RESEARCH ARTICLE

Immunomodulating Effects of Bleomycin and its Derivatives, Peplomycin and Liblomycin on Murine Antitumour Effector Cells

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ABSTRACT

We investigated the immunomodulating effects of bleomycin and its derivatives, peplomycin and liblomycin on natural killer (NK) cells and lymphokine-activated killer (LAK) precursor cells from the spleens of C57BL/6 mice. Results showed that bleomycin and peplomycin can increase *in vitro* NK and LAK precursor cell activities both per spleen and per unit number (1×10^6) of the spleen cells as compared with normal mice from day 1 to 9, while the number of spleen cells did not increase. Meanwhile, a single administration of liblomycin caused a decrease in the number of spleen cells and the activity of LAK precursor cells per mouse spleen from day 1 and they recovered to normal levels by day 9. NK activity, which was also suppressed by liblomycin, recovered slowly but failed to reach the complete restoration by day 9. Liblomycin showed no effect on LAK precursor cell activity per unit number of spleen cells. Further study showed that these immunomodulating effects of bleomycin and its derivatives are apparently at least partially mediated by the endogenous cytokine release.

KEYWORDS bleomycin, peplomycin, liblomycin, natural killer cell, lymphokine-activated killer precursor cell, cytokine

INTRODUCTION

Bleomycin (BLM) is an antitumour agent, which is well known for its various immunoaugmenting effects[1] such as the enhancement of interleukin-2 (IL-2) production by mitogen-stimulated spleen cells[2], activation of macrophages[3] and the elimination of suppressor T-lymphocyte activity which might aid the host in overcoming immunosuppression[4]. Its clinical use, however, is limited by toxicity to the lungs in the form of lung fibrosis[5]. Peplomycin (PEP), an analogue of BLM, has a lower pulmonary toxicity than BLM, but this toxicity remains a major dose-limiting factor of this drug. Liblomycin (LIB) is a novel analogue of BLM and exhibits a broader antitumour spectrum as well as an extremely weaker pulmonary toxicity than BLM and PEP[6] (Fig. 1). Furthermore, it has been widely reported that LIB is generally more effective against some BLM or PEP–unresponsive tumour lines, both *in vitro* and *in vivo*. LIB also exhibits such properties as a greater cellular drug uptake and resistance to BLM hydrolase[7].

The previous study has reported that BLM and PEP increase IL-2, tumour necrosis factor (TNF) and interferon (IFN) release during mitogen-stimulated rat spleen cell culture, whereas, cytokine release decreased after treatment with LIB[8]. Therefore, in this study, we compared equivalent doses of LIB, PEP and BLM for their effects on mouse natural killer (NK) and lymphokine-activated killer (LAK) precursor cell activities and also for cytokine production *in vivo*. The results indicate that while BLM has an immunoenhancing effect in the mouse, PEP has a negligible effect while LIB causes immunosuppression. These immunosuppressive effects of LIB may be attributed to the efficacy of the antitumour agent as a drug.

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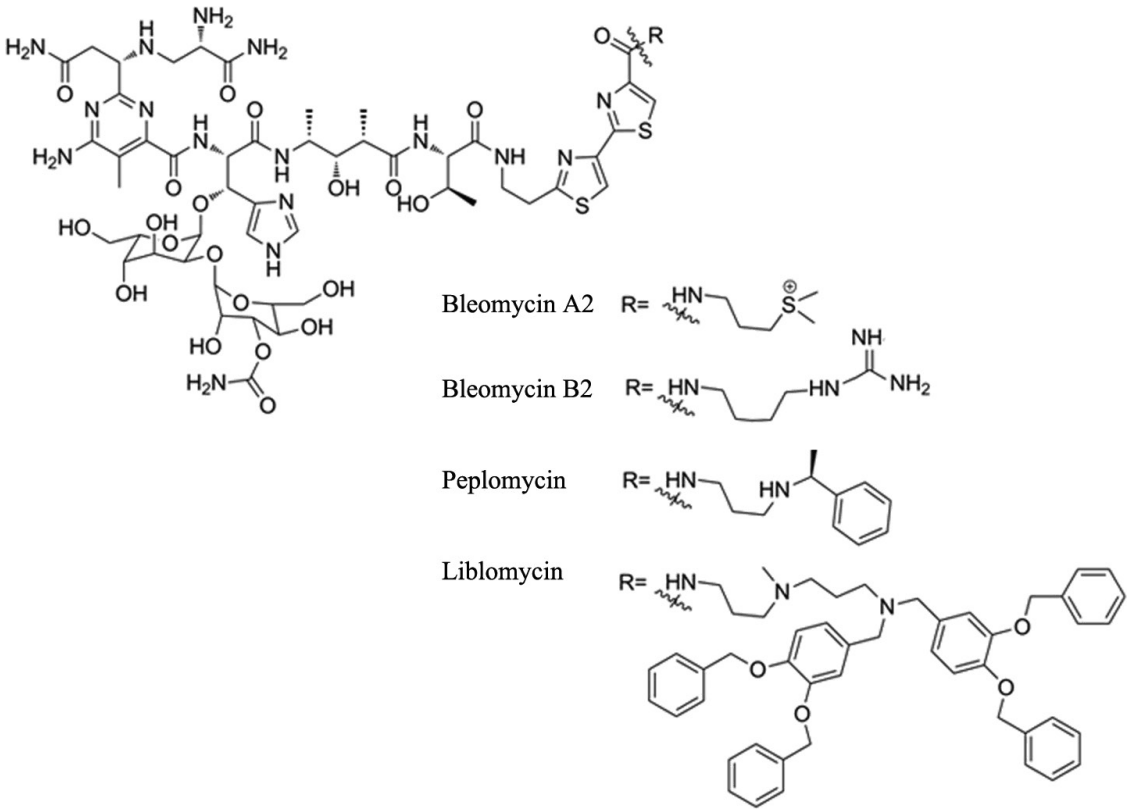

Fig. 1 Chemical structures of BLM and its derivatives.

MATERIALS AND METHODS

Chemicals

BLM, PEP and LIB were generously provided by Nippon Kayaku Co., Ltd., Tokyo, Japan, and dissolved in phosphate-buffered saline (PBS) solution immediately before use. Recombinant human IL-2 (rIL-2) was a generous gift of Shionogi Pharmaceutical Co., Ltd., Osaka, Japan. Concanavalin A (Con A; SIGMA, Saint Louis, America) was dissolved in RPMI-1640 medium and sterilised by filtration through 0.22 μm cellulose acetate membrane filters (Millipore, Massachusetts, America) and diluted to a concentration of 5 μg/ml before use in spleen cell cultures for the production of IL-2. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; SIGMA, Saint Louis, America) was dissolved in PBS to a working solution of 5 mg/ml.

Animals

Female C57BL/6 mice were obtained from the laboratory division of the Shizuoka Agriculture Cooperative Association, Hamamatsu, Japan. The experimental mice were 8 weeks old and weighed 18–20 g. They were kept in a specific pathogen-free animal room.

Experimental groups

The experimental animals were given BLM of 20 mg/kg, PEP of 20 mg/kg and LIB of 20, 10 or 5 mg/kg diluted in PBS solution or PBS only by tail-vein injection during 1, 3, 5, 7 and 9 days prior to examination. The splenocytes were obtained from the control (PBS only) and experimental animal groups as previously described. The spleens were removed under sterile conditions and the cells were then incubated with buffered ammonium chloride for 2 min to lyse the erythrocytes. The splenocytes were then centrifuged and washed 3 times with 1% foetal calf serum (FCS) RPMI 1,640 medium prior to culture.

Cell culture medium

RPMI 1,640 medium, supplemented with 10% FCS, 0.3% fresh L-glutamine, 0.1 mM non-essential amino acids, 100 μg/ml streptomycin and 100 U/ml penicillin. Eagle's minimum essential medium containing 8% FCS, 0.1 mM sodium pyruvate, 0.1 mM non-essential amino acids, 0.3% fresh L-glutamine, 100 μg/ml streptomycin and 100 U/ml penicillin. Complete medium consisted of RPMI 1640 supplemented with 10% FCS, 0.3% fresh L-glutamine, 0.1 mM non-essential amino acids, 0.3% N-2-hydroxyethylpiperazine-N′-2-ethanesulphonic acid and streptomycin and penicillin.

Cell lines

The YAC-1 tumour is a moloney virus-induced mouse T cell lymphoma of A/SN origin. The YAC-1 cell line was cultivated in RPMI 1640 medium, at 37°C in a humidified 5% CO_2 before testing cytotoxic activity on NK activity. BMT-11 is a transplantable fibrosarcoma induced by 3-methylcholanthrene in a C57BL/6 mouse. The BMT-11 cell line was cultivated in Eagle's minimum essential medium at 37°C in a humidified 5% CO_2. The cells were routinely cultured and passaged using 0.25% trypsin or 0.02% EDTA solution to detach the cells for application in LAK cell cytotoxicity assays. CTLL-2 is an IL-2-dependent cytotoxic T cell line. The cell line was kindly provided by Dr. K. Kumagai (Tohoku University, Japan). The CTLL-2 cells were cultured in RPMI 1640 medium containing rIL-2 25 U/ml for IL-2 activity assay with passage once for every 2 days.

NK cell activity

1×10^7/ml YAC-1 target cells were placed in vials containing 0.9 ml RPMI 1,640 medium and labelled with 100 µl of ^{51}Cr-sodium chromate (100 µ Ci, NEN, Boston) by incubation for 1 h at 37°C in a 5% CO_2 atmosphere. After centrifugation and washing, the spleen cells in 100 µl of culture medium were mixed at different effector/target cell rations with 1×10^5/ml ^{51}Cr-sodium chromate YAC-1 cells in 100 µl of RPMI 1640 medium in 96-wells round bottom microwell plates (Corning, New York, America). The maximum isotope release was produced by the incubation of target cells with 100 µl of 1 N hydrochloric acid. The spontaneous isotope release was produced by the incubation of target cells in RPMI 1,640 medium alone. Assay for each effector/target cell ratios were performed in triplicate. After incubation for 6 h at 37°C in 5% CO_2 atmosphere, the specific ^{51}Cr release was calculated using the formula:

$$\text{Lysis (\%)} = (\text{experimental release} - \text{spontaneous release}) / (\text{maximum release} - \text{spontaneous release}) \times 100$$

The LU30 was arbitrarily defined as the number of effector cells necessary to give a 30% of specific ^{51}Cr release. The NK activities per unit number of spleen cells (LU30/10^6 cells) and per spleen were calculated as follows:

$$\text{LU30}/10^6 = \text{No. of cell for 1 LU30}/1 \times 10^6 \text{ spleen cells}$$

$$\text{LU30/spleen} = \text{No. of cell for 1 LU30}/\text{No. of spleen cells}$$

LAK precursor activity

To generate LAK cells, the splenocytes obtained as described above were incubated in 100 × 20 mm tissue culture dishes containing 2.5×10^6 cells/ml in 15 ml complete medium with 1,000 U/ml of rIL-2, for 5 days at 37°C in 5% CO_2 atmosphere. After being harvested, the LAK cells were washed and resuspended in RPMI 1,640 medium for cytotoxic activity assay. LAK cell cytotoxicity was determined by a 4-h chromium-release assay against NK-resistant BMT-11 cells as described above.

Preparation of IL-2 containing supernatants and activity assay

The spleens obtained from animals in each treatment group were pooled and a cell suspension in medium was prepared as described above. Erythrocytes were lysed by Tris-NH_4Cl/PH 7.3 phosphate buffer treatment and washed three times with RPMI medium. The cells were resuspended at 5×10^6 cells/ml in wells of a 24-well culture plate with 2 ml of RPMI 1,640 medium supplemented with 10% FCS, and 5 µg/ml of Con A. The plates were incubated for 24 h in a 37°C in 5% CO_2 humidified atmosphere. The supernatants were obtained by centrifugation at 3,000×*g* for 10 min and kept frozen at −20°C until use. The IL-2 activity in the supernatants was determined by a standard microassay based on the IL-2-dependent proliferation of CTLL-2 as follows. Cytotoxic T cell line (CTLL) cells of 1×10^5/ml in RPMI 1,640 medium were seeded in 100 µl volumes in 96 flat-bottomed microwell plates together with a 2-fold dilution of Con A-stimulated mouse spleen cell culture supernatant samples in triplicate. After 24 h of incubation, viable cell number was determined by an MTT assay. Briefly, 16 µl of an MTT solution in PBS (5 mg/ml) were added to each well and the plates were incubated for 4 h. At the end of the incubation time, the microwell plates were centrifuged at a high speed and the supernatants were discarded. Dimethylsulphoxide (DMSO, 150 µl) was added to each well to dissolve the formazan crystals. The absorbance was measured at 570 nm with an ELISA reader. One unit of IL-2 was taken to be equivalent to that amount of IL-2, which causes a 50% proliferation of that CTLL-2 line.

RESULTS

Effects of BLM and its derivatives on murine antitumour effector cells

As shown in Fig. 2A, there is an insignificant effect on the total number of spleen cells after treatment with BLM and PEP at 20 mg/kg i.v. However, LIB (20 mg/kg i.v.) markedly reduced the spleen cell number ($P < 0.01$). The total spleen cell number decreased from day 1 to 7 and the cell number increased on the day 9. As shown in Fig. 2B, the NK cell activity per unit number (1×10^6) of spleen cells showed a slight increase after treatment with BLM and PEP. However, LIB reduced the NK activity per unit number on day 5 and 7, ($P < 0.05$). In Fig. 2C, the NK cell activity per spleen was observed to increase after treated with either BLM or PEP ($P < 0.05$). LIB suppressed NK cell activity per spleen from

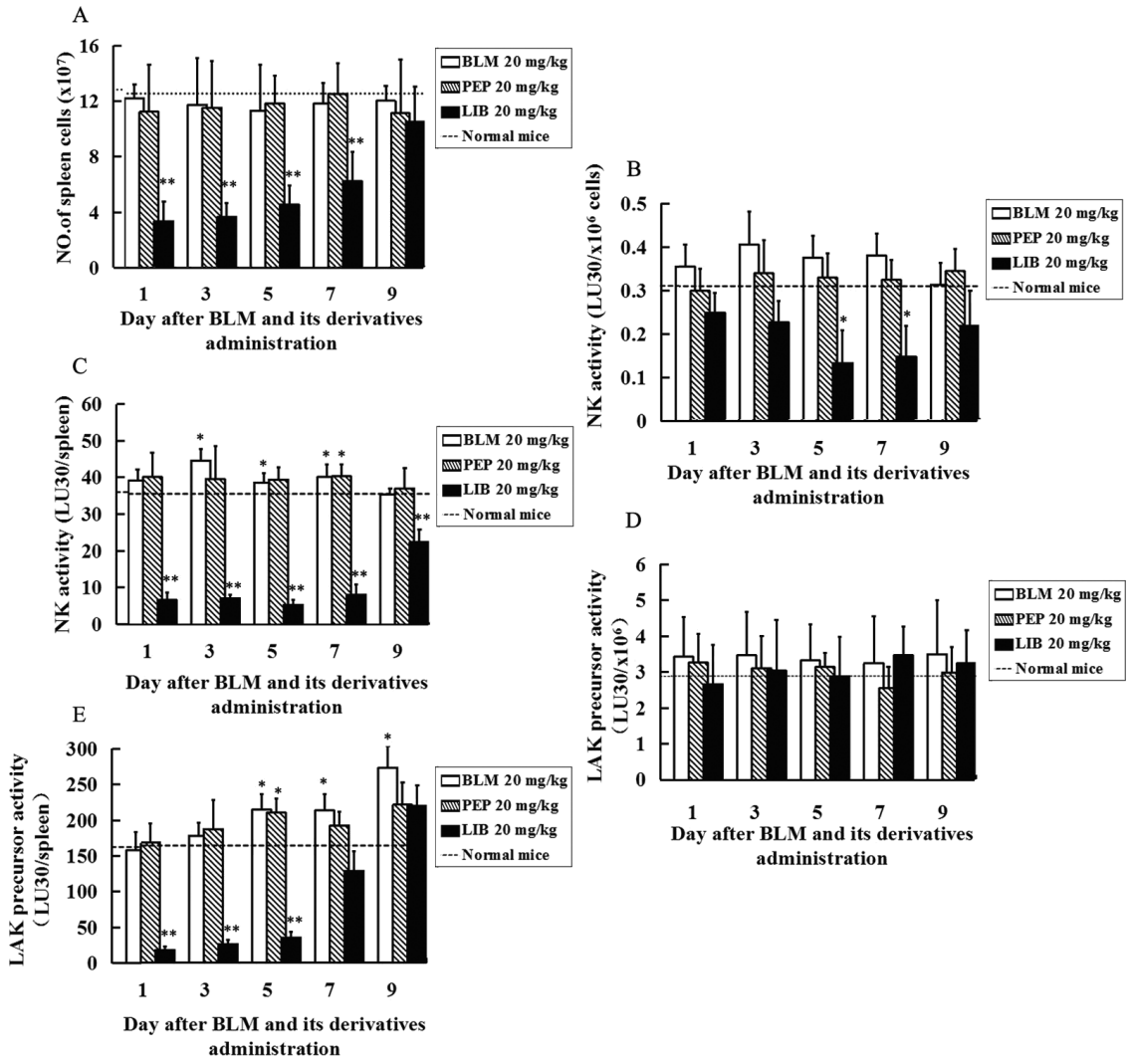

Fig. 2 Effects of BLM and its derivatives on murine antitumour effector cells. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with normal mice.

day 1 to 9 ($P < 0.01$). BLM enhanced the LAK precursor cell activity per unit number of spleen cells, but similar effects were not observed after treatment with PEP or LIB (Fig. 2D). In Fig. 2E, BLM significantly increased the LAK precursor cell activity from day 5 to 9 after treatment ($P < 0.05$) while PEP significantly increased LAK precursor cell activity on day 5 ($P < 0.05$). The LAK precursor cell activity per spleen was decreased from day 1 ($P < 0.01$) and recovered on day 7.

Effects of BLM and its derivatives on IL-2 release

As shown in Fig. 3, IL-2 release during mitogen-stimulated spleen cell culture after treatment with BLM *in vivo* was enhanced when compared with normal mice, and the maximum release of IL-2 was observed to occur on day 7 after the drug administration ($P < 0.05$). PEP did not have any significant effect on IL-2 release. LIB decreased the IL-2 release from day 1 to 3 ($P < 0.05$); the IL-2 release recovered from day 5 and reached to normal level on day 7.

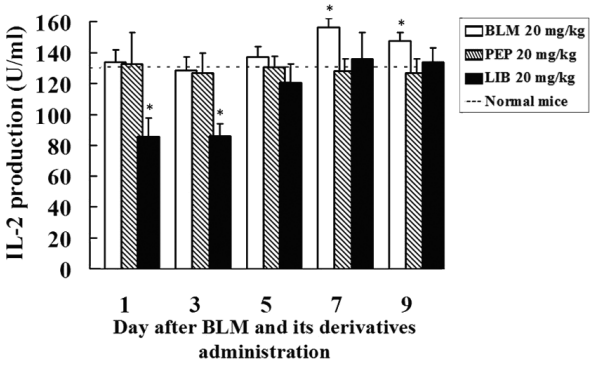

Fig. 3 Effects of BLM and its derivatives on IL-2 release. $^{*}P < 0.05$ compared with normal mice.

Effects of various doses of LIB on murine antitumour effector cells

LIB at 20 and 10 mg/kg significantly reduced the spleen cell numbers on day 1 after the treatment ($P < 0.01$), but a dose of 5 mg/kg did not affect spleen cell numbers when compared with normal mice (Fig. 4A). LIB at

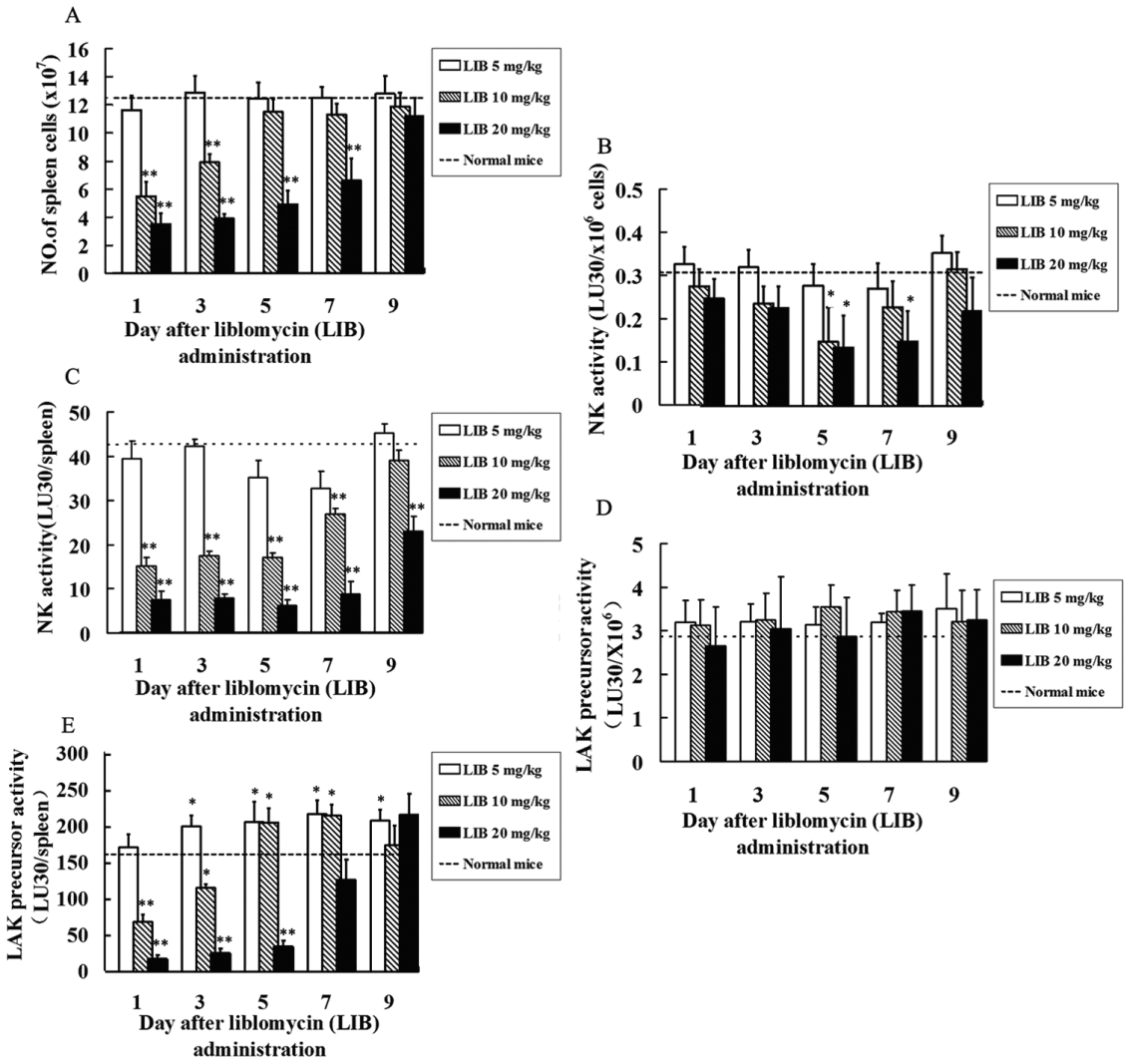

Fig. 4 Effects of various doses of LIB on murine antitumour effector cells. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with normal mice.

20 and 10 mg/kg doses suppressed NK activities per unit number of spleen cells from day 5 ($P < 0.05$) and per spleen from day 1 ($P < 0.01$) when compared to normal mice (Fig. 4B, C). As shown in Fig. 4D and E, the LAK precursor cell activity per unit number of spleen cells was unaffected after the administration with various doses of LIB. LIB at 10 or 20 mg/kg, suppressed LAK cell activity per spleen from day 1 ($P < 0.01$). Interestingly, the dose of 10 mg/kg increased the LAK precursor activity per spleen on day 5 ($P < 0.05$), the LAK cell activity per spleen increased after treatment with LIB at 5 mg/kg from day 3 ($P < 0.05$).

Effects of various doses of LIB on IL-2 activity

As shown in Fig. 5, the IL-2 activity in Con A-stimulated spleen cell culture supernatants was below that in control cultures after treatment with LIB 20 and 10 mg/kg ($P < 0.05$), while a dose of 5 mg/kg showed no significant changes in IL-2 release by spleen cells when compared with normal mice.

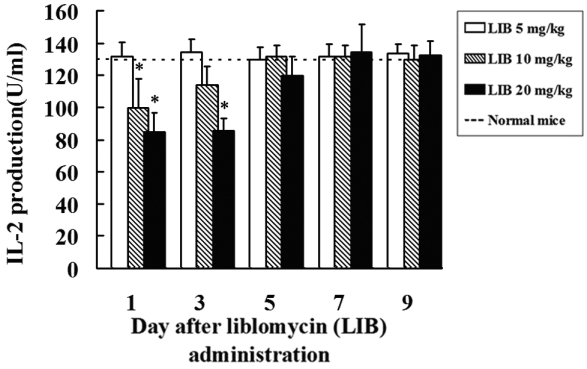

Fig. 5 Effects of various doses of LIB on IL-2 activity. $^{*}P < 0.05$ compared with normal mice.

DISCUSSION

In this study, we have demonstrated that BLM (20 mg/kg) augments antitumour effect or activities including NK activity, LAK precursor activity and the total number of spleen cells, and although the same dose of PEP did not

augment these antitumour effects when expressed per unit number (1×10^6) of spleen cells, but increased NK and LAK precursor cell activities per spleen. LIB significantly suppressed all of these activities under the same conditions.

The BLM, an antitumour antibiotic glycopeptide was produced in 1977[9], and it could cause breaks in DNA which is similar to radiotherapy[10]. Others have reported that, BLM was effectively used for cancer tests and Hodgkin disease[11,12]. BLM had produced an antitumour effect by inducing the immunogenic cell death and regulating the proliferation of T cells[13]. PEP and LIB were the analogues of BLM. The previous study showed that the immunoregulatory cytokine (IFN, TNF and IL-2) release in rat spleen cell was reportedly increased after treatment with BLM and PEP; however, the administration of LIB could reduce cytokine levels[8]. It had proved that LIB was associated with less pulmonary toxicity and with more potent antitumour activity than BLM in animal tumours[14]; for another thing, LIB was more resistant to the enzyme BLM hydrolase[15]. PEP, the BLM derivate antibiotic, it has been proved to produce cytotoxicity of BLM by inducing G1-phase specific apoptosis in Bel-7402 cell line[16]. The PEP was useful for treating cutaneous squamous cell carcinoma by inducing immunomodulatory effects[17].

On analysis of the chronological changes in mouse immune effector cells and IL-2 release after the administration of BLM and its derivatives *in vivo*, the results indicated that a single administration of BLM or PEP (20 mg/kg) enhances mouse immune cell antitumour properties when compared with untreated controls, but LIB markedly decrease the total mouse spleen cell number, suppresses the NK cell activity per unit number of spleen cells and also decreases the IL-2 release by mitogen-stimulated spleen cells from as early as day 1 after the drug administration. The restoration of the NK cell activity was slow when compared with either the recovery of spleen cell number or IL-2 release; however, the LAK precursor cell activity per unit number of spleen cells was almost unaffected by the treatment with LIB. To determine an end point for the effects of LIB, we examined the immunomodulating effects of various doses of LIB and found that at a dose of 5 mg/kg libliomycin did not affect the mouse spleen cell number but suppressed the NK cell activity from day 5 after treatment. In this study, although we are unable to understand the mechanisms of action of LIB, the NK cell activity of the mouse spleen appears to be very susceptible to toxicity by LIB when compared to either BLM or PEP. As a result of the modification of the chemical structure of BLM to produce LIB, undoubtedly changes in drug metabolic pathways and drug function may occur as well as changes in immunomodulating properties such as reported for daunorubicin and its immunoaugmenting derivative doxorubicin[18].

This study describes the effects of the BLM group of antitumour antibiotics on NK and LAK precursor cell activities, in which the activities are considered to be the most effective immune cell-mediated cytotoxic events. Since LIB exhibits a broad *in vitro* antitumour spectrum as well as an extremely weaker *in vivo* pulmonary toxicity than either BLM or PEP, it is a promising drug for using clinically; however, immunosuppression, especially in the form of decreased NK cell activation, is a problem which requires caution to be exercised when such antitumour agents are used.

REFERENCES

1. Cragg GM, Kingston DGI, Newman DJ. Anticancer agents from natural products, 2nd ed., Boca Raton: CRC Press LLC; 2005.
2. Abdul Hamied TA, Parker D, Turk JL. Potentiation of release of interleukin-2 by bleomycin. Immunopharmacology. 1986; 12(2):127–134.
3. Hillel AT, Samad I, Ma G, Ding D, Sadtler K, Powell JD, et al. Dysregulated macrophages are present in bleomycin-induced murine laryngotracheal stenosis. Otolaryngol Head Neck Surg. 2015;153(2):244–250.
4. Xu ZY, Hosokawa M, Morikawa K, Hatakeyama M, Kobayashi H. Overcoming suppression of antitumor immune reactivity in tumor-bearing rats by treatment with bleomycin. Cancer Res. 1988;8(23):6658–6663.
5. Williamson JD, Sadofsky LR, Hart SP. The pathogenesis of bleomycin-induced lung injury in animals and its applicability to human idiopathic pulmonary fibrosis. Exp Lung Res. 2015;41(2):57–73.
6. Takita T, Ogino T. Peplomycin and liblomycin, a new analogues of bleomycin. Biomed Pharmacother. 1987;41(5):219–226.
7. Takahashi K, Ekimoto H, Minamide S, Nishikawa K, Kuramochi H, Motegi A, et al. Liblomycin, a new analogue of bleomycin. Cancer Treat Rev. 1987;14(3–4):169–177.
8. Micallef M, Hosokawa M, Shibata T, Nakane A, Yang ZB, Minagawa T, et al. Immunoregulatory cytokine release in rat spleen cell cultures after treatment with bleomycin and its analogues *in vivo*. Cancer Immunol Immunother. 1991; 33(1):33–38.
9. Ryzhkov VK, Zeidlits VN, Tikhonov KB. Use of protamine sulfate as a vasodilator in liver and spleen angiography. Vestn Rentgenol Radiol. 1977;(6):41–45.
10. Dresp J, Schmid E, Bauchinger M. The cytogenetic effect of bleomycin on human peripheral lymphocytes *in vitro* and *in vivo*. Mutat Res. 1978;56(3):341–353.
11. Meyer RM, Gospodarowicz MK, Connors JM, Pearcey RG, Wells WA, Winter JN, et al. ABVD alone versus radiation-based therapy in limited-stage Hodgkin's lymphoma. N Engl J Med. 2012;366:399–408.
12. Williams SD, Birch R, Einhorn LH, Irwin L, Greco FA, Loehrer PJ. Treatment of disseminated germ-cell tumors with cisplatin, bleomycin, and either vinblastine or etoposide. N Engl J Med. 1987;316:1435–1440.
13. Bugaut H, Bruchard M, Berger H, Derangère V, Odoul L, Euvrard R, et al. Bleomycin exerts ambivalent antitumor immune effect by triggering both immunogenic cell death and proliferation of regulatory T cells. PLoS One. 2013;8(6):e65181.

14. Yoshida T, Ogawa M, Ota K, Yoshida Y, Wakui A, Oguro M, et al. Phase II study of NK313 in malignant lymphomas: an NK313 malignant lymphoma study group trial. Cancer Chemother Pharmacol. 1993;31(6):445–448.
15. Lazo JS, Braun ID, Labaree DC, Schisselbauer JC, Meandzija B, Newman RA, et al. Characteristics of bleomycin-resistant phenotypes of human cell sublines and circumvention of bleomycin resistance by liblomycin. Cancer Res. 1989;49(1): 185–190.
16. Chen HY, Zheng CY, Zou GL, Xie DX, Gong JP. Peplomycin induces G1-phase specific apoptosis in liver carcinoma cell line Bel-7402 involving G2-phase arrest. Acta Pharmacol Sin. 2004;25:1698–1704.
17. Fujimura T, Kambayashi Y, Furudate S, Kakizaki A, Haga T, Hashimoto A, et al. Immunomodulatory effects of peplomycin on immunosuppressive and cytotoxic cells in the lesional skin of cutaneous squamous cell carcinoma. Dermatology. 2015;230(3):250–255.
18. Schwartz HS, Grindey GB. Adriamycin and daunorubicin: a comparison of antitumor activities and tissue uptake in mice following immunosuppression. Cancer Res. 1973;33(8): 1837–1844.