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Potentiation of Antitumor Effect of Bleomycin by Fusogenic Lipid-Surfactant Mixed Micelles. II. Tumor-Neutralizing Assay for Inherently Bleomycin-Resistant Murine Leukemia

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We investigated whether fusogenic lipid-surfactant mixed micelles (MM), a solubilized lipid system, composed of linoleic acid (LA) and polyoxyethylated (60 mol) hydrogenated castor oil (HCO60) as a nontoxic solubilizer, could potentiate the antitumor activity of bleomycin (BLM) against inherently BLM-resistant murine L1210 leukemia. In a Winn-type tumor-neutralizing assay in which mice were inoculated intraperitoneally with L1210 leukemia cells preincubated with test materials, treatment with BLM plus LA-HCO60 MM showed a significant prolongation of mouse survival time as compared with BLM alone or MM alone. Further, combined use of BLM and concentrated MM accomplished total tumor cell killing, and all animals survived tumor-free. From these results, we presume that MM can make BLM-resistant tumor cells sensitive to BLM.

Keywords——cancer chemotherapy; bleomycin; drug resistance overcoming; fusogenic lipid-surfactant mixed micelle; murine leukemia; tumor-neutralizing assay

It is well recognized that a difference of sensitivity or the acquisition of resistance of tumor cells to a variety of antitumor drugs is closely related to the extent of drug penetration through the tumor cell membrane and drug accumulation in the cell.[1-3] Therefore, if we could enhance the intracellular level of an antitumor drug by increasing the permeability of the tumor cell membrane, we would expect potentiation of the antitumor effect of the drug and also sensitization of drug-resistant cells. Previous reports from our laboratory have shown that lipid-surfactant mixed micelles (MM) greatly promoted the gastrointestinal absorption of non absorbable drugs such as streptomycin,[4] bleomycin (BLM),[5] heparin[6-8] and interferons.[9-12] Among the lipids used in MM, those having polar heads and flexible long acyl a chains such as oleic acid and linoleic acid (LA) exhibited remarkable enhanced effects on the permeability of biomembranes in the alimentary canal[13,14] and bladder.[15] Interestingly, those lipids are identical to the fusogenic lipids which cause erythrocyte fusion.[16]

We have also reported that this MM composed of LA and polyoxyethylated (60 mol) hydrogenated castor oil (HCO60), a nontoxic surfactant, greatly potentiated the antitumor activity of BLM against BLM-sensitive mouse Ehrlich ascites tumor and rat hepatoma AH 66 in *in vivo* therapy and tumor-neutralizing assay.[17] In this work, we employed the Winn-type tumor-neutralizing assay,[18] to examine whether LA-HCO60 MM could potentiate the antitumor activity of BLM against murine L1210 leukemia, an inherently BLM-resistant tumor.

Experimental

Materials——BLM was kindly supplied by Nippon Kayaku Co., Ltd., Tokyo, Japan. LA of 99.0% purity

(Nippon Oil & Fats, Co., Ltd., Tokyo, Japan) and macromolecular surfactant HCO60 (approximately mean molecular weight estimated from its structural formula, 3500; Nikko Chemicals Co., Ltd., Tokyo, Japan) were used. All other chemicals were commercial products of reagent grade.

Solutions of MM were prepared by dispersing LA and HCO60 (approximate molar concentration ratio, LA : HCO60 = 16 : 1) in Hanks' balanced salt solution (HBS, Research Institute for Microbial Disease, Osaka University, Osaka, Japan) followed by sonication at 37 °C using a sonicator (model 5202, Ohtake Works Co., Ltd., Tokyo, Japan) at 100 W for 4 min. The MM of this component ratio greatly promoted the gastrointestinal absorption of poorly absorbable drugs.[9-11] Other test solutions were prepared by dissolving BLM (0.1 or 2 mg/ml) in HBS and BLM (0.1 or 2 mg/ml) in the aforementioned MM solution. Male CDF_1 mice weighing 18—20 g were purchased from Shizuoka Agricultural Association for Laboratory Animals, Hamamatsu, Japan. The animals were given a pellet diet (CE-2, Clea Japan, Inc., Tokyo, Japan) and water *ad libitum*. The L1210 leukemia cells were kindly supplied by Shionogi Pharmaceutical Co., Ltd., Osaka, Japan. A Winn-type tumor-neutralizing assay[18] was performed by incubating L1210 leukemia cells with BLM (0.1 or 2 mg/ml) alone, MM_1 (100 μM LA + 6 μM HCO60) alone, MM_2 (150 μM LA + 9 μM HCO60) alone and BLM (0.1 or 2 mg/ml) plus MM_1 or plus MM_2 in HBS. After incubation at 37 °C for 60 min, L1210 cells were washed 3 times, suspended in HBS (1×10^5 cells/mouse), and injected into 8 CDF_1 male mice in each group. The tumor-neutralizing activity was expressed as $T/C \times 100$ (%) where T is the mean survival time of dead mice in the treated group and C is that in control mice group. Survival data were analyzed statistically by using the two-tailed Student's t-test.

Results and Discussion

Winn's tumor-neutralizing assay[18] was performed to assess the potentiation by LA-HCO60 MM of the antitumor effect of BLM against murine L1210 leukemia by following the survival of mice. When the L1210 cells were preincubated with BLM (0.1 mg or 2 mg/ml) alone, remarkable prolongation of the survival time of mice was not observed (Figs. 1 and 2). The combined use of BLM and MM, however, greatly extended the survival time with 7/8 mice being cured by BLM_1 (0.1 mg/ml) + higher concentration MM_2 treatment (Fig. 1) in particular, all 8 mice survived tumor-free after treatment with BLM_2 (2 mg/ml) plus MM_2 (Fig. 2). On the other hand, MM alone did not show any marked prolongation of survival time (Fig. 3). Therefore, the strong tumor-neutralizing activity arising in the case of combined use (Figs. 1 and 2) should not be caused by the cytocidal action of MM itself at the concentrations used. The values of mean survival as a percentage calculated from the data of dead animals in the treated group relative to that in the control group ($T/C \times 100$) were 113.0 (BLM_1), 120.3 (BLM_2), 118.1 ($BLM_1 + MM_1$), 170.4 ($BLM_2 + MM_1$, $p < 0.05$), 350.8 ($BLM_1 + MM_2$), 100.6 (MM_1) and 117.9 (MM_2). These data indicate that addition of MM prolonged the survival time of animals compared to the treatment with BLM alone, and furthermore, the combined use of BLM with a higher concentration of MM showed complete tumor-neutralizing activity, that is, total tumor cell killing.

It is generally difficult to evaluate the direct cytocidal action of a test compound against inoculated tumor cells in *in vivo* therapy because of the host-mediated action against tumor cells. On the other hand, tumor-neutralizing assay is a method to examine the cytocidal action of test material on tumor cells under conditions similar to those of *in vivo* therapy.[18] Lipid-

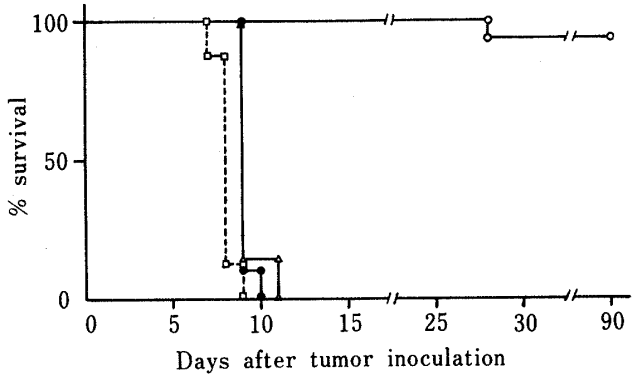

Fig. 1. Tumor-Neutralizing Activity against L1210 Leukemia

BLM_1 (●), $BLM_1 + MM_1$ (△) and $BLM_1 + MM_2$ (○).

Concentrations of test materials: BLM_1 (0.1 mg/ml), MM_1 (LA 100 μM + HCO60 6 μM) and MM_2 (LA 150 μM + HCO60 9 μM). Mice inoculated with L1210 cells (preincubated in HBS alone) served as the control (□). Survival was followed for 90 d. Winn-type tumor neutralizing assay was performed as described in Materials and Methods.

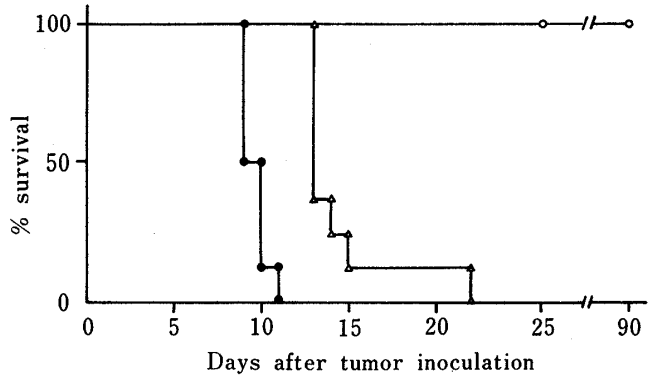

Fig. 2. Tumor-Neutralizing Activity against L1210 Leukemia

BLM_2 (2 mg/ml, ●), $BLM_2 + MM_1$ (△) and $BLM_2 + MM_2$ (○).
Concentrations of test materials were as in Fig. 1.

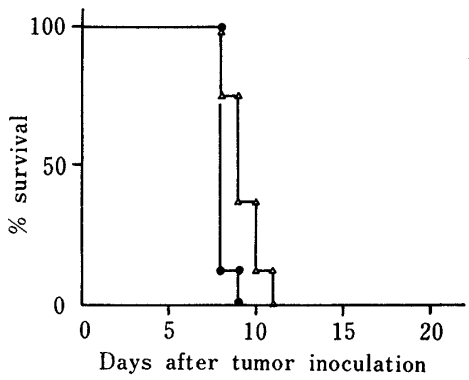

Fig. 3. Tumor-Neutralizing Activity against L1210 Leukemia

MM_1 (●) and MM_2 (△).
Concentrations of test materials were as in Fig. 1.

surfactant MM itself at the concentration used in this study did not exert direct cytocidal action on L1210 leukemia cells (Fig. 3), though there are some reports that polyunsaturated fatty acids such as LA have antitumor activity.[19-21] Further, Bégin *et al.* have reported that LA or linolenic acid selectively acts against tumor cells compared to normal cells.[22] Therefore, we can presumably expect to obtain a selective synergic antitumor effect of BLM and MM by regulating the dose of MM.

We have been investigating the effect of MM as a gastrointestinal absorption enhancer for poorly absorbable and macromolecular drugs. In this series of studies, we found that MM could greatly increase the absorption of poorly absorbable drugs such as BLM[5] and interferons.[9-12] However, neither surfactant alone nor the lipid alone in an insoluble state (emulsion) could enhance the absorption. We speculate that the enhancement mechanism by MM is as follows[23]; the fusogenic lipid solubilzed by MM and incorporated into the membrane interact with the polar head groups of membrane lipids, increasing the membrane fluidity, and resulting in enhanced membrane permeability to non absorbable drugs. We also found by using the *in vivo* and tumor-neutralizing assay, that LA-HCO60 MM could potentiate the therapeutic effect of BLM against originally BLM-sensitive tumors, although, HCO60 alone and emulsified LA alone could not enhance the effect of BLM.[17] From the results of our studies on the promotion of BLM absorption from the alimentary canal[5] and bladder,[15] we speculate that MM potentiated the antitumor activity of BLM by enhancing BLM transport through the membrane of tumor cells. The precise mechanism of potentiation of the antitumor effect of BLM by MM is now under study.

It was reported that some surfactants increase the tumor cellular uptake of anticancer drugs.[24] On the other hand, we found that the surfactant employed in this work, HCO60, did not enhance BLM transport through the mucosa of the alimentary canal[5] and bladder,[15] and it is known that HCO60 is essentially nontoxic. Thus, we consider that HCO60 can not enhance the tumor cellular transport of poorly permeable drugs, and in our experimental system, the MM-solubilized lipid is the effective permeability enhancer. It is known that BLM has no antitumor effect against murine L1210 leukemia in *in vivo* therapy, and also in this work, the high concentration of BLM alone did not show tumor-neutralizing activity. The results in this study indicate that MM can potentiate the antitumor effect of BLM against not only BLM-sensitive but also naturally BLM-insensitive tumor cells. It appears that with the aid of this MM system we can make inherently resistant tumor cells drug-sensitive or we can overcome acquired drug resistance.

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