Cytotoxic Activity of Lymphocytes Isolated from Mouse Liver Involved into Tumor Process

N. K. Akhmatova, E. N. Kuzovlev*, O. V. Lebedinskaya, F. V. Donenko**, I. Zh. Shubina**, A. I. Makashin, and M. V. Kiselevskii****

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We studied cytotoxic activity and immunophenotype of mononuclear cells isolated from the liver of mice after implantation of ovarian cancer cells into the liver parenchyma. The isolated cells exhibited higher natural killer cell activity and possessed higher cytotoxic potential against autologous tumor cells compared to spleen lymphocytes. The ratio of $CD3^+$ lymphocytes and natural killer cells was high in the liver with tumor metastases. Lymphoid cells were practically absent in the liver of intact animals. Natural killer cells and T cells from liver tumor tissue play an important role in antitumor immunity and can be used for local and regional adjuvant immunotherapy of liver metastases.

Key Words: *liver metastases; ovarian cancer; immunophenotype*

The liver is one of the primary sites for metastatic dissemination of various tumors. Liver metastases are found in 20% patients after surgery for colorectal cancer and often cause patient's death due to the development of liver failure [1,3]. Previous experiments identified a special subpopulation of lymphocytes designated as liver-associated natural killer (NK) cells. They were phenotypically classified to NKT cells due to expression of markers for T cells and NK cells on the membrane [2,4-8]. NKT cells have antimetastatic activity. Activation is followed by a significant increase in cytotoxic potential of these NK cells. For example, systemic administration of LPS derivatives is accompanied by regression of liver metastases in mice [9]. The antimetastatic effect is associated with activation of liver NK cells due to enhanced interleukin-12 (IL-12) secretion by LPS-stimulated dendrite cells in the liver [10].

Here we studied activity of NK cells and cytotoxic properties of liver-associated lymphocytes in mice with implanted CaO-1 tumor.

MATERIALS AND METHODS

Experiments were performed on male CBA mice weighing 20-25 g and obtained from the Stolbovaya nursery.

A suspension of mouse ovarian cancer cells (300,000 cells, 20 ml) was administered into liver tissue to induce growth of tumor nodes. The experimental and control groups consisted of 8 animals.

In *in vitro* experiments, the spleen and liver were isolated 14 days after tumor implantation and cell suspension was prepared. Hepatocytes and erythrocytes were separated from mononuclear leukocytes (MNL) by centrifugation at 50*g* for 5 min. MNL were isolated by centrifugation in Ficoll-Verografin density gradient (PanEko, 1.088 g/cm^3). The cells were washed 2 times with medium 199 and

I. I. Mechnikov Institute of Vaccines and Sera, Russian Academy of Medical Sciences; *N. N. Blokhin Russian Cancer Research Center, Russian Academy of Medical Sciences, Moscow; **Perm State Medical Academy, Russian Ministry of Health. ***Address for correspondence:*** kisele@inbox.ru; anelly@mail.ru. N. K. Akhmatova

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resuspended in complete culture medium RPMI 1640 containing 10% fetal bovine serum, 2 mM glutamine, 5000 U/ml streptomycin, and 5000 U/ml penicillin.

The cells suspension isolated from the tumor node was washed 2 times with Hanks solution and resuspended in complete nutrient medium at 37°C and 4.5% CO_2. The logarithmic growth phase was maintained by passaging the culture at 2-3-day intervals.

Cytotoxic activity of MNL was determined by reduction of 3-(4,5-dimethylthiazole-2-il)-2,5-diphenyltetrazolium bromide (MTT test) using NK-dependent cells of mouse YAC-1 lymphoma and autologous tumor cells. Tumor cells (3×10^4 cells/ml) were incubated in the culture medium with MNL in the 1:5 ratio. Incubation was performed in flat-bottom 96-well microplates (Costar) at 37°C and 4.5% CO_2 for 18 h. Vital dye MTT (Sigma) was put in wells. Optical density at 540 nm was measured on a MS multiscan (Labsystem). The percent of lysed tumor cells was calculated (percentage of cytotoxicity).

MNL immunophenotype (expression of surface molecules) was estimated using monoclonal antibodies against the corresponding antigens (Caltag Lab.). The study was performed by the method of flow cytofluorometry on a FACScan flow cytometer (Becton Dickinson). Expression of differentiation antigens on MNL was studied by double labeling with CD3-FITC and NK1.1-PR. The cell population gate was estimated from forward and lateral light scattering and size of cells (10,000 events per gate were calculated). The results were analyzed by means of WINMDI 2.8 software.

Expression of surface markers was studied using monoclonal CD3-FITC and NK1.1-PE antibodies (Caltag Lab.). MNL were incubated with monoclonal antibodies at 4°C for 30 min and washed 2 times in phosphate buffered saline by centrifugation at 100*g* for 10 min. The cell suspension was placed on glass slides covered with polylysine. Vital fluorescence microscopy of cells was performed under an Axioplan 2 luminescence microscope using an Axiovision 4.2 digital system for image recording and analysis (Zeizz). Expression of the T cell CD3 antigen served as a marker of NK cells.

The results were analyzed by Student's *t* test (standard software, Windows 98).

RESULTS

Oval tumor nodes (diameter 3-15 mm) invading surrounding tissues were found in the liver 14 days after implantation of tumor cells into the liver.

MNL isolated from liver tumor tissue had high spontaneous NK activity. These cells were 2-fold more potent than spleen lymphocytes in producing the killer effect (Table 1). As differentiated from spleen MNL, liver MNL were characterized by the ability to lyse autologous tumor cells. Cytotoxicity of effector cells was 18 and 43%, respectively.

We failed to isolate sufficient number of lymphocytes from the liver of control mice to perform the cytotoxic test.

MNL isolated from liver tumor tissue expressed membrane antigens of T lymphocytes (CD3) and NK markers (Fig. 1). The population of spleen MNL included $CD3^+$ T lymphocytes, but no NK^+ cells. Lymphocytes from liver tumor tissue were divided into 2 subpopulations of T lymphocytes (35%) and NK cells (18%), respectively. In both populations the ratio of NKT cells carrying markers of T cells and NK cells varied from 1 to 3%. Spleen lymphocytes from intact mice and animals with implanted tumor expressed only surface $CD3^+$ antigen. The number of NK cells did not exceed 2.5%.

The formation of tumor nodes in mouse liver is followed by infiltration of the parenchyma with MNL. They not only exhibited high spontaneous NK activity in the test with NK-sensitive YAC-1 cell line, but also had higher cytotoxic potential against autologous tumor cells (compared to spleen lymphocytes). These cells were practically absent in the liver of intact animals. Infiltration of the liver parenchyma with lymphocytes during tumor growth is considered as a local immune reaction to malignant cells. Published data show that these lymphoid cells are immunophenotypically classified to NKT cells. They express surface markers of T cells and NK cells. NKT cells are formed in the liver due to local secretion of IL-12 by resident macrophages (dendrite cells) involved in presentation of tumor-associated antigens [1,3,10]. Our results indicate that liver-associated lymphocytes can be divided into 2 subpopulations. Each population mainly includes NK cells or T cells. Only a small number of lymphocytes can be attributed to NKT cells. These differences in immunophenotypic characteristics of MNL from mouse liver can be determined by the

TABLE 1. NK Activity and Cytotoxicity of MNL from Mouse Liver and Spleen ($M\pm m$, %)

Organ	Target cells	
	YAC-1	autologous tumor cells
Liver	61±14*	43±11*
Spleen	37±10	18±8

Note. *$p<0.05$ compared to spleen MNL.

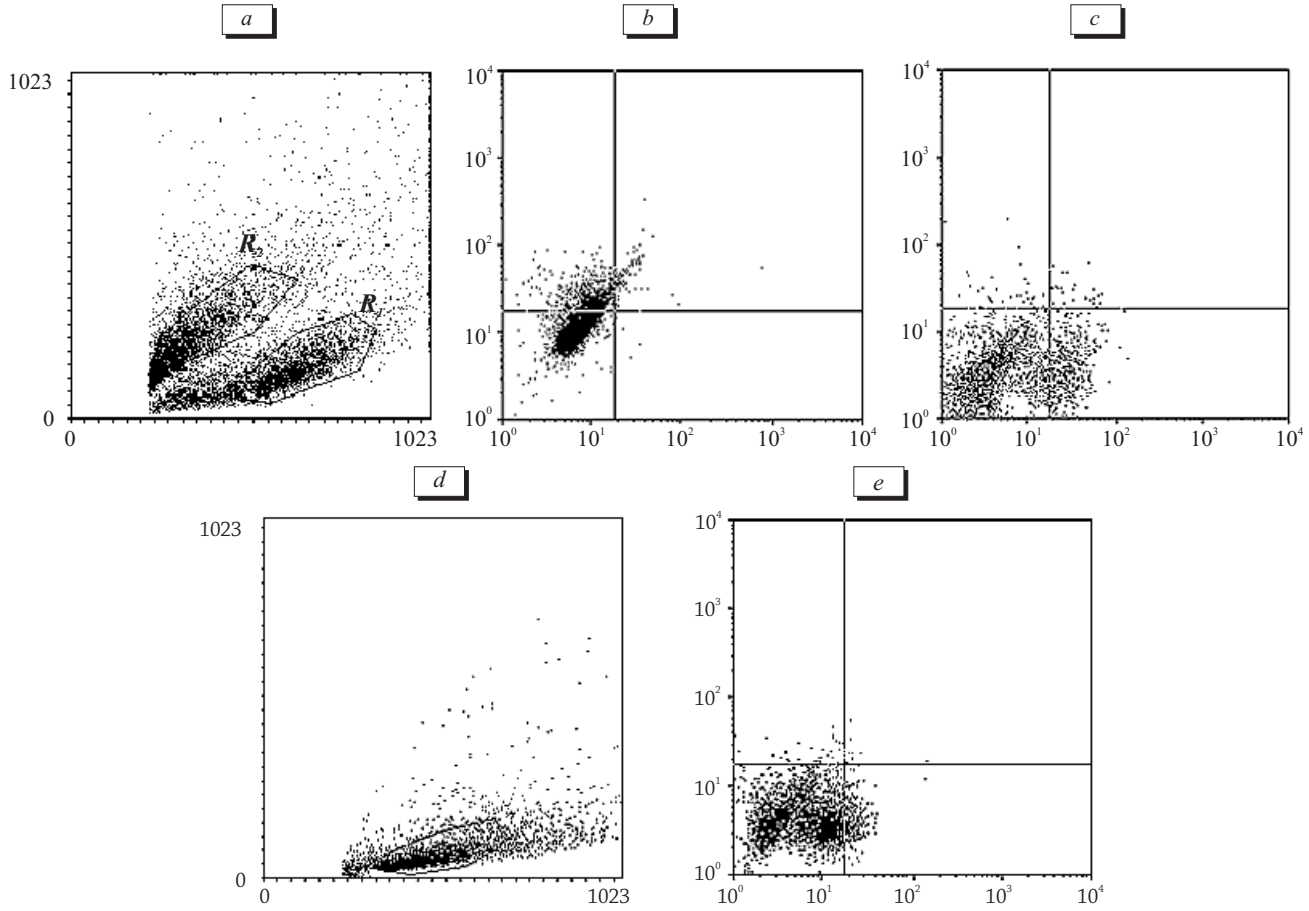

Fig. 1. Cytofluorograms characterizing expression of surface CD3 molecules, natural killer cells (NK; staining with FITC and phycoerythrin, respectively), and mononuclear leukocytes (MNL) from the liver (*a*-*c*) and spleen (*d*-*e*) of mice with liver tumor. *a*) Distribution of MNL in the liver; *b*) distribution of MNL in the liver (CD3/NK) during double vital staining in region R_2; *c*) distribution of MNL in the liver (CD3/NK) during double vital staining in region R_1; *d*) distribution of MNL in the spleen; *e*) distribution of MNL in the spleen (CD3/NK) during double vital staining in region R_1.

fact that in previous experiments ocean sponge polysaccharide (α-galactosamine) was injected intravenously to mice with liver metastases as a NKT inductor [3].

Our findings indicate that liver-associated lymphocytes have particular characteristics of immunophenotype and functional activity. They probably play an important role in local antitumor immunity.

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