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EFFICACY OF DIFFERENT IMMUNOTHERAPY APPROACHES TOWARD TREATMENT OF DOXORUBICIN-RESISTANT AND DOXORUBICIN-SENSITIVE TRANSPLANTABLE RHABDOMYOSARCOMA

O.B. Belova, U.D. Vinnichuk, V.A. Shlakhovenko, N.M. Berezhnaya*
R.E. Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology,
National Academy of Sciences of Ukraine, Kyiv 03022, Ukraine

Aim: To evaluate the efficacy of different variants of immunotherapy, namely, adoptive LAK-therapy, vaccine therapy and their combination *in vivo* using transplantable murine MC-rhabdomyosarcoma resistant and sensitive to doxorubicin (Dox). *Materials and Methods:* The study was carried out on BALB/c mice bearing Dox-sensitive and Dox-resistant transplantable murine MC-rhabdomyosarcoma. LAK-therapy (using lymphocytes from lymph nodes of syngenic mice) was performed starting from day 7 after tumor cell transplantation for 5 days; LAK (3×10^6 cells in 0.2 ml medium) were injected in the region of tumor. The vaccine prepared on the base of tumor cell glycopeptides was administered intraperitoneally at the volume of 0.2 ml before or after tumor transplantation. Efficacy of immunotherapy was evaluated by tumor growth inhibition and life span of animals. *Results:* By the indexes of tumor growth inhibition and average life span, for animals bearing Dox-sensitive tumors vaccine therapy was the most effective, whilst adoptive LAK-therapy was the most effective for mice bearing Dox-resistant tumors. All applied variants of therapy — adoptive LAK-therapy, vaccine therapy and their combination were effective for treatment of mice bearing Dox-sensitive and Dox-resistant transplantable murine MC-rhabdomyosarcoma. *Conclusion:* The obtained data demonstrated that Dox-sensitive and Dox-resistant tumors differ by the sensitivity to different types of immunotherapy.
Key Words: transplantable MC-rhabdomyosarcoma, resistance, doxorubicin, vaccine therapy, LAK-therapy.

It is known that chemoresistance of tumors is among the major problems of modern oncology because it leads to failure of therapy. That's why it is necessary to find the way to overcome drug resistance. The related approach is to study the sensitivity of resistant tumors to the action of immunological mechanisms of antitumor defense [1].

Among known types of immunotherapy (therapy with the use of monoclonal antibodies, cancer vaccines, cytokines, adoptive immunotherapy etc) adoptive immunotherapy with the use of lymphocytes activated with cytokines occupies a special place [2–5]. However, the data on its use for the treatment of chemoresistant tumors are limited, and mostly they are obtained in vitro evidencing on a pronounced sensitivity of the cells of resistant tumors to the action of LAK [6–9]. The data on the use of vaccine therapy or combined therapy (vaccine + LAK) of chemoresistant tumors are nearly absent. There is some positive experience on the combined use of LAK-therapy with other types of immunotherapy and chemotherapy [10, 11].

As we have shown earlier, chemoresistant human tumors (soft tissue sarcoma, epithelial tumors) and doxorubicin-resistant animal tumors (B16 melanoma, transplantable murine MC-rhabdomyosarcoma) demonstrated elevated sensitivity to the action of LAK both *in vitro* and *in vivo* [12, 13].

At the same time it remains unknown what is the sensitivity of chemoresistant tumors to other types of immunotherapy, in particular, to vaccination with the use of cancer vaccines prepared on the base of different tumor antigens. The different technologies for preparation of such vaccines are used, in particular,

the vaccines prepared on the base glycopeptide carbohydrate antigens [14–17].

The aim of the present work was to evaluate the efficacy of different types of immunotherapy (LAK-therapy, vaccine therapy and their combination) toward doxorubicin (Dox)-sensitive and Dox-resistant transplantable murine MC-rhabdomyosarcoma (MC-RMS).

MATERIALS AND METHODS

In the study, BALB/c 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) Dox-resistant MC-RMS cells; 2) Dox-sensitive MC-RMS cells.

Lymphokin-activated cells (LAK) were obtained by incubation of lymphocytes from lymph nodes of tumor-bearing mice (n = 30) with recombinant IL-2 (3×10^6 cells with 1000 MU IL-2) for 2 h at 37 °C; lymphocytes were isolated from syngeneic tumor-bearing mice at the days 7–11 after tumor transplantation accounting the level of expression of IL-2 receptor (CD25), analyzed by the method of indirect immunofluorescence. LAK-therapy was performed starting from day 7 after tumor cell transplantation (at the stage of appearance of tumor node) for 5 days; LAK (3×10^6 cells in 0.2 ml of medium) were injected in the region of tumor.

For vaccination, an autovaccine prepared by the method of controlled proteolytic hydrolysis of tumor tissue by its treatment with the filtrate of culture medium of Bacillus subtilis with the next fractionation by DEAE-chromatography and purification of polypeptides that carry polysaccharide residues. Determination of polysaccharide component was done using electrophoresis in polyacrylamide gel. Immunogenicity of the

vaccine was expressed in relative units equivalent to the number of tumor cells. The technology of preparation of glycopeptide vaccine is patented in Ukraine, and it is allowed for trials [18]. Earlier, the efficacy of this vaccine has been evaluated in other *in vivo* models [19].

The vaccine was administered intraperitoneally at the volume of 0.2 ml by two different schemes: 1) vaccination prior to tumor transplantation (triple injection with 5 days intervals); tumor was transplanted 3 days after the last injection; 2) vaccination at the day 7 after tumor transplantation — triple injection with 5 days intervals.

Animals bearing resistant and sensitive tumors were housed in the next groups (20 animals per group) according to applied therapy: 1) adoptive LAK-therapy; 2) vaccine therapy; 3) combined immunotherapy (adoptive LAK-therapy and vaccine therapy); 4) control group (no therapy). All researches on animals were carried out in accordance with Bioethic standards for study on animals approved in Ukraine.

RESULTS AND DISCUSSION

The study was initiated from the search for optimal scheme and dose of vaccine because this vaccine was not tested in MC-RMS model. The efficacy of 3 doses — 5000, 50 000 and 100 000 relative units (r. u. are equivalent to the number of tumor cells used for vaccine preparation) administered at two regimens mentioned above was studied. We have shown that for treatment of MC-RMS, the optimal dose is 50 000 r. u. and prophylactic scheme of vaccination, and exactly this dose and scheme of vaccination were used in present study; the percent of tumor growth inhibition, tumor volume and life span of enimals served as the main indexes for evaluation of the efficacy of the therapy [20].

For Dox-sensitive MC-RMS, we have shown that all applied types of immunotherapy were effective. At the day 9 after tumor transplantation, tumor growth inhibition differs significantly in all immunized animals compared to the control, but not in mice that received LAK-therapy. In vaccinated animals, the percent of tumor growth inhibition reached its maximum at the day 14 after tumor transplantation and was 62.9 ± 0.62% versus 36.5 ± 1.37% ($p < 0.01$) for LAK-therapy and 51.6 ± 0.76% ($p < 0.001$) in the case of combined therapy (Fig. 1).
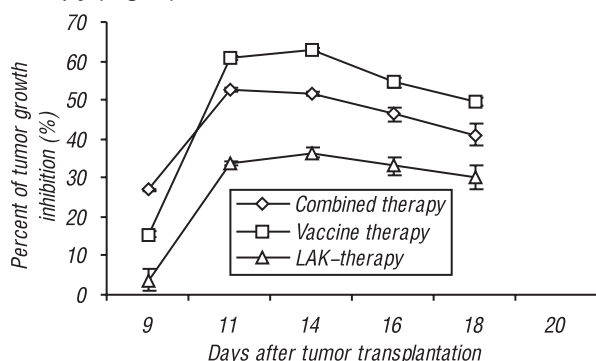
Fig. 1. Inhibition of tumor growth in BALB/c mice bearing Dox-sensitive MC-RMS upon the influence of different types of immunotherapy

Analysis of average life span (Fig. 2) of animals bearing Dox-sensitive tumors also demonstrated significant benefit of vaccination compared to LAK-therapy and combined therapy.
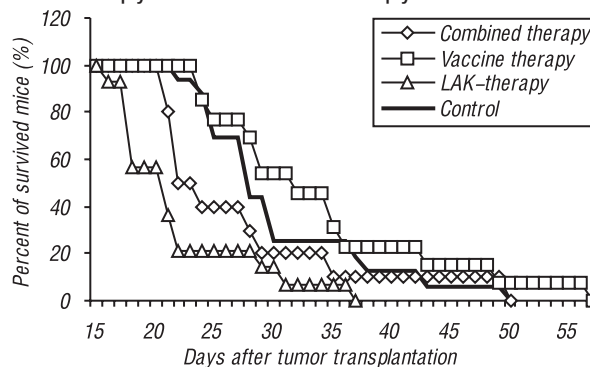
Fig. 2. Life span of BALB/c mice bearing Dox-sensitive MC-RMS upon the influence of different types of immunotherapy

So, vaccine therapy was more effective for treatment of animals bearing Dox-sensitive MC-RMS than LAK- and combined therapy.

Analogous studies were carried out on Dox-resistant MC-RMS model, and it has been revealed that upon use of all mentioned types of immunotherapy, significant increase of the percent of tumor growth inhibition was observed in animals from all groups without significant differences till the day 21 after tumor transplantation (57–60%). However, starting from the days 22–23, LAK-therapy was found to be more effective than vaccine therapy or combined therapy; At the day 23, 28, 30 after tumor transplantation in animals that received LAK-therapy, the percent of tumor growth inhibition was 61.4 ± 1.84%; 70.3 ± 4.94%; 66.2 ± 6.24% versus 48.8 ± 3.85% ($p < 0.001$); 47.6 ± 8.91% ($p < 0.05$); 31.8 ± 15.91% ($p < 0.05$) for vaccinated animals, and 41.2 ± 3.74% ($p < 0.001$); 42.6 ± 8.71% ($p < 0.001$); 45.7 ± 10.33% ($p < 0.1$) in animals that received combined therapy respectively (Fig. 3).
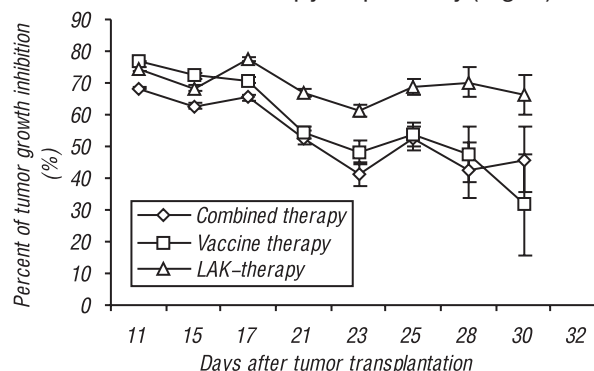
Fig. 3. Inhibition of tumor growth in BALB/c mice bearing Dox-resistant MC-RMS upon the influence of different types of immunotherapy

LAK-therapy was shown to be more effective than vaccination and combined therapy also by the index of life span of experimental animals: whilst average life span in control group was 32 days, animals treated by LAK survived up to 46–49 days (Fig. 4).
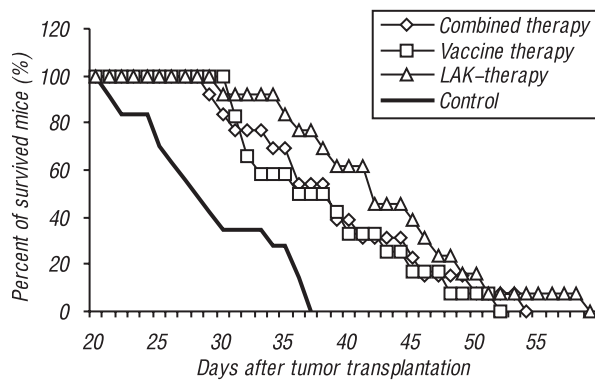
Fig. 4. Life span of BALB/c mice bearing Dox-resistant MC-RMS upon the influence of diffent types of immunotherapy

So, one may conclude that LAK-therapy is the most effective type of treatment of mice bearing Dox-resistant MC-RMS.

Finally, we have performed comparative analysis of the efficacy of different types of immunotherapy in mice bearing Dox-sensitive and Dox-resistant MC-RMS (Fig. 5). Firstly, all types of therapy were found to be more effective for treatment of Dox-resistant tumor. Secondly, for Dox-sensitive tumor, vaccine therapy was the most effective, whilst in the case of Dox-resistant tumor adoptive LAK-therapy resulted in the highest life span of animals and highest tumor growth inhibition. Thirdly, the combined therapy in the cases of both resistant and sensitive tumors occupies intermediate place between vaccination and LAK-therapy.
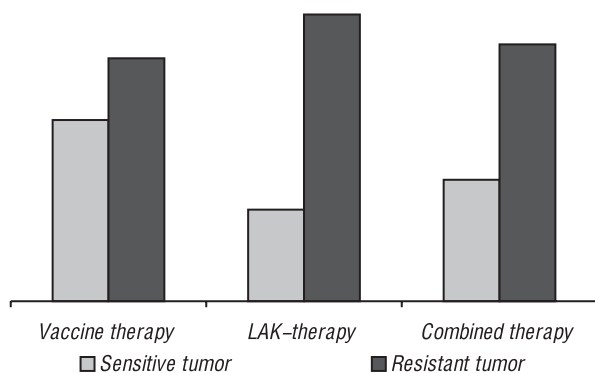
Fig. 5. Comparative analysis of the efficacy of different types of immunotherapy for treatment of BALB/c mice bearing Dox-sensitive and Dox-resistant MC-RMS

The discussion of the presented results is complicated by the lack of information on the mechanisms of the development of drug resistance, on phenomenon of elevated sensitivity of chemoresistant tumors to the action of immune system, but there is an opinion that in these processes different surface molecules expressed on tumor cells may be involved. In particular, it was reported that the development of drug resistance is associated with expression of survivin — protein that inhibits apoptosis. Survivin was found on the cells of a number of tumors (lung, stomach, mammary gland, liver, neuroblastoma), and its high level of expression correlates with tumor progression [21, 22]. It was shown that surviving is involved in the formation of radio- and chemoresistance via direct or mediated inhibition of caspases [21].

There are data showing that the development of acquired resistance (in particular, to paklitaxel) in ovarian carcinoma cells is associated with expression of Bub R1 — protein that belongs to the family of proteins веретена скручивания [23]. The study of antiapoptotic molecules by chemoresistant tumor cells is in spite of interest too: in resistant bladder cancer cells, elevation of Bcl-2 expression upon inhibition of Bax translocation has been demonstrated [24].

At the same time, P-gp — the product of mdr1 gene, is in the focus of studies. It was shown that upon the influence of chemopreparations, there are alterations in epigenetic modification of mdr 1 locus and methylation of promoter of mdr 1, that is accompanied by the development of drug resistance [25].

As it is known, P-gp protein may play physiologic role as well: it is expressed by a number of normal cells (intestinal epithelium, some hepatic cells, renal канальцев etc) where it plays a correcting role in organogenesis [26]. Moreover, P-gp is expressed in natural killer cells, dendrite cells, T- and B-lymphocytes, but its role in these cells remains unknown [26]. In some studies the authors tried to analyze whether expression of P-gp correlates with elevated sensitivity of resistant tumor cells to effectors of immune system [1], but till now no significant correlation was found. Analyzing the data on the role of P-gp in the development of drug resistance, one may conclude that P-gp is not universal protein associated with chemoresistance (it is not expressed in all tumor cells), and it could not block completely apoptotic mechanisms in tumor cells, because P-gp blocks only caspase-dependent apoptosis, whilst ability of cells to undergo caspase-independent apoptosis is restored as well as their lysis by cytotoxic T-lymphocytes with the involvement of granzim B and perforin [27–29].

It is necessary to discuss why our data showed that for therapy of Dox-resistant tumors LAK-therapy is the most effective, and Dox-sensitive MC-RMS-vaccine therapy. Taking into account that the mechanisms of antitumor action for mentioned types of therapy are principally different [30, 31], it could be proposed that in animals with resistant tumors there could occur changes of immune system that affect vaccination efficacy. It was shown that upon the development of drug resistance in Dox-resistant human melanoma cells (the cells did not express P-gp) cocultivated with allogeneic cells of peripheral blood, the production of IFN-γ in the latest decreased as well as proliferation rate; the authors supposed that this phenomenon may occur *in vivo* as well [32].

In experiments with L1210 cells resistant to cysplatin it was shown that the expression of class II MHC antigens decreased on their surface [33]. So, upon formation of the resistance the events impeding the recognition with the involvement of class II MHC molecules may take place, when this process occupies the central place in the induction of immune response upon vaccination.

There is different situation upon LAK-therapy: the lysis of tumor cells is performed by cytotoxic cells activated by IL-2 *in vitro*, where expression of adhesion molecules by LAK and increase of cytokine production is of special importance. Not only IL-2 possesses such ability, but other cytokines as well in particular IL-12, IL-15, IL-18 etc. [1, 34–36]. That's why LAK-therapy does not require active involvement of immunologic mechanisms of the host and is mainly dependent on the patterns of interacting lymphocytes and tumor cells, especially expression of adhesion molecules, significantly affecting the efficacy of LAK-therapy. For chemoresistant tumors this problem is not studied *in vivo* yet; it was shown that elevated expression of ICAM-1 by tumor cells leads to promotion of their lysis by natural killers [34]. It's necessary to add that adoptive transfer even of non-activated cytotoxic cells may lead to the lysis of resistant tumor cells by different ways [37]. The study of molecular mechanisms of interaction between lymphocytes and tumor cells will allow understand the phenomenon of elevated sensitivity of resistant tumors to immunotherapy.

In conclusion, our data evidence on higher efficacy of all applied types of immunotherapy toward treatment of mice bearing resistant tumors than sensitive ones. Comparative analysis of the efficacy of all types of therapy has shown that in the case of resistant tumors, LAK-therapy using autologous lymphocytes has the highest efficacy, when in the case of sensitive tumors vaccine therapy is of higher efficacy.

ACKNOWLEDGEMENT

REFERENCES

1. **Berezhnaya NM, Chekhun VF.** System of interleukins and cancer. Kyiv: DIA, 2000; 224 p (In Russain).

2. **Hanai N, Nakamura K, Shitara K.** Recombinant antibodies against ganglioside expressed on tumor cells. Cancer Chemother Pharmacol 2000; **46**: 13–7.

3. **Hoose A, Parmiani G, Hege K, et al.** A clinical development paradigm for cancer vaccines and related biologics. J Immunother 2007; **30**: 1–15.

4. **Demidov LV, Kharkevich GYu, Timofeev IV.** Success and failure of the use of cytokines in chemotherapy of some solid tumors. Prakt Onkol 2003; **4**: 140–7 (In Russain).

5. **Toh U, Yamana H, Kido K, et al.** Autologous tumor specific immunotherapy of refractory cancers with ex vivo-generated T cells stimulated by autologous tumor cell. Gan To Kagaku Ryoho 2003; **30**: 1566–70.

6. **Quan WD Jr, Walker PR, Quan FM, et al.** Activity of continuous infusion plus pulse interleukin-2 with famotidine in patients with metastatic kidney cancer or melanoma previously treated with interleukin-2. Cancer Biother Radiopharm 2006; **21**: 437–42.

7. **Zlatnik EIu, Golotina LIu.** The use of roncoleukin for LAK therapy in ovarian cancer. Vopr Onkol 2005; **51**: 680–4.

8. **Zheng YJ, Zhang J, Wang SM, et al.** Morphological observation on NK/LAK cell-mediated lysis of human oral carcinoma cells. Di Yi Jun Yi Da Xue Xue Bao 2004; **24**: 392–6.

9. **Ozkan A, Ayhan A, Fiskin K.** Combined effect of epirubicin and lymphokine-activated killer cells on the resistant human breast cancer cells. Cell Biol Toxicol 2004; **20**: 261–71.

10. **Kiselevskii MV.** Adoptive immunotherapy in malignant tumors. Vestn Ross Akad Med Nauk 2003; **1**: 40–4.

11. **Kiselevskii MV.** Immunotherapy in malignant tumors. Together against cancer 2005; **3** (In Russain).

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

13. **Berezhnaya NM, Vinnichuk UD, Konovalenko VD, 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.

14. **Liakatos A, Kunz H.** Synthetic glycopeptides for the development of cancer vaccines. Curr Opin Mol Ther 2007; **9**: 35–44.

15. **Nava-Parada P, Forni G, Knutson KL, et al.** Peptide vaccine given with a Toll-like receptor agonist is effective for the treatment and prevention of spontaneous breast tumors. Cancer Res 2007; **67**: 1326–34.

16. **Sharav T, Wiesmuller KH, Walden P.** Mimotope vaccines for cancer immunotherapy. Vaccine 2007.

17. **Parmiani G, De Filippo A, Novellino L, et al.** Unique human tumor antigens: immunobiology and use in clinical trials. J Immunol. 2007; **178**: 1975–9.

18. Patent 7A 61K 38/01, 38/17, 35/12; № 57608 Ukraine. The means for preparation of cancer autovaccine / VO Shlyakhovenko, GP Potebnya, VS Mosienko et al. Publ 16.03.2003. Bul № 6 (In Ukrainian).

19. **Shlyakhovenko VO, Mosienko VS, Kazak VV, et al.** Cancer autovaccine on the base of glycopeptides of tumor cells. Oncologiya 2004; **6**: 180–4 (In Ukrainian).

20. **McKenzie RC, Oran A, Dinarello CA, et al.** Interleukin-1 receptor antagonist inhibits subcutaneous B16 melanoma growth *in vivo*. Anticancer Res 1996; **16**: 437–41.

21. **Zaffaroni N, Daidone MG.** Survivin expression and resistance to anticancer treatments: perspectives for new therapeutic interventions. Drug Resistance Updates 2002; **5**: 65–72.

22. **Altieri DC.** The molecular basis and potential role of survivin in cancer diagnosis and therapy. Trends Mol Med 2001; **7**: 542–7.

23. **Fu Y, Ye D, Chen H, et al.** Weakened spindle checkpoint with reduced BubR1 expression in paclitaxel-resistant ovarian carcinoma cell line SKOV3-TR30. Gynecol Oncol 2007; **105**: 66–73.

24. **Cho HJ, Kim JK, Kim KD, et al.** Upregulation of Bcl-2 is associated with cisplatin-resistance via inhibition of Bax translocation in human bladder cancer cells. Cancer Lett 2006; **237**: 56–66.

25. **Baker EK, Johnstone RW, Zalcberg JR, et al.** Epigenetic changes to the MDR1 locus in response to chemotherapeutic drugs. Oncogene 2005; **24**: 8061–75.

26. **Smyth MJ, Krasovskis E, Sutton VR, et al.** The drug efflux protein, P-glycoprotein, additionally protects drug-resistant tumor cells from multiple forms of caspase-dependent apoptosis. Proc Natl Acad Sci USA 1998; **95**: 7024–9.

27. **Ruefli AA, Smyth MJ, Johnstone RW.** HMBA induces activation of a caspase-independent cell death pathway to overcome P-glycoprotein-mediated multidrug resistance. Blood 2000; **95**: 2378–85.

28. **Ruefli AA, Tainton KM, Darce PK, et al.** P-glycoprotein inhibits caspase-8 activation but not formation of the death inducing signal complex (disc) following Fas ligation. Cell Death Differ 2002; **9**: 1266–72.

29. **Johnstone RW, Tainton KM, Ruefli AA, *et al*.** P-glycoprotein does not protect cells against cytolysis induced by pore-forming proteins. J Biol Chem 2001; **276**: 16667–73.

30. **Berezhnaya NM, Kovalchuk EV.** LAK-phenomenon (cell phenotype, mechanism of action and conditions for its realization). Immunologiya 1995; **2**: 12–6 (In Russain).

31. **Berezhnaya NM, Chekhun VF.** Immunology of malignant growth. Kyiv: Naukova Dumka, 2005; 792 p (In Russain).

32. **Micallef MJ, Darmanin S, Buhagiar JA, *et al*.** Interferon gamma induction in human melanoma cells/allogeneic leukocyte co-cultures is enhanced by interleukin 18 but drug resistant melanoma cell poorer inducers of IFN-γ. Int Immunopharmacol 2001; **1**: 295–305.

33. **Bhushan A, Kuperman JL, Stone JE, *et al*.** Drug resistance results in alterations in expression of immune recognition molecules and failure to express Fas (CD95). Immunol Cell Biol 1998; **76**: 350–6.

34. **Liebau C, Merk H, Schmidt S, *et al*.** Interleukin-12 and interleukin-18 change ICAM-I expression, and enhance natural killer cell mediated cytolysis of human osteosarcoma cells. Cytokines Cell Mol Ther 2002; **7**: 135–42.

35. **Abraham C, Griffith J, Miller J.** The dependence for leukocyte function-associated antigen-1/ICAM-1 interactions in T cell activation cannot be overcome by expression of high density TCR ligand. J Immunol 1999; **162**: 4399–405.

36. **Miles DW, Happerfield LC, Bobrow LG, *et al*.** Adhesion molecule expression and leucocyte trafficking following immunotherapy with recombinant interleukin-2. Histopathology 1996; **28**: 301–8.

37. **Wu JY, Ernstoff MS, Hill JM, *et al*.** *Ex vivo* expansion of non-MHC-restricted cytotoxic effector cells as adoptive immunotherapy for myeloma. Cytotherapy 2006; **8**: 141–8.

ЭФФЕКТИВНОСТЬ РАЗЛИЧНЫХ ВИДОВ ИММУНОТЕРАПИИ ПО ОТНОШЕНИЮ К РЕЗИСТЕНТНОЙ И ЧУВСТВИТЕЛЬНОЙ К ДОКСОРУБИЦИНУ ПЕРЕВИВНОЙ МХ-РАБДОМИОСАРКОМЕ

Цель: изучить влияние различных видов иммунотерапии, а именно адоптивной ЛАК-терапии, вакцинотерапии, а также их комбинации, дать оценку их эффективности в условиях перевивной МХ-рабдомиосаркомы мышей, резистентной и чувствительной к доксорубицину. ***Материалы и методы:*** исследования проведены на мышах линии BALB/c с чувствительной и резистентной к доксорубицину перевивной МХ-рабдомиосаркомой. ЛАК-терапию (лимфоцитами лимфатических узлов сингенных мышей) проводили начиная с 7 сут после перевивки опухолевых клеток на протяжении 5 дней; ЛАК вводили в область опухоли в количестве 3 млн в 0,2 мл среды. Вакцину, полученную на основе гликопептидов опухолевых клеток, вводили мышам интраперитонеально в объеме 0,2 мл по двум схемам: до перевивки и после перевивки опухоли. Влияние иммунотерапии оценивали по проценту торможения роста опухоли и выживаемости животных. ***Результаты:*** данные проведенной иммунотерапии свидетельствуют, что у мышей с чувствительной к доксорубицину опухолью наиболее эффективна вакцинотерапия, а при резистентной опухоли — адоптивная ЛАК-терапия, что подтверждалось наибольшей протяженностью жизни и наиболее выраженным торможением роста опухоли у животных этой группы. Кроме того, сравнительный анализ результатов применения ЛАК-терапии, вакцинотерапии и их сочетания показал, что все ее виды эффективны у мышей как с чувствительной, так и резистентной к доксорубицину МХ-рабдомиосаркомой. ***Выводы:*** полученные данные свидетельствуют о том, что резистентные и чувствительные к доксорубицину опухоли отличаются чувствительностью к различным видам иммунотерапии, что, по всей вероятности, объясняется различными механизмами их действия.

Ключевые слова: перевивная МХ-рабдомиосаркома, резистентность, доксорубицин, вакцинотерапия, ЛАК-терапия.