Exp Oncol 2008
30, 1, 52–55

Experimental ONCOLOGY

THE USE OF DOXORUBICINE AT LOW DOSES FOR ELEVATION OF LAK-ACTIVITY TOWARD EXPLANTS AND CELLS OF MC-RHABDOMYOSARCOMA AND B16 MELANOMA RESISTANT TO DOXORUBICIN

N.M. Berezhnaya, Yu.D. Vinnichuk, O.B. Belova*

R.E. Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology, NAS of Ukraine, Kyiv, Ukraine

Aim: **To study the influence of doxorubicin at low doses on antitumor action of activated (LAK) and non-activated lymphocytes from lymph nodes toward tumor cells of mice bearing doxorubicin-resistant and doxorubicin-sensitive transplantable MC-rhabdomyosarcoma and B16 melanoma.** ***Materials and Methods:*** **The study was carried out on BALB/c mice bearing MC-rhabdomyosarcoma and C57BL/6 mice bearing B16 melanoma. Explants, tumor cells and lymphocytes were cultivated in diffusion chambers, filters were stained with hematoxylin by Karachi, and morphology of preparations was examined.** ***Results:*** **At the day 7 of tumor growth in mice bearing resistant MC-rhabdomyosarcoma, non-activated lymphocytes pretreated with low-dose doxorubicin possess the highest antitumor activity, and in mice bearing doxorubicin-resistant B16 melanoma the highest antitumor activity was detected for lymphocytes after combined cultivation with IL-2 and doxorubicin. At the day 14 of tumor growth, LAK obtained from lymphocytes pretreated with doxorubicin possess the highest cytotoxic activity toward resistant tumor cells both of MC-rhabdomyosarcoma and B16 melanoma. There was no such effect in the case of sensitive tumors.** ***Conclusion:*** **To elevate antitumor activity of LAK toward MC-rhabdomyosarcoma and B16 melanoma cells, low doses of doxorubicin could be used at certain conditions of LAK generation.**

Key Words: **transplantable MC-rhabdomyocarcoma, melanoma, resistance, doxorubicin, LAK.**

It is known that tumors resistant to anticancer preparations in the majority of cases demonstrate marked aggressiveness and ability to quick progression. Combination of chemoresistance with the resistance to radio- and phototherapy additionally complicates the treatment of the patients with chemoresistant tumors [1]. The problem of therapy of the patients with chemoresistant tumors requires not only improvement of the therapy with already existing preparations and the development of new ones, but also the search for new approaches for treatment of such patients.

In our earlier studies we have shown that the cells and explants of human chemoresistant tumors (soft tissue tumors of locomotor system, and epithelial tumors of female reproductive system) and animal tumors (B16 melanoma and transplantable MC-rhabdomyosarcoma) demonstrate elevated sensitivity to the action of LAK [2, 3]. This fact has been proved not only in the study in vitro, but also upon immunotherapy of mice bearing doxorubicin-resistant MC-rhabdomyosarcoma [4]. To study the sensitivity of explants of human tumors, we have used the method developed by us for determination of individual sensitivity to preparations widely used for chemotherapy (doxorubicin, methotrexate, vincristine, carboplatin, cyclophosphan). It is necessary to note that the phenomenon of elevated sensitivity of resistant tumors to the action of LAK was observed even if there was resistance only to a single preparation [3].

So, the question arises — is it possible to elevate antitumor action of LAK against chemoresistant tumors? As one approach, we have used doxorubicin (Dox) at the low doses — according to the data of literature, this drug and a number of other chemopreparations possess immunomodulating effect at the low doses.

For example, low doses Dox may promote functions of the cells of immune system, in particular peritoneal exudate (macrophages, neutrophils, lymphocytes) and elevate production of INFγ, TNFα [5, 6]. The use of low (subtoxic) doses of chemopreparations returns to lymphocytes the ability for cytotoxic action toward chemoresistant renal and prostate tumors that is realized via perforin-dependent and perforin-independent mechanisms [7]. The authors concluded that the use of the low doses of chemopreparations results in "sensibilization" of chemoresistant tumors.

Dox at the low doses may promote effect of TNFα, as it was shown in the experiments with EL4-resistant murine lymphoma cells. Antitumor activity of cytotoxic splenocytes and thymocytes increased, and such increase correlated with elevated survival of mice. The effect was observed also for spleen macrophages and LAK [8]. Doxorubicin possesses ummunomodulatory action, and if it is used as a component of co-polymer Dox-immunoglobulin, activity of LAK and natural killer cells was higher [6].

There are other preparations able to promote tumor cell lysis; for example, mitomycin and cysplatin may elevate the sensitivity of colon cancer cells of SW480 line to apoptotic signals [9]. The treatment of human ovarian cancer cells of SVO3 line with paclitaxel elevated their sensitivity to LAK-mediated lysis [10].

Unfortunately, the number of studies aimed on evaluation of antitumor activity of LAK against resistant tumor cells using low doses of Dox or other anticancer preparations is limited. That's why the present research was directed on the development of optimal

Received: October 15, 2007.

*Correspondence: E-mail: berezh@onconet.kiev.ua

Abbreviations used: Dox – doxorubicin; LAK – lymphokine-activated killer cells.

conditions for elevation of antitumor activity of LAK against Dox-resistant tumors.

MATERIALS AND METHODS

In the study, BALB/c and C57BL/6 mice weighting 15–20 g bred in the vivarium of R.E. Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology, NAS of Ukraine (Kyiv, Ukraine) were used. As experimental tumor models, we have used the strains generated by us: 1) B16 melanoma and transplantable MC-rhabdomyosarcoma cells resistant to Dox; 2) B16 melanoma and transplantable MC-rhabdomyosarcoma cells sensitive to Dox.

The studies were performed on tumor explants and isolated tumor cells and lymphocytes from lymph nodes of experimental animals.

Lymphokine-activated cells (LAK) were obtained by incubation of lymphocytes of lymph nodes of tumor bearing mice with recombinant IL-2 (3×10^6 cells with 1000 MU RIL-2) for 2 h at 37 °C.

Lymphocytes and LAK were treated with 0.4 μg/ml doxorubicin (Ebeve, Austria) for 24 h in complete RPMI-1640 medium at 37 °C. The optimal dose and conditions of cultivation were estimated prior to experiments (calibration of preparation dose at the base of therapeutic one accounting animals weight, has been done).

The following systems of cultivation in diffusion chambers were used: 1) cultivation of lymphocytes, Dox-pretreated, with tumor cells or explants; 2) cultivation of LAK, Dox-pretreated, with tumor cells or explants; 3) cultivation of tumor cells or lymphocytes after combined treatment with Dox and IL-2. Controls were as follow: 1) tumor growth at standard conditions; 2) the growth of tumor cells with non-activated lymphocytes; 3) the growth of tumor cells with LAK. Evaluation of antitumor action of lymphocytes was performed at the base of tumor growth morphology [05]. All experiments were done with the use of the cells and explants of both Dox-sensitive and Dox-resistant MC-rhabdomyosarcoma and B16 melanoma.

RESULTS AND DISCUSSION

The study of anticancer activity of LAK received from lymphocytes from lymph nodes of mice was carried out in the dynamics of tumor growth (at the days 7 and 14 after tumor transplantation). At the day 7, tumor explants were studied because it was impossible to receive required quantity of tumor cells (at this period tumors were too small (0.06–0.09 g)), and at the day 14 isolated tumor cells and explants were examined.

At the day 7 after tumor transplantation, the comparative study of antitumor action of non-activated lymphocytes and LAK from animals bearing transplantable MC-rhabdomyosarcoma resistant and sensitive to Dox upon the influence of low doses of the drug has shown the following: upon cultivation of lymphocytes from mice bearing resistant MC-rhabdomyosarcoma it has been shown that pretreatment of non-activated lymphocytes with Dox elevated their cytotoxicity to the level of LAK; pretreatment of LAK with Dox did not alter their antitumor action. Lymphocytes cultivated with IL-2 and Dox possess the same antitumor activity. In the case of sensitive tumor, pretreatment of lymphocytes with Dox did not alter antitumor action of non-activated lymphocytes, but elevated antitumor activity of LAK (Fig. 1).

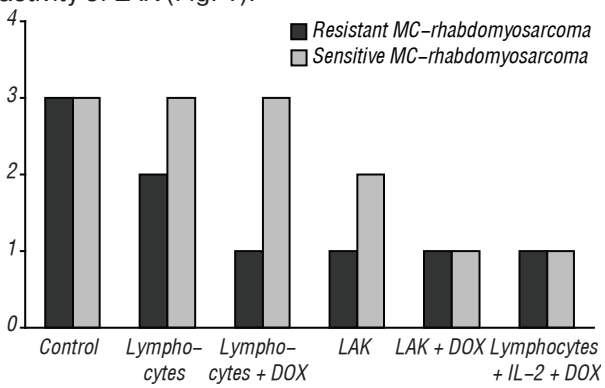

Fig. 1. Antitumor action of lymphocytes of mice bearing transplantable MC-rhabdomyosarcoma resistant and sensitive to doxorubicine at day 7 after tumor transplantation. 1 — migration of single cells, initial stages of formation of the monolayer; 2 — intense formation of monolayer; 3 — single conglomerates

In analogous way, the study was performed for lymphocytes and tumor cells of mice bearing melanoma B16 sensitive and resistant to Dox. In the case of resistant tumor explants, the highest antitumor activity was observed for lymphocytes cultivated with IL-2 and Dox. However, pretreatment with Dox did not alter cytotoxic activity of lymphocytes and LAK (which activity was higher than that of lymphocytes). In the case of sensitive tumor explants, pretreatment of lymphocytes with Dox led to elevation of their cytotoxic activity to the level of that of LAK; the influence of pretreatment of LAK with Dox or its combination with IL-2 was not observed (Fig. 2).

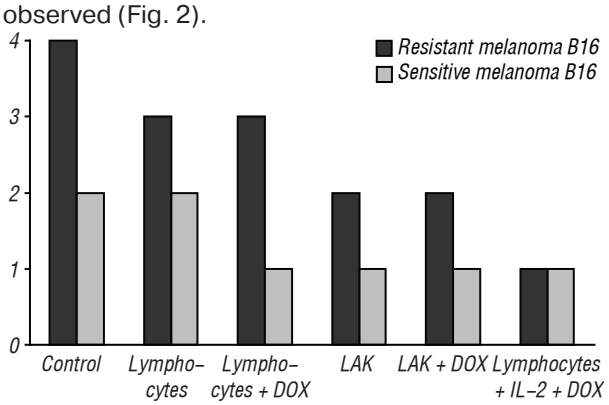

Fig. 2. Antitumor action of lymphocytes of mice bearing melanoma B16 resistant and sensitive to doxorubicine at day 7 after tumor transplantation. 1 — migration of single cells, initial stages of formation of the monolayer; 2 — intense formation of monolayer; 3 — single conglomerates; 4 — intense formation of conglomerates

Comparison of the results of antitumor activity of activated and non-activated lymphocytes received at different conditions against the explants of the resistant B16 melanoma and transplantable MC-rhabdomyosarcoma revealed some differences; in particular, the pretreatment of non-activated lymphocytes with Dox at low doses elevated their antitumor activity upon cultivation with MC-rhabdomyosarcoma explants, but

not in the case of melanoma cells. The highest antitumor activity against melanoma cells was observed for lymphocytes incubated with IL-2 and Dox. It is necessary to note that in the case of MC-rhabdomyosarcoma cells, antitumor activity of lymphocytes elevated after using all variants of pretreatment (see Fig. 1, 2).

At the day 14 after tumor transplantation, in the case of resistant MC-rhabdomyosarcoma the highest antitumor action was registered for LAK obtained from lymphocytes pretreated with Dox. The pretreatment with Dox did not influence the activity of non-activated lymphocytes; cultivation of lymphocytes with IL-2 and Dox also did not elevate their antitumor action (Fig. 3). For resistant melanoma B16 cells, the highest activity was registered for LAK obtained from lymphocytes pretreated with Dox; Dox-pretreatment of non-activated lymphocytes also elevated their antitumor potency. Similarly to MC-rhabdomyosarcoma model, combined treatment did not influence antitumor action of lymphocytes (Fig. 4).

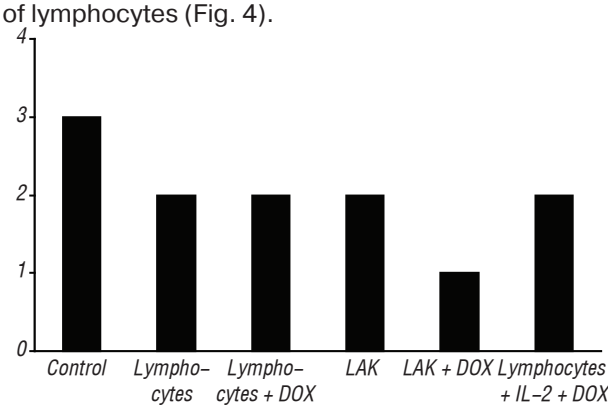

Fig. 3. Antitumor action of lymphocytes of mice bearing transplantable MC-rhabdomyosarcoma resistant and sensitive to doxorubicine at day 14 after tumor transplantation. 1 — migration of single cells, initial stages of formation of the monolayer; 2 — intense formation of monolayer; 3 — single conglomerates

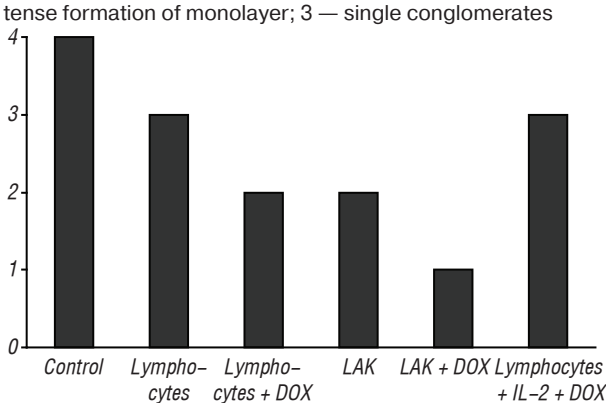

Fig. 4. Antitumor action of lymphocytes of mice bearing melanoma B16 resistant to doxorubicine at day 14 after tumor transplantation. 1 — migration of single cells, initial stages of formation of the monolayer; 2 — intense formation of monolayer; 3 — single conglomerates; 4 — intense formation of conglomerates

The common pattern of lymphocytes from mice bearing sensitive MC-rhabdomyosarcoma and B16 melanoma was the absence of influence of Dox-pretreatment on their antitumor activity.

As it was mentioned above, the task of present research was the development of approach for elevation of antitumor activity of LAK against chemoresistant tumors (MC-rhabdomyosarcoma, B16 melanoma) using low doses of Dox. The use of different systems for cultivation of lymphocytes from mice bearing Dox-sensitive and Dox-resistant tumors allowed to show that, firstly, the conditions for elevation of activity of LAK against Dox-resistant tumors differ from these for Dox-sensitive mice; secondly, the influence of low doses of Dox on antitumor activity of lymphocytes from mice with Dox-resistant MC-rhabdomyosarcoma and B16 melanoma is also different, especially at early stages of tumor growth.

Such effect of Dox one could explain by its ability for imunomodulating action. It is known that the total population of LAK is heterogeneous and is presented by different subpopulations of killer cells [11]. According to the data of literature, Dox may elevate the activity of different cells of immune system. In particular, the data showing that upon the development of resistance to adriamycin, increased expression of I class antigens of histocompatibility complex (A, B, C) in ovarian cancer cells promoting their lysis, point on the possibility of activation of cytotoxic T-lymphocytes [12]. The influence of adriamycin is not limited by cytotoxic T-lymphocytes, but involves also natural killer cells and LAK; this fact has been shown upon the study of bladder cancer cells, lymphocytes of peripheral blood and lymphocytes infiltrating tumor [13]. The authors consider that the treatment of tumor cells with low doses of adrsamycin induces their elevated sensitivity to the action of different killer cells. The similar point of view has been documented in another study where elevated expression of ICAM-1 in ovarian cancer cells resistant to packlitaxel considered as a main cause of elevated sensitivity of the cells to LAK action, has been reported [10]. The data on positive influence of low dose Dox on Dox-resistant tumor cells are in accordance with our earlier results for in vivo and in vitro studies that showed that simultaneous introduction of Dox and LAK to mice bearing Dox-resistant MC-rhabdomyosarcoma promoted the effect of LAK [2].

In summary, one may conclude that the effect of low dose Dox is a result of both elevated activity of different killer cells and increased sensitivity of the tumor to their action. The presented data are supporting the reported earlier results and show that low dose Dox may be used for treatment of tumor cells and for generation of LAK possessing higher activity, and these effects are complementary. That's why one may consider that the low doses of anticancer drugs may be used in regular chemotherapy, and immunomodulating effect of Dox will not be suppressed [14].

The results of the present study have shown that for elevation of antitumor activity of LAK toward Dox-resistant MC-rhabdomyosarcoma and B16 melanoma cells, low dose Dox could be used upon special conditions of LAK generation. These data make reasonable the further study of the processes responsible for elevated sensitivity of resistant tumor cells to cytotoxic action of lymphocytes and of optimizing effect of low dose Dox; the research of expression of surface molecules

on lymphocytes and resistant tumor cells will be especially important.

REFERENCES

1. **Koppold B, Sauer G, Buening H, *et al.*** Chemotherapeutic agents enhance AAV2-mediated gene transfer into breast cancer cells promoting CD40 ligand-based immunotherapy. J Cancer Res Clin Oncol 2006; **132**: 787–94.

2. **Berezhnaya NM, Kovalchuk EV, Spivak SI, *et al.*** Antitumor action of lymphocytes in the dynamics of formation of the resistanse to doxorubicine in mice bearing transplantable MC-rhabdomyosarcoma. Exp Oncol 2002; **24**: 292–5 (In Russian).

3. **Berezhnaya NM, Vinnichuk YD, Konovalenko VF, *et al.*** The sensitivity of chemioresistant human tumor explants to lysis by activated and nonactivated autological lymphocytes: a pilot study. Exp Oncol 2005; **27**: 303–7.

4. **Berezhnaya NM, Kovalchuk EV, Vinnichuk YD, *et al.*** Experimental immunotherapy of mice with transplated MC-rhabdomiocarcoma resistant to doxorybicin. Exp Oncol 2004; **26**: 63–7.

5. **Eralp Y, Wang X, Wang JP.** Doxorubicin and paclitaxel enhance the antitumor efficacy of vaccines directed against HER 2/neu in a murine mammary carcinoma model. Breast Cancer Res. 2004; **6**: 275–83.

6. **Rihova B, Strohalm J, Prausova J.** Cytostatic and immunomobilizing activities of polymer-bound drugs: experimental and first clinical data. J. Control Release 2003; **91**: 1–16.

7. **Frost P, Caliliw R, Belldegrun A, *et al.*** Immunosensitization of resistant human tumor cells to cytotoxicity by tumor infiltrating lymphocytes. Int J Oncol 2003; **22**: 431–7.

9. **Ehrke MJ, Verstovsek S, Maccubbin DL, *et al.*** Protective specific immunity induced by doxorubicin plus TNF-alpha combination treatment of EL4 lymphoma-bearing C56BL/6 mice. Int J Cancer 2000; **87**: 101–9.

10. **Zhu Q, Liu JY, Yang CM, *et al.*** Influence of antitumor drugs on the expression of Fas system in SW480 colon cancer cells. Eur J Gastroenterol Hepatol 2006; **18**: 1071–7.

11. **Law KS, Chen HC, Liao SK.** Non-cytotoxic and sublethal paclitaxel treatment potentiated the sensitivity of cultured ovarian tumor SKOV-3 cells to lysis by lymphokine-activated killer cells. Anticancer Res 2007; **27**: 841–50.

12. **Bereznaya NM, Koval'chuk EV.** LAK-phenomenon (cell phenotype, mechanism of action and conditions for its realization). Immunologiya 1995; **2**: 12–6 (In Russian).

13. **Fisk B, Ioannides CG.** Increased sensitivity of adriamicin-selected tumor lines to CTL-mediated lysis results in enchanced drug sensitivity. Cancer Res 1998; **58**: 4790–3.

14. **MizutaniY, Yoshida O, Miki T.** Adriamycin-mediated potentiation of cytotoxicity against freshly isolated bladder cancer cells by autologous non-activated peripheral blood lymphocytes and tumor infiltrating lymphocytes. J Urol 1999; **162**: 2171–5.

15. **Zagozdzon R, Golab J.** Immunomodulation by anticancer chemotherapy: more is not always better (review). Int J Oncol 2001; **18**: 417–24.

ИСПОЛЬЗОВАНИЕ МАЛЫХ ДОЗ ДОКСОРУБИЦИНА ДЛЯ УСИЛЕНИЯ АКТИВНОСТИ ЛАК В ОТНОШЕНИИ ЭКСПЛАНТАТОВ И ОПУХОЛЕВЫХ КЛЕТОК МХ-РАБДОМИОСАРКОМЫ И МЕЛАНОМЫ В16, РЕЗИСТЕНТНЫХ К ДОКСОРУБИЦИНУ

Цель: **изучить влияние малых доз доксорубицина на противоопухолевое действие активированных (ЛАК) и неактивированных лимфоцитов лимфатических узлов по отношению к опухолевым клеткам мышей с резистентными и чувствительными к доксорубицину перевивной МХ-рабдомиосаркомой и меланомой В16.** ***Материалы и методы:*** **исследования проведены на мышах линии BALB/c с МХ-рабдомиосаркомой и C57BL/6 с меланомой В16. Эксплантаты, опухолевые клетки и лимфоциты культивировали в диффузионных камерах и окраской фильтров гематоксилином по Караччи. Исследовали морфологическую картину полученных препаратов с учетом критериев роста опухоли.** ***Результаты*****: сопоставление результатов противоопухолевой активности активированных и неактивированных лимфоцитов, полученных в различных условиях, показало следующее. На 7-е сутки у мышей з резистентной МХ-рабдомиосаркомой наибольшей противоопухолевой активностью обладали неактивированные лимфоциты, предварительно обработанные малыми дозами докорубицина, а у мышей з резистентной меланомой В16 — лимфоциты после сочетанного культивирования с ИЛ-2 и доксорубицином. На 14-е сутки роста опухоли наибольшей цитотоксической активностью обладали ЛАК, полученные из лимфоцитов, предварительно обработанных доксорубицином, в отношении резистентных опухолевых клеток как перевивной МХ-рабдомиосаркомы, так и меланомы В16. Относительно чувствительных клеток указанных опухолей такой эффект не отмечался.** ***Выводы*****: для усиления противоопухолевой активности ЛАК по отношению к клеткам МХ-рабдомиосаркомы и меланомы В16, резистентных к доксорубицину, могут быть использованы малые дозы доксорубицина при соблюдении определенных условий получения ЛАК.**

Ключевые слова: **перевивная МХ-рабдомиосаркома, меланома В16, резистентность, доксорубицин, ЛАК.**