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# Enhancement of Murine Tumor Cell Lysability by Interleukin-2 Activated Killer Cells After Treatment with Mitomycin C

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## ABSTRACT

The cytotoxicity of interleukin-2 activated killer cells with antitumor drug, mitomycin C (MMC) against murine tumor cell lines with acquired drug resistance was evaluated *in vitro* by a $^{51}Cr$ release cytotoxicity assay. Tumor cell lines, a mouse fibrosarcoma cells (MCA-F) and its individual metastatic lung clones (MCA-F-M1, 2, 3, and 4) have been established *in vitro*. Furthermore, using a soft agar cloning technique, MMC resistant clones (MCA-F-M2-1, 2, 4, 8, and 10) and sensitive clones (MCA-F-M1-3, MCA-F-M3-8, 9, MCA-F-M4-9, and 10) were established and their lysability was examined with or without MMC against lymphokine-activated killer (LAK) cells, demonstrating that LAK cells showed high % cytotoxicity against 2 resistant clones (MCA-F-M2-1 and 8) by the 7 day-exposure of 1.0 $\mu g/ml$ MMC concentration in culture but with a low % cytotoxicity in the case of only 3 day-MMC exposure. The other resistant clones showed high % cytotoxicity at 3 day-MMC exposure. On the other hand, all the sensitive clones showed high % cytotoxicity against LAK cells with the only 1 day-exposure of MMC. Thus, the combination of LAK cells and MMC treatment had a synergistic effect on MMC resistant clones as well as sensitive clones and these results suggested that the lysability of MMC resistant clones might be due to the altered susceptibility to LAK cells by use of MMC time-dependently.

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[2] The abbreviations used are: rIL-2, recombinant interleukin-2; LAK, lymphokine-activated killer; MMC, mitomycin C; MCA-F, 3-methylcholanthrene-induced mouse fibrosarcoma; MCA-F-M1 through MCA-F-M4, lung metastatic clones of MCA-F; E/T ratio, effector/target cell ratio; CTL, cytotoxic T-lymphocytes; NK, natural killer; PE, plating efficiency.

**Key words:** Tumor cell lysability, LAK cells, Mitomycin C

## INTRODUCTION

The emergence of a drug resistance in tumor cells is a serious clinical problem to curative chemotherapy. Recently many inventigators have reported various approaches to eradicate the drug resistant cells remaining after chemotherapy (3, 5, 12, 23). On the other hand, since the discovery of interleukin-2 (IL-2[2]), the rapid expansion of T-lymphocytes *in vitro* by IL-2 made it possible to be applied in adoptive treatments of tumor bearing mice (2, 9). Furthermore, such lymphokine-activated killer (LAK) cells possess a novel cytotoxic function against autologous tumor cells and have been used in the adoptive immunotherapy of human neoplastic disease (16). However, most of the adenocarcinomas are generally thought to be less sensitive to lysis by LAK cells than other tumors such as melanomas, sarcomas, and some kidney tumors (15). Allavena *et al.* reported that human colon cancer cell lines with pleiotropic drug resistance established by continuous exposure to adriamycin more efficiently killed LAK cells than were the parental tumor cells in some experiments (1). Moreover, Leroux *et al.* reported that treatment with some anticancer drugs enhances the sensitivity of murine lymphoma cells to the killing action of both LAK cells and cytotoxic T-lymphocytes (8).

Previously, we established 4 spontaneous lung metastatic tumor cell lines, MCA-F-M1 through MCA-F-M4, and examined their clonal difference of tumorigenicity by measuring % PE and their cytotoxic drug sensitivity using MMC in soft agar assays, resulting in that there existed a small population of drug resistant or sensitive clones within metastatic lesions (19).

In the study, we evaluated the cytotoxicity of LAK cells against MMC resistant or sensitive mouse fibrosarcoma cell clones with or without MMC treatment and investigated the feasibility of clinical application of the combination of LAK cells and antitumor drugs to develop methods of overcoming acquired drug resistance.

## MATERIALS AND METHODS

*Cell lines and cell preparation*

The murine fibrosarcoma (MCA-F) was induced by 3-methylcholanthrene in female C3H/HeJ mice (14), and was used in the 7th *in vivo* passage generation. Tumors were maintained by serial subcutaneous passage in 4-6 week old specific-pathogen free female C3H/HeN (MTV$^-$) mice (Charles River, Kingston, NY)

as already described (7).

$5\times10^5$ of MCA-F cell lines were injected into the right hind footpad (i. f. p.) of mice. When the growing subcutaneous tumors reached an average diameter of 1.0 cm, the tumor-bearing leg including the popliteal lymph node was resected at midfemur. Three weeks after resections, spontaneous metastatic lung colonies of 1-2 mm diameter were aseptically removed. Of the same mouse, 4 colonies were isolated from each individual lung nodules and single cell suspensions were prepared respectively, as described previously (19). The spontaneous lung metastatic cell lines, designated as MCA-F-M1 through MCA-F-M4 were cloned twice *in vitro* by the limiting dilution technique as reported previously (17). Cultures were incubated in 5% $CO_2$ at 37℃. Two weeks later, the cultures showing predominant cell growth were harvested by brief incubation in 0.05% trypsin−0.02% EDTA and a second single cell cloning was done for each clone. Using a clonogenic assay, drug resistant or sensitive cell clones were examined and maintained *in vitro* after cloning by soft agar culturing (19).

*Clonogenic assay*

A modification of the *in vitro* double layer soft agar assay described by Hamburger and Salmon's method (4) was undertaken. $1\times10^3$ cells/m*l* in a tube for clones were incubated with MMC at 0, 0.1, and 1.0 $\mu$g/m*l* concentrations for 1 h, then the cells were washed 3 times with Hank's balanced salt solution (HBSS). The effect of MMC on the anchorage-independent growth of resultant cells was studied for $10^3$ cells/m*l* inocula in 35 mm Petri dishes (Corning 25000, Corning, NY) in which was the seeder layer that was adjusted to a 1 m*l* of 0.3% bacto-agar (Difco Laboratories, Detroit, MI) in Eagle's minimal essential medium (MEM) supplemented 10% heat-inactivated fetal bovine serum (FBS). The underlayer as a feeder on which the cells were plated consisted of 1 m*l* of 0.6% agar in 2 fold concentration of MEM and FBS. The anchorage-independent growth capability of cells was scored microscopically at 2 to 3 weeks after plating, and the colony formation was expressed as the % PE: (number of cluster - number of original cell aggregates) $\times100$ / number of viable nucleate cells plated. Cells forming clusters appeared as early as the 6th day of culture and grew to form colonies with more than 30 cells until 14th day after plating. Prior to plating, the cell viability was checked by trypan blue exclusion and also cell aggregation was examined, resulting is the fact that all suspensions exhibited more than 95% cell viability and no aggregation. All assays were undertaken in triplicate. After estimation of clonal drug sensitivity, MMC resistant and sensitive tumor cell clones were isolated from the soft agar beds respectively and propagated in MEM with 10% FCS for 7 days.

*Cytotoxicity assay*

The $^{51}Cr$ release assay for the determination of cytotoxic activity of LAK cells was carried out as described previously (17, 18, 20). Briefly, target tumor cells were labeled by 100 $\mu$Ci of $^{51}Cr$ Sodium chromate (New England Nuclear, Boston, MA) and were incubated for 3 h at 37℃. The cells were washed 5 times with PBS, and $1\times10^4$ target cells in 0.1 m*l* MEM were seeded into U-bottomed microtiter plates (Costar 3799, Cambridge, MA). Thereafter, 0.1 m*l* of the effector cell suspension as a predetermined dose was added, and the plates were centrifuged at 200×g for 5 min. After 6 h incubations at 37℃ in 5% $CO_2$ incubator, 0.1 m*l* of culture supernatant was harvested and the radioactivity was counted with a liquid gamma scintillation counter. The percent of lysis was calculated as follows: % cytotoxicity = (Experimental release − Spontaneous release)×100 / (Maximal release − Spontaneous release). To determine the maximal release, 0.1 m*l* of 0.1 N hydrochloric acid was added to the appropriate wells. Spontaneous release was assessed by incubation of target cells with the medium alone, and it was usually below 15% in the experiment. All determinations were made in triplicate, and the data were represented as the mean±SE.

*Preparation of LAK cells*

For the preparation of LAK cells, non-adherent lymphocytes were isolated from the spleen of C3H/HeN mice. Adherant cells were removed by preincubation in a plastic flask at 37℃ for 4 h in an atmosphere of 5% $CO_2$ incubator. The T-lymphocytes were then obtained, passed over Lympholyte-M (Cedarlane Laboratories Ltd., Hornby, Ontario, Canada) to remove dead cells, and washed 3 times with HBSS before pasing the cells through a nylon wool columm. This procedure was repeated twice more and obtained cells phenotypically showed approximately 96% Thy-1 (monoclonal antibody reacted with T-lymphocytes) positive by indirect immunofluorescence. Collected lymphocytes were cultured in RPMI-1640 medium supplemented 10% FBS with 1000 Units/m*l* of rIL-2 and $2\times10^{-5}$ Mol/m*l* of 2-mercaptoethanol (sigma chemical Co., St. Louis, MO) for 7 days. Before the experiment, the number of LAK cells was adjusted to an E/T ratio of 25. rIL-2 produced by recombinant DNA technology was provided by Biogen Research Corp., MA.

*Drug*

MMC was purchased from Sigma Chemical Co., St. Louis, MO. Two mg of drug powder were reconstituted in 10 m*l* of 0.9% NaCl solution, and appropriate drug concentration were made with MEM.

*Statistics*

Statistical defferences in % PE of clone cells and their parental cells were determined by the Student's t test.

## RESULTS

1. *Clonogenic assay for metastatic lung clones*

Four spontaneous metastatic clones in lung, namely MCA-F-M1 through MCA-F-M4, were produced by a single i. f. p. of MCA-F parent cells and consequently 10 daughter clones derived from each of them were studied in clonogenic assay to determine their MMC sensitivity. According to the results of previous clonogenic assay (19), the MMC sensitivity of these clones were heterogeneous and dose-dependent. MCA-F-M2 clone cells exhibited considerably high resistance against MMC and, most of all 5 daughter clone (MCA-F-M2-1, 2, 4, 8, and 10) out of MCA-F-M2 were more resistant than the others. On the other hand, in several MMC sensitive clones such as MCA-F-M1, MCA-F-M3, and MCA-F-M4, more sensitive daughter clones (MCA-F-M1-3, MCA-F-M3-8, 9, MCA-F-M4-9, and 10) were demostrated (Table 1).

2. *Cytotoxicity of LAK cells against MMC resistant cell clones*

The $^{51}Cr$ release assay was used to measure cytotoxic activity of LAK cells against MMC resistant (MCA-F-M2-1, 2, 4, 8, and 10) or sensitive cell clones (MCA-F-M1-3, MCA-F-M3-8, 9, MCA-F-M4-9, and 10). As shown in Fig. 1, the results of this assay showed that there was no statistically significant

**Table 1** *Effect of MMC on Colony Formation of Metastatic Clones*

| MMC ($\mu g/ml$) | % Plating Efficiency at 2nd week | | | | |
|---|---|---|---|---|---|
| | MCA-F-M2-1 | MCA-F-M2-2 | MCA-F-M2-4 | MCA-F-M2-8 | MCA-F-M2-10 |
| 0 | 25.6±0.8 | 26.6±1.8 | 32.8±1.4 | 28.8±2.6 | 33.3±2.9 |
| 0.1 | 19.8±3.3 | 23.8±1.9 | 19.5±1.3 | 18.9±1.2 | 18.9±3.8 |
| 1.0 | 1.9±0.6 | 2.7±1.9 | 2.1±0.9 | 2.1±0.9 | 4.2±0.7 |
| | MCA-F-M1-3 | MCA-F-M3-8 | MCA-F-M3-9 | MCA-F-M4-9 | MCA-F-M4-10 |
| 0 | 28.3±4.3 | 33.8±1.6 | 30.3±2.3 | 37.4±2.7 | 32.2±1.8 |
| 0.1 | 0.9±0.8 | 3.7±1.2* | 2.8±0.3* | 4.3±1.6 | 4.8±1.8 |
| 1.0 | 0 | 0 | 0 | 0 | 0 |

$10^3$ cells were inoculated into a 0.3% agar in triplicate cultures and % PE were calculated by the formula described in Materials and Methods (mean±SE). Statistically the differences between mean % PE of MCA-F parent clones (n=20) (20.4±3.8 at 0 $\mu g/ml$ of MMC, 3.1±1.6 at 0.1 $\mu g/ml$, and 0.7±0.8 at 1.0 $\mu g/ml$) and each % PE of clones were significant ($P<0.001-0.05$). * No statistical significance.

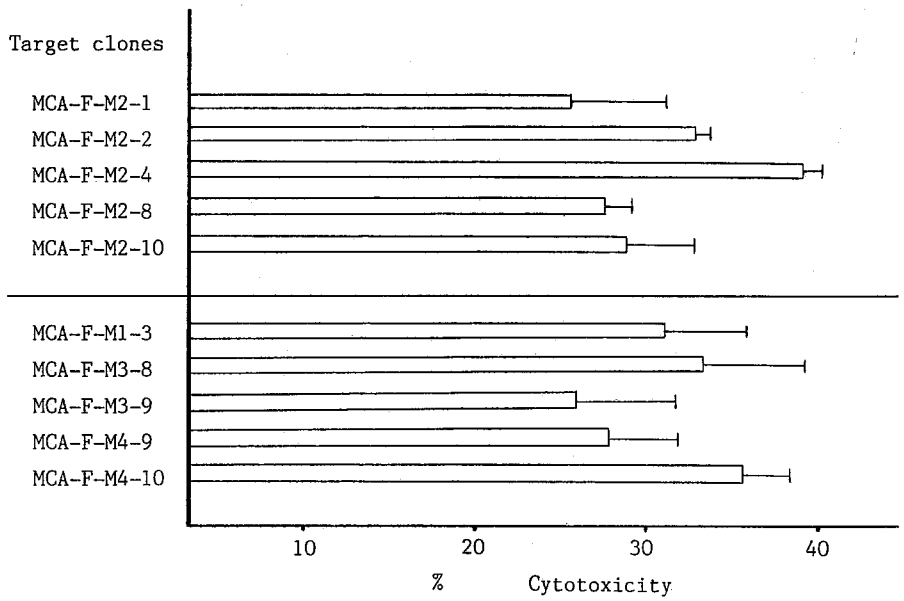


**Fig. 1** Cytotoxicity of LAK cells against MMC resistant or sensitive daughter clones of metastatic lung clones (MCA-F-M1 through MCA-F-M4). LAK cells were generated from spleen cells of C3H/HeN mice, culturing with rIL-2 for 7 days. Incubation time was 6 h at an E/T ratio of 25. Columns indicate percent cytotoxicity and bars represent SE.

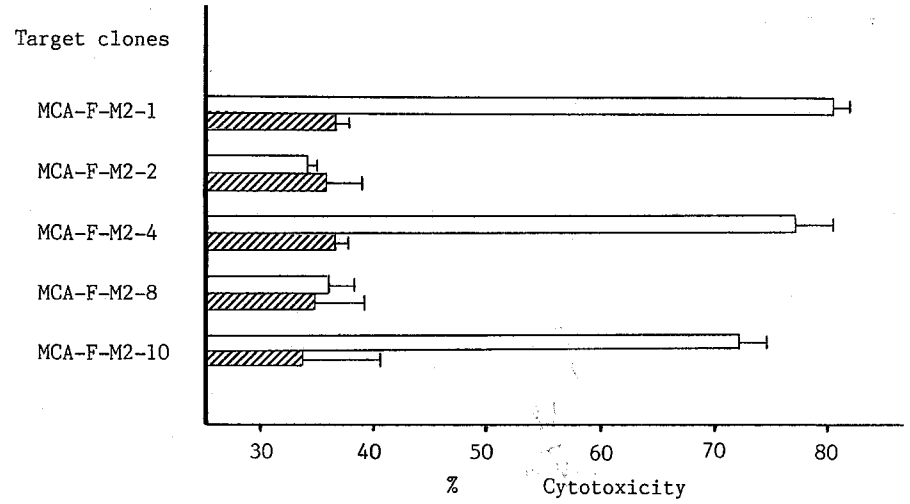


**Fig. 2** Cytotoxicity of LAK cells against resistant daughter clones with (□) or without (▨) 1.0 $\mu$g/m*l* MMC in 3 day-culture. Incubation time was 6 h at an E/T ratio of 25. Columns indicate percent cytotoxicity and bars represent SE.

difference among the clones in cytotoxic activity (26.8−34.9% cytotoxicity) by LAK cells.

3. *Cytotoxicity of LAK cells against MMC resistant cell clones under the addi-*

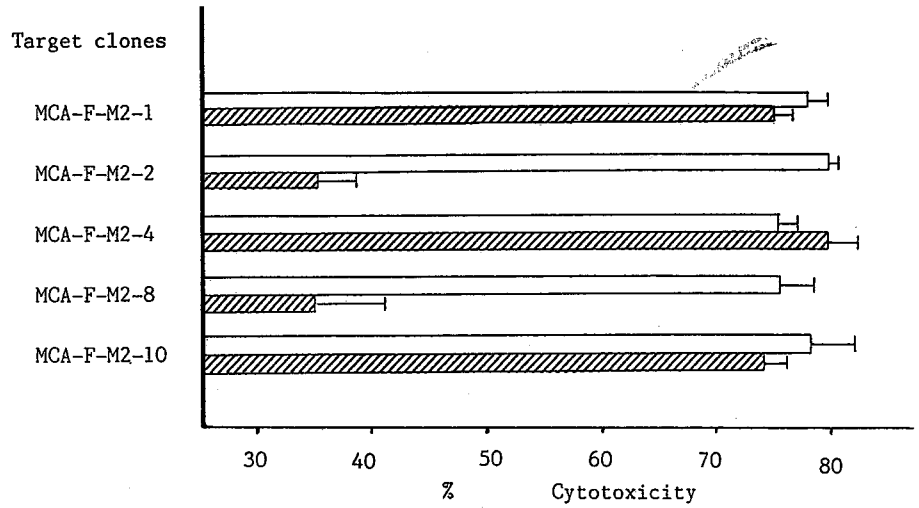


**Fig. 3** Cytotoxicity of LAK cells against resistant daughter clones with 1.0 $\mu$g/m*l* MMC in 7 day-culture (□) and 3 day-culture (▨). Incubation time was 6 h at an E/T ratio of 25. Columns indicate percent cytotoxicity and bars represent SE.

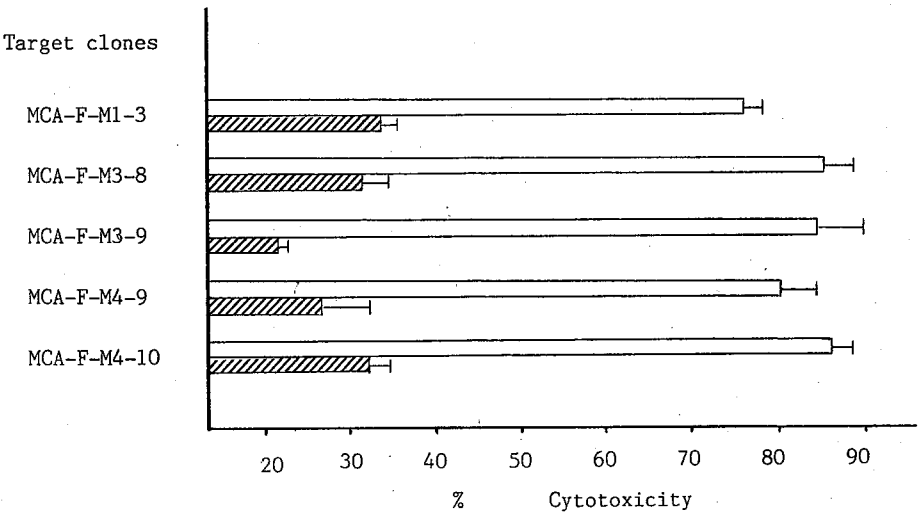


**Fig. 4** Cytotoxicity of LAK cells against sensitive daughter clones with (□) or without (▨) 1.0 $\mu$g/m*l* MMC in 1 day-culture. Incubation time was 6 h at an E/T ratio of 25. Columns indicate percent cytotoxicity and bars represent SE.

*tional condition of 3 or 7 day-MMC exposure*

In the case of MMC resistant clones under the 3 days more MMC exposure in culture, LAK cells showed low % cytotoxicity against 2 resistant clones, MCA-F-M2-2 (35.2%) and 8 (38.2%), meanwhile high % cytotoxiciy (72.8–81.2%) was shown in the other clones (MCA-F-M2-1, 4, and 10) (Fig. 2). However, in the case of 7 day-MMC exposure, these 2 clones showed high % cytotoxicity respectively (80.4% and 76.3%) like the other clones (Fig. 3).

4. *Cytotoxicity of LAK cells against MMC sensitive cell clones under the additional condition of 1 day-MMC exposure*

As schemed in Fig. 4, the results of this assay showed that LAK cells showed high % cytotoxicity (78.6–86.4%) against all sensitive clones, MCA-F-M1-3, MCA-F-M3-8, 9, MCA-F-M4-9, and 10 under the condition of only 1day-MMC exposure in culture. On the other hand, in case of no additional MMC exposure as a control study, LAK cells showed low % cytotoxicity (22.8–34.7%) against all clones.

## DISCUSSION

Drug resistance is a common clinical observation that tumors initially sensitive to chemotherapeutic agents become resistant, and relapse occurs following an initial response despite continuing treatment. In recent years, various approaches to overcome acquired resistances *in vitro* have been reported (3, 5, 12, 23). Many reports have demonstrated that tumor cells resistant to antitumor drugs such as vincristine or adriamycin achieved multi-drug resistance and this resistance could be overcome significantly by calcium antagonists, calmodulin inhibitors, or immunosuppressive drugs (6). On the other hand, Leroux *et al.* reported that treatment with some antitumor drugs enhances the sensitivity of tumor cells to the killing action of LAK cells (8). Furthermore, Ohtsu *et al.* found a marked increase in sensitivity to LAK cells in the cisplatin (CDDP) resistant cell lines, and a synergistic effect between LAK cells and CDDP in CDDP resistant cell line (11). Such results suggested that LAK cells might have some value in the therapy of tumors with acquired drug resistance.

MMC has been known to show a broad spectrum of activity against various tumors and significant clinical antitumor effects with a high response rate. Previously we established MMC resistant or sensitive murine tumor cell clones derived from metastatic lung tumors (19). Using these clones, in the present study, we examined the possibility of an immunological approach using LAK cells to circumvent drug resistance.

Consequently, our results in the present study showed that some of the clones

resistant to MMC were much more susceptible to killing by LAK cells in case of additional MMC treatment for 7 days but no response was seen in case of 3 day-treatment. On the other hand, in case of MMC sensitive clones, the cytotoxicity of LAK cells in combination with additional MMC treatment were sufficiently high in the only one day treatment. Therefore we obtained results that even drug resistant tumor cells might have to possibility to be eradicated by LAK cells with sufficient antitumor drug, suggesting that LAK therapy might be a useful therapy for drug resistant tumor. However, further examination with various drug resistant cells should be done to evaluate the efficacy of LAK therapy. As to drug sensitive cells, LAK therapy in combination with drug seems to have more desirable prognosis in patients. We previously demonstrated the heterogeneity with respect to cytotoxic susceptibility of autologous tumor cells against CTL and NK cells (21). LAK cells however did not show any significant heterogeneity in cytotoxic activity against target tumor cell clones. Similar results have recently been reported by Papa *et al.* (13). The basis for such the observed differences in LAK susceptibility of the drug resistant cells, it has been considered that the fatty acid composition of the cell membranes and its modification by antitumor drugs play an important role in the efficiency of the " lethal hit " in cell destruction by some immunological promoter such as specific antibody and complement *in vivo* (22). Further, antitumor drug has been shown to modify the expression of some cell surface receptors (10), which might also influence the expression of putative LAK cell receptors. Our results with MMC resistant cell clones suggest that MMC may cause such a modification on the surface of tumor cells and enhance their sensitivity to LAK cells.

In conclusion, we have found that *in vitro* experiments with MMC can increase very significantly the lysability of tumor cells, if it were drug sensitive or resistant, in LAK cells. This could be advantageously used in chemoimmunotherapy protocols using LAK cells, a type of adoptive immunotherapy which seems presently to have some limitations. Further assessment for various antitumor drugs along this line should be needed to improve the therapeutic effect in adoptive immunotherapy of LAK cells.

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