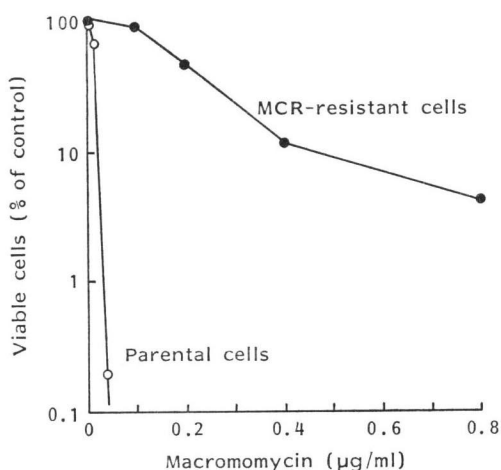


Table 1. Drug sensitivity of MCR-resistant subline of L5178Y cells in comparison with that of the parental cells.

Antibiotic	IC ₅₀ (µg/ml) for		Degree of resistance*
	Parental	Resistant	
Macromomycin	0.021	0.35	17
Neocarzinostatin	0.029	0.46	16
Mitomycin C	0.049	0.80	16
Adriamycin	0.064	0.93	15
Aclarubicin	0.035	0.12	3
Bleomycin A ₂	1.6	3.0	2
Neothramycin	0.3	0.37	1

Parental and MCR^R cells were cultured at various concentrations of the drugs in regular medium in short test tubes (2 ml/tube, 20,000 cells/ml at 0 day) and cell number was determined after 3 days in a Coulter counter. IC₅₀ was obtained from dose-response curve of each drug.

* The degree of resistance was expressed as ratio of IC₅₀ values for resistant to parental cell line.

antibiotics (data not shown).

Determination of Enzyme Activities Associated with Plasma Membrane

We have already reported that enzyme activities associated with plasma membrane of ADM^R, ACR^R and BLM^R sublines are altered²¹. Crude plasma membranes were prepared from lysate of parental and MCR^R cells by discontinuous sucrose density gradient centrifugation. By this procedure, the specific activity of alkaline phosphodiesterase increased 4.2- and 6.4-fold in the plasma membrane fractions of

parental and MCR^R cells, respectively, compared with that in their lysate, and that of 5'-nucleotidase 17- and 14-fold, respectively. About 0.5% of total protein of the lysate was recovered in the membrane fraction. As presented in Table 2, the MCR^R cells showed about 4-fold higher alkaline phosphodiesterase activity and slightly higher activity of 5'-nucleotidase than those of the parental cells. A marked increase of alkaline phosphodiesterase activity is also observed in ADM^R, ACR^R and BLM^R cells of L5178Y²¹.

Uptake and Efflux of [³H]ADM

Since the MCR^R cells showed cross-resistance to ADM in a similar degree to MCR, [³H]ADM was used to examine for a permeability change to drug in the MCR^R cell line. To cell suspensions of the parental and MCR^R cells (10⁷ cells/ml), 4 μg/ml of [³H]ADM was introduced and the radioactivity incorporated into the cells was determined as described in Materials and Methods. The uptake of [³H]-

Fig. 4. Uptake of [³H]ADM by parental and MCR^R L5178Y cells.

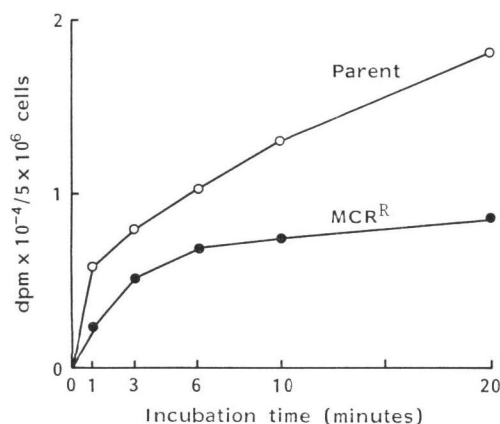


Fig. 5. Efflux of [³H]ADM from parental and MCR^R L5178Y cells.

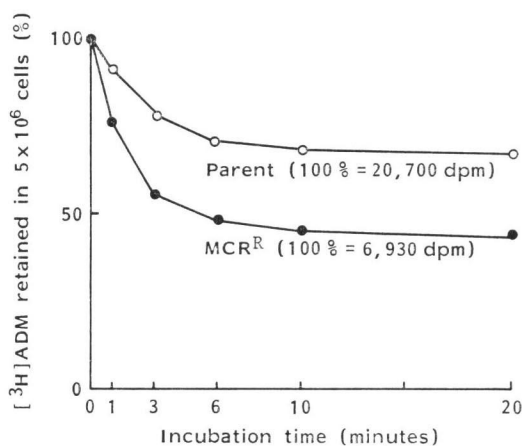


Table 2. Enzyme activities of the plasma membrane fraction isolated from parental and MCR^R L5178Y cells.

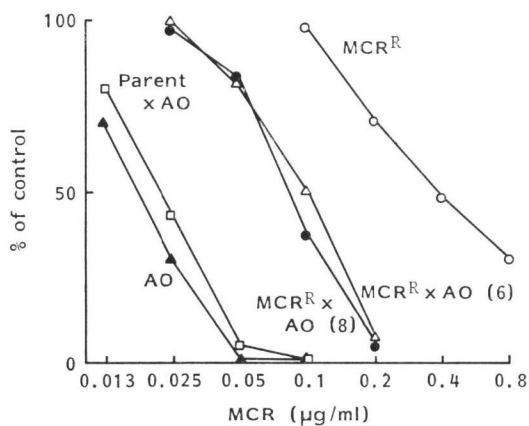
Enzyme	Parent	MCR ^R
Alkaline phosphodiesterase (pmol/minute/mg protein)	35.6 (100)	138.1 (388)
5'-Nucleotidase (dpm × 10 ⁻³ /minute/mg protein)	676 (100)	824 (122)

Table 3. Chromosome number of parental, drug-resistant and hybridoma cell lines of L5178Y cells.

Cell line	Mode	Range
Parent	42	37~45
MCR ^R	65, 66	56~71
AO	44	38~48
AO × MCR ^R clone 6	98	96~103
AO × MCR ^R clone 8	99	93~104
AO × parent	86	76~89

Fig. 6. Sensitivity of hybridomas to MCR.

Cells (20,000 viable cells/ml at day 0) were cultured in MCR-containing medium with various concentrations in a CO₂ incubator and viable cells were determined by trypan blue method after 3 days.



ADM by the parental cells increased almost linearly for 20 minutes, after a rapid initial incorporation during the first minute. In contrast to the parental cells, the resistant cells rapidly achieved a near-plateau of incorporation such that, by 20 minutes, there was a 2-fold difference in incorporation between the 2 cell lines (Fig. 4). To carry out an efflux experiment, a higher amount of [3 H]ADM was added to the medium of the MCR^R cell suspension than to that of the parental cell suspension. Even though $\times 2$ [3 H]ADM was added to MCR^R cell suspension, the radioactivity in MCR^R cells was lower than that in parental cells, after 20 minutes incubation with [3 H]ADM and brief washing with cold PBS; 6,930 dpm in MCR^R cells and 20,700 dpm in parental cells of 5×10^8 cells. In this condition, the resistant cells released about 55% of the [3 H]ADM and the parental cells released about 35% by 20 minutes, as shown in Fig. 5. It is suggested, therefore, that the resistance of MCR^R cells is attributed to both decreased incorporation and retention of the drug by resistant cells.

Cell-to-cell Hybridization Studies

To test for the dominant or recessive nature of the MCR-resistant trait, MCR^R cells were hybridized with MCR-sensitive AO cells, which bear a dominant marker for ouabain resistance and a recessive marker for 8-azaguanine resistance. The hybridomas were recovered as colonies in soft agar medium containing HAT and 1 mM ouabain. A hybridoma resulting from the fusion of AO cells with the parental cells was obtained as a control. To confirm that the colonies obtained were hybridomas, the chromosome numbers were examined. The mode for the number of metaphase chromosomes was 42 for the parental, 65, 66 for the MCR^R and 44 for AO cells, and every hybridoma clone selected had a nearly additive number of chromosomes compared to that of the parental cells before fusion (Table 3).

Dose-response curves were determined by incubation of cells at 37°C for 3 days in a CO₂ incubator with various concentrations of MCR. The MCR^R and AO cells were cultured in RPMI1640 medium supplemented with 10% fetal calf serum, and hybridomas were cultured in the same medium except that HAT and ouabain were added. The results with clones No. 6 and 8 are presented in Fig. 6, as typical examples of hybridomas resulting from fusion between MCR^R and AO cells. The dose-response curves of clones No. 6 and 8 appeared midway between those of MCR^R and MCR-sensitive AO cells, whereas a hybridoma formed by fusion between L5178Y and AO cells showed a sensitivity to MCR similar to that of AO cells. These results suggest that gene responsible for MCR-resistance is functioning codominantly in the hybridomas.

Discussion

A MCR-resistant subline of mouse lymphoblastoma L5178Y cells was obtained by successive treatment of tumor-bearing mice with the antibiotic for 7 generations, when the therapeutic effect of MCR disappeared. Of several DNA-interacting antitumor antibiotics examined, the resistant cells showed cross-resistance to NCS, MMC and ADM (Table 1). The uptake and efflux studies with [3 H]ADM showed that the resistance may be due to a permeability change of the plasma membrane (Figs. 4 and 5). Activity of alkaline phosphodiesterase in a crude membrane fraction increased markedly in MCR^R cells, compared with that in parental cells (Table 2). This is similar to ADM^R, ACR^R and BLM^R L5178Y cells we had obtained previously²¹⁾, although the relationship between alkaline phosphodiesterase activity and drug resistance remains to be determined.

A permeability change of the plasma membrane is commonly thought to be responsible for pleiotropic cross-resistance to unrelated drugs in various drug-resistant mammalian cells and in some cases, altered cell surface proteins appeared in resistant cell lines²²⁻²⁵⁾. We obtained a monoclonal antibody to the plasma membrane of an ACR^R subline of L5178Y cells, which reacts strongly with the ACR^R cells,

slightly with MCR^R cells, but not significantly with ADM^R, BLM^R and parental cells²⁰⁾. The monoclonal antibody identified the appearance of a 230 kilodalton plasma membrane protein in the ACR^R cells and the protein seemed to be responsible for the drug resistance. Whether the 230 kilodalton membrane protein is involved in resistance of MCR^R cells or another membrane component participates is under investigation.

Resistance of various methotrexate-resistant mammalian cells was reported to be due to amplification of the gene for dihydrofolate reductase^{27,28)}. Gene amplification was also suggested in murine neuroblastoma cells which showed multidrug resistance accompanying a permeability change²⁹⁾. In this connection, it is of interest that the chromosome number was markedly increased in MCR^R cells (Table 3). Difference in sensitivity to MCR in hybridomas of MCR^R × AO and parent × AO cells excludes the possibility that the resistance of MCR^R × AO is simply due to an increased amount of DNA, which is the target of MCR, in hybridomas, and indicates that MCR resistance is a codominant trait (Fig. 6).

Acknowledgments

The current work was supported in part by a Grant-in-Aid for Cancer Research from the Ministry of Education, Science and Culture, Japan. The authors express their deep thanks to Dr. HAMAO UMEZAWA, Institute of Microbial Chemistry, for his generous advice and cooperation.

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