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Nemeth-Kellner Lymphoma Is a Valid Experimental Model in Testing Chemical Agents for Anti-Lymphoproliferative Activity*

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ABSTRACT

Scheme of detection and investigation of antitumor activity of chemical substances by use of experimental murine Nemeth-Kellner lymphoma (NK/Ly) are described. It includes monitoring of growth of NK/Ly ascites by everyday weighting of animals in course of intraperitoneal application of tested substance in 24 or 48 hours after inoculation of 10^7 tumor cells. The inhibitory effect became distinct on the 6th - 8th day of experiment, and 3 types of response were observed: 1) complete suppression of ascites development, corresponding to high antitumor activity of tested substance; 2) partial suppression of ascites growth (weak antitumor activity); 3) insignificant effect on ascites growth (lack of activity, at least towards hematoblastoses). As compared with leukemia L1210 or P388 lymphoma NK/Ly provides more distinct daily increment of body weight due to tumor growth. Clinical pattern of tumor development was supported by cytomorphological investigations of tumor cells which included: 1) cells count in ascites; 2) measuring cell dimensions and ratio of enlarged cells in a population (diameter over 17 μm in case of NK/Ly); 3) quantitative evaluation of tumor cells damage by smears count after staining with azure-eosin, bromophenol blue and hematoxylin. In case of complete suppression of ascites growth the cytological investigation was conducted by delayed application of tested agent to animals with the developed ascites. After treatment with vinblastine or doxorubicin NK/Ly cells represent a convenient object for fractionation of cell population, as demonstrated by separation of giant and small tumor cell subpopulations.

Keywords: Nemeth-Kellner Lymphoma; Antitumor Substances; Screening

1. Introduction

Nemeth-Kellner lymphoma (NK/Ly) was developed in 1961 at National Oncological Institute in Budapest (Hungary) by an intraperitoneal inoculation of leukemia cells from spleen of an old inbred mice with spontaneous leucosis to a newborn mouse and subsequent transplantation of developed tumor as ascites, thereafter as solid tumor [1,2]. Defined initially as lympholeukosis, after transformation tumor displayed properties of lymphosarcoma [3]. During 1960-1990 the usefulness of this tumor model in screening antitumor substances was proved and confirmed by numerous investigations [4-9]. However, nowadays it is rarely used in experimental oncology. Our long-term work with mentioned tumor model demonstrated its convenience in testing antitumor activity of different chemical substances. The versatile approach consists of registering the dynamics of ascites growth at the initial stage and characterizing cytomorphological changes of tumor cells under the influence of tested substance.

Besides we used lymphoma ascitic cells for fractionation of cell populations. Here we demonstrated separation of heterogeneous population of NK/Ly cells which is developed during treatment with doxorubicin into pure subpopulations of "giant" and "small" cells for further investigation of their properties.

The aim of present communication is to demonstrate the validity of NK/Ly model for detection and characterization of antitumor activity of chemical substances in *in vivo* experiments as well as its usefulness for studies of tumor cell biology and as a source of large amount of

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conditioned biomaterial.

2. Material and Methods

2.1. Tumor Strains and *in Vivo* Experiments

NK/Ly lymphoma and L1210 leukemia were obtained from the collection of tumor strains at R. E. Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology, NAS of Ukraine (Kyiv). Tumors were transplanted in ascitic form by the intra-abdominal inoculation aliquots of ascites containing 10^7 NK/Ly cells or 10^6 L1210 cells, as described in [3,10]. Samples of ascites for transplantation were taken on the 7^{th} - 8^{th} day of tumor growth. Experiments were performed on C57 black mice, 3 - 5 animals in control and experimental groups were processed simultaneously. Requirements of the European Convention on protection of vertebrate animals (November 13, 1987) and The Law of Ukraine, on protection of animals from cruel behaviour" (March 28, 2006) were kept.

The following substances were used for experimental treatment of lymphoma: Vinblastine sulphate ("Gedeon Richter", Hungary), Doxorubicin ("Arterium", Ukraine), sapogenin-like substance, sanguinarine + chelerythrine (sanguerythrine) alkaloids from the Greater celandine were home prepared, as described [11,12]. Tested substances were dissolved and stored as stock solutions in 50% alcohol at rate of 1 dose in 5 or 10 µl (applied doses are indicated in the figures). Just before application, stock solution was diluted with sterile distilled water or saline and injected intraperitoneally. Treatment was started in 24 or 48 hours after inoculation of tumor cells, as recommended for detection of antitumor activity [10].

Ascites growth was monitored by everyday weighting of animals during 30 days. Change in body mass was expressed in percents relatively to initial value, accepted as 100%, in order to adopt data for statistical analysis.

Cytological analysis included measurement of cell diameter performed by using AxioVision release 2007 program (Karl Zeiss) on images of native cells in hemocytometric chamber taken with Canon A590 digital camera. A plot of distribution of cells according to dimensions was constructed on the basis of obtained data. A ratio of damaged cells in a population was determined by counting images of smears stained with azure-eosin (Romanovsky-Giemsa method), bromophenol blue for evaluating cell protein content [13,14], and hematoxylin for characterizing structure of nucleus and chromatin. As damaged, cells which differed from standard image of NK/Ly cell in the early stage of tumor growth (shape, dimension, intensity of staining, nuclei fragmentation, presence of cytoplasm vesicles) were defined. No less than 300 cells were counted on the images from each smear.

2.2. Isolation of Subpopulations of NK/Ly "Giant" and "Small" Cells

For induction of giant cells production mice were treated with vinblastine or doxorubicin the next day after evacuation of ascites (the 2^{nd} - 4^{th} drainage). Drug was applied intraperitoneally in 2 - 3 injections (single dose— 1 mg/kg) at 48 hours interval. The resulting ascites was taken 3 days after the last injection using EDTA as anticoagulant to prevent coagulation of fibrinogen. Ascites with not less then 20% of giant cells was subjected to centrifugation 1 min at 150 g, sediment of giant cells was retained, supernatant was centrifuged at 1500 g for 5 min, and sediment was retained for isolation of small cells. Supernatant was clarified by centrifugation at 2000 g for 10 min, and clear supernatant (cell free ascitic fluid) was collected and used as a medium for processing cell fractions. Giant cells were purified by suspending in cell free ascitic fluid and centrifugation for 1 min at 150 g. This procedure was repeated three times. Small sized cells were purified by centrifugation of cell suspension in ascitic fluid and collecting cells which sediment between 150 g for 3 min and 1000 g for 5 min. The purity of cell subpopulations was checked in the hemocytometric chamber.

2.3. Statistical Analysis

All statistical calculations were performed using Microsoftt Office Excel 2003 program. Significance of difference between groups of data was determined by using Student's ***t*** criterion, and significance was defined as $p < 0.05$.

3. Results

3.1. Effect of Tested Substances upon Growth of NK/Ly Ascites

Three types of response in plots of body mass dynamics during treatment of animals with tested agents were observed (**Figure 1**). Routinely used antitumor drugs vinblastine and doxorubicin completely inhibited the development of ascites (**Figure 1(a)**), and thus may be accepted as standard in evaluation of potential antitumor drugs. The sample of sapogenin-like substance isolated from seeds of Greater celandine exhibited partial inhibition effect towards NK/Ly ascites growth (**Figure 1(b)**). Similar effect was observed in application of purified antibodies developed by immunization of rabbits with microvesicles obtained from aged ascites. Mixture of sanguinarine and chelerythrine alkaloids isolated from Greater celandine showed no significant inhibition of NK/Ly ascites growth (**Figure 1(c)**), although *in vitro* they exhibited a toxic effect towards tumor cell lines

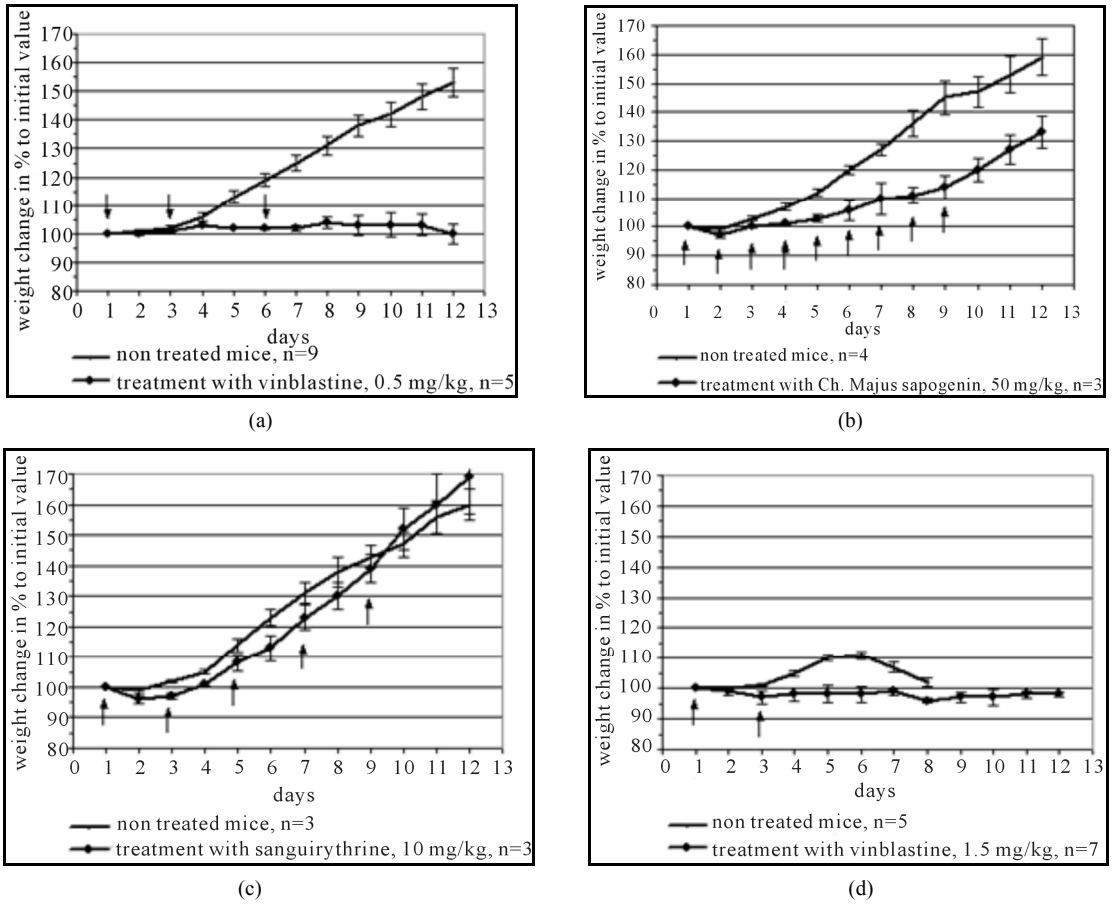


Figure 1. Principal types of development of NK/Ly ascites ((a)-(c)) under treatment with different substances and comparison with L1210 leukemia (d). (a) Complete suppression of ascites development at treatment with vinblastine or doxorubicin applied intraperitoneally (i/p) as indicated by arrows (single dose 0.5 mg/kg). (b) Partial suppression of ascites growth at treatment with sapogenin-like substance isolated from Greater celandine, applied i/p as indicated by arrows (single dose 50 mg/kg). (c) Non-significant effect on ascites growth at treatment with sanguerythrine isolated from greater celandine, applied i/p as indicated by arrows (single dose 1.5 mg/kg). (d) Complete suppression of L1210 ascites growth at treatment with vinblastine, applied i/p as indicated by arrows (single dose 2.5 mg/kg). In all graphs the scale is the same for comparison of body mass dynamics during development of NK/Ly and L1210 ascites.

MT-4 and SEM-T [15].

It should be noted that daily increment of body mass due to the development of NK/Ly ascites was expressed much more distinctly as compared with L1210 leukemia (**Figure 1(d)**).

After obtaining information about antitumor activity of tested agent (10 - 12 days) further investigation was continued for studying animal survival. Accumulated ascites was periodically evacuated in control and treated animals at the same day for analysis of its characteristics (volume, total cell number and cytological pattern). In cases of complete suppression of development of NK/Ly ascites high probability of recovery or considerable prolongation of life of experimental animals was expected. Recovery is assumed when mouse survive for 90 days with no visible signs of tumor [10]. In our studies death in about 60 days was usually caused by development of solid tumor nodes in loci of injection. In contrary, suppression of L1210 leukemia ascites development was rarely followed by the recovery due to the appearance of endotoxicosis, which is a characteristic feature of this tumor strain, and which can be retarded but not totally eliminated even in suppression of ascites growth.

3.2. Effect of Tested Substances on Cytological Pattern of NK/Ly Cells

The results of monitoring tumor growth agreed with ascites characteristics (volume of ascites, total number of cells, and quantitative cytomorphological evaluation of cell damage). In block of ascites production by strong antitumor agents these investigation can be performed in special experiments with delayed application of tested substance to well developed ascites, *i.e.* on the 7^{th} - 9^{th} day after inoculation of NK/Ly cells or even later after

evacuation of ascites. Using this approach it was revealed that NK/Ly cells are massively transformed into "giant" cells under treatment with vinblastine or doxorubicin (**Table 1)**. Substances with moderate antineoplastic activity (e.g. sapogenin-like substance from the Greater celandine) provided a lower percent of "giant" cells, and in case of inactive substances the level of "giant" cells did nor differ from that in untreated mice.

Cytological analysis of NK/Ly ascites smears demonstrated changes in cell morphology which were the best detected by staining with azure-eosin (**Table 2**). The score of cell damage based on the evaluation of cell protein content was much lower than one at staining with azure-eosin. The reason of that might be more intensive staining of many "giant" cells due to normal or increased intracellular protein content. Thus these cells were not registered as damaged, while according to the results of azure-eosin staining they differed significantly from typical parental cells and were classified as damaged. Hematoxylin staining was shown to be of low informative validity, and its score was in close resemblance to the results of Romanovsky-Gimsa staining.

The level of cell alterations based on measuring cell dimensions and changes in their morphology correlated with the cytotoxic efficacy of tested agent.

Highly active antitumor substances of potential practical significance induced highly significant ($p < 0.001$) changes in studied cytological indices versus control, while substances with moderate or weak activity has a significance at the level of $0.001 < p < 0.01$.

3.3. Isolation of Subpopulations of "Giant" and "Small" NK/Ly Cells

Generation of a high percentage of greatly enlarged tumor cells, defined as "giant" cells, under the influence of vinblastine or doxorubicin is an interesting property of NK/Ly experimental tumor model. Vinblastine was shown to induce higher quantity of "giant" cells that in some cases reached 90%, than doxorubicin, while the last induced a more uniform subpopulation of "giant" cells. A significant difference in size of "giant" cells and the rest of the parental and other "small" cells permits separating these subpopulations by using low speed differential centrifugation. Light microscopy image of separated "giant" and "small" cells subpopulations is presented on **Figure 2**. Fraction of "giant" cells is rather uniform in size, while fraction of "small" cells is heterogeneous and contains small parental and degraded NK/Ly cells as well as mononuclears. Investigation of "giant" cells can be of interest in sense of their tumorogenic properties, as it has been shown that these polyploid cells can revert to pseudodiploid condition and cause a relapse of tumor growth [16-18]. Besides, fraction of "small" cells can be used for isolation of lymphocytes and further investigation of their cytotoxicity towards NK/Ly cells *in vivo*. We have

Table 1. Effect of tested substances on dimensional characteristics of NK/Ly cells.

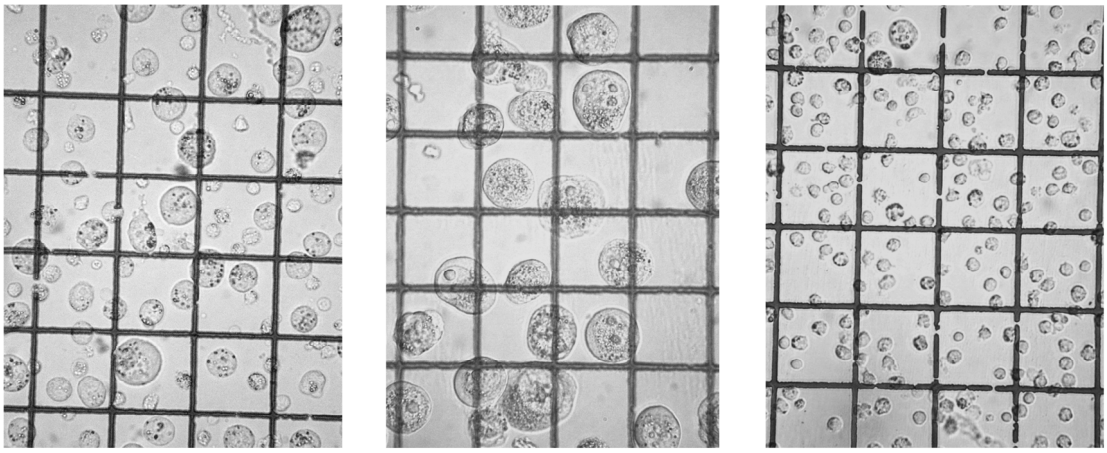
Mode of treatmentof animals	N	Cell diameter, μm (M ± m)	Percent of cells over 17 μm (M ± m)
Control (no treatment)	10	13.6 ± 0.3	6.3 ± 0.5
Treatment with vinblastine	8	29.2 ± 1.7*	78.4 ± 9.4*
Treatment with doxorubicin	10	17.8 ± 0.4*	41.8 ± 13*
Treatment with sapogenin-like substance from Greater celandine	5	16.5 ± 0.3*	29.8 ± 2.3*
Treatment with sanguerythrine	4	13.4 ± 0.3	8.2 ± 1.4

*$p < 0.01$ vs. control.

Table 2. Cytological idexes of NK/Ly cells damage in treatment with tested substances.

Mode of treatment of animals	N	% of damaged cells in population after staining with		
		Azure-eosin	*Bromophenol blue*	*Hematoxylin*
Control (no treatment)	8	26.8 ± 3.9	18.5 ± 1.5	13.2 ± 1.2
Treatment with vinblastine	8	80.2 ± 3.2**	51.9 ± 4.5**	71.3 ± 4.3**
Treatment with doxorubicin	5	83.8 ± 4.5**	27.4 ± 1.7**	78.5 ± 5.0**
Treatment with sapogenin-like substance from Greater celandine	4	70.2 ± 4.8*	27.3 ± 2.3*	63.0 ± 5.1*
Treatment with sanguerythrine	3	35.6 ± 4.4	23.0 ± 2.5	18.3 ± 2.3

*$0.001 < p < 0.01$ vs. control, **$p < 0.001$ vs. control.



(a) (b) (c)

Figure 2. Separation of "giant" and "small" cell subpopulations from NK/Ly ascites developed after treatment with doxorubicin. (a) Initial ascites, mean diameter of cells 15.3 ± 0.4 μm, ratio of cells over 17 μm 34.1%; (b) Subpopulation of giant cells, mean diameter of cells 33 ± 0.5 μm, ratio of cells over 17 μm 100%; (c) Subpopulation of small cells, mean diameter of cells 8.3 ± 0.1 μm, amount of cells over 17 μm 0%, cells below 12 μm 95.2%. Images are taken in the hemocytometric unit, square side corresponds to 50 μm and is used as a scale.

detected a massive infiltration of the abdominal cavity fluid with lymphocytes after reinoculation of tumor cells to mice, which have recovered after the first inoculation of NK/Ly cells and treatment with vinblastine or doxorubicin.

4. Discussion

The scheme is proposed on testing antitumor activity that permits answering a question if the tested substance exhibits antitumor activity *in vivo* or not. Obtaining positive answer (**Figure 1(a)**) predicts the expediency of further studies in a complex preclinical investigation of the potential antitumor agent (therapeutic effect, scheme of application, toxicity and side effects, drug technology etc.). Negative result (**Figure 1(c)**) may be interpreted as absence of activity towards lymphoproliferative malignancies, and, thus, testing on tumors of another origin is reasonable. The results of investigation of substance activity on a panel of tumor cell lines can be used as a guide in choosing the type of tumor target. Besides, the used lymphoma NK/Ly permits revealing substances with moderate antitumor activity (**Figure 1(b)**), which is not possible at employment of L1210 leukaemia.

There are several advantages of NK/Ly model, in particular much better expressed increment of body mass due to tumor growth comparing that in L1210 leukemia.

In this property NK/Ly lymphoma resembles Ehrlich carcinoma which was also used in monitoring tumor growth [19,20]. Now we modified this approach by introducing relative values of weight expressed in percent of the initial weight value accepted as 100% in order to subject the data obtained in individual animals to statistical analysis.

A complete suppression of growth of NK/Ly ascites in early stages of tumor development permits expecting with high probability of a recovery or considerable prolongation of life in the treated animals. In L1210 leukaemia it is not possible due to the development of endotoxicosis, a characteristic feature of this murine tumor strain, and in suppression of ascites growth it can be retarded but not stopped.

Here is reasonable to mention the data of Prihozhina and Vendrov [21] who revealed the karyotype identity of NK/Ly lymphoma, S-180 and S-37 sarcomas with Ehrlich carcinoma according to chromosome number and set of chromosomal markers. This was interpreted as substitution of the former three tumor strains with Ehrlich carcinoma and, consequently, to regard these tumors as the sub-strains of Ehrlich carcinoma. We suggest that it may be also another interpretation of those results in sense that identity of karyotypes of mentioned tumors indicates on a possibility of obtaining similar, yet not identical, results in testing activity of the antitumor agents. This thesis can be supported by comparison of sensitivity of NK/Ly lymphoma and Ehrlich carcinoma to a number of chemotherapeutic drugs [10]. Besides resemblance in response to many drugs Ehrlich carcinoma is more sensitive to 5-fluorouracile, cis-platine, hydroxyurea, and less sensitive to cytarabine then NK/Ly lymphoma.

Not excluding the validity of Ehrlich carcinoma and L1210 and P388 acute leukaemias as models for screening of antitumor drugs we consider that NK/Ly lymphoma should be also included to the list of experimental tumors recommended for employment in screening of

drugs towards nonacute forms of hemato-/lymphoproliferative malignancies.

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