just as in the writers' previous investigation [4], that the ability of bone marrow NK cells to undergo activation is exhausted, whereas this activity of NK cells is preserved in the blood and spleen of the same animals. The results can be taken as evidence of active release of NK cells and their precursors from the bone marrow tissue of tumor-bearing animals.

The results of the competitive inhibition of CTT test in vitro showed that HETR-MLN-8 cells, which can inhibit NR of the host to tumors in vivo, also have much greater ability to inhibit the cytotoxicity of normal NK cells in vitro compared with the parental HETR cells. The results of this series of experiments suggest that this effect may perhaps be dependent on the degree of malignancy of the tumor cells.

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EXPRESSION OF ANTIGENS IMMUNOLOGICALLY RELATED TO MOUSE MAMMARY GLAND CARCINOMA VIRUS ANTIGENS IN HUMAN BREAST TUMOR TISSUE

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Because of the similarity between certain features of neoplastic processes in the human and mouse mammary gland, frequent attempts have been made to discover an agent analogous to mouse mammary tumor virus (MMTV) in man. Virus particles morphologically similar to MMTV have been found in human milk, and in ultrathin sections through cells sedimented from it [5]. However, their budding from the cell surface has not been observed, nor have any cell systems capable of growing them been discovered. The principal searches by investigators have been aimed at finding nucleotide sequences homologous with sequences of MMTV in the genome of human cells and at studying antigens cross-reacting with MMTV antigens in human material. The results of research in this direction cannot be unequivocally interpreted, because they are very contradictory [6]. In our view the reason for this is the inadequacy of the methods used and, in particular, their lack of sensitivity.

In the present investigation two types of indirect solid-phase immunologic tests were used to look for antigens immunologically related to MMTV antigens in human breast tumor (HBT) tissues.

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EXPERIMENTAL METHOD

Enzyme-linked immunosorption assay (ELISA) on plastic plates and a modification of the radioimmunoblotting technique on nitrocellulose filters were used.

Samples of HBT and other tumors were obtained from the Department of Pathomorphology, All-Union Oncologic Scientific Center. A piece of tumor, 1-3 cm^3 in volume, was homogenized in 3 M KCl solution to obtain extracts of cell surface proteins. The homogenate was shaken vigorously for 24 h at 4°C, clarified at 10,000 rpm, then recentrifuged for 2 h at 40,000 rpm. Protein in the extracts was determined by Lowry's method. Extracts of human embryonic tissue (stomach, liver, lungs, heart muscles of embryos aged 8-28 weeks) and normal breast tissue were obtained in the same way. The samples were taken from cadavers of persons dying from various physical diseases.

Antisera against the principal viral membrane glycoprotein gp-52 were obtained in the writers' laboratory by immunization of rabbits in the popliteal lymph nodes followed by reimmunization intravenously and intramuscularly with purified gp-52 [1]. Polyvalent antiserum was obtained against MMTV proteins by immunizing rabbits with an MMTV concentrate (500 μg protein/ml) from milk of C3H mice, intraconjunctivally (0.1 ml daily for 1 week in both eyes alternately), followed by reimmunization every 7 days: intramuscularly 3 times at 4 points, intradermally once at 2 points (total dose 4.1 ml of the MMTV preparation). The sera were tested by radioimmunodiffusion for specificity of precipitation of viral proteins from the virus preparation and from a culture of MM5 cells (mammary gland carcinoma of C3H mice) (Fig. 1).

Serum against rabbit immunoglobulins was obtained from the N. F. Gamaleya Institute of Epidemiology and Microbiology, Academy of Medical Sciences of the USSR. Its globulin fraction was precipitated with ammonium sulfate, then conjugated with peroxidase (from Reanal, Hungary) by the method in [4] or labeled with ^{125}I by the chloramine T method.

Concentrates of Moloney, Rauscher, and MMTV viruses were obtained from the National Cancer Institute (USA).

The immunoperoxidase test was conducted in Falcon 3040 plates. After concentration of the test extracts to obtain relatively standard levels of protein concentration (about 5-8 mg/ml) they were dialyzed against 0.2 M carbonate buffer, pH 9.8, and added in volumes of 0.2 ml to the wells in the plate in several parallel tests with positive and negative controls. The MMTV preparation and also a lysate of hormone-treated F-2 cells (clonal cell line of mouse mammary gland carcinoma gR with strictly hormone-dependent expression of the virus), and lysate of MM5 cells.

The negative controls were lysates of HeLa and Hep-2 cells, F-2 cells not treated with hormones, concentrate of Moloney and Rauscher viruses, disintegrated sheep's red blood cells, and human albumin (from Reanal). Pure carbonate buffer (as a control of possible nonspecific sorption of the antiviral serum) was added to some wells.

After incubation for 24 h at 4°C the wells were washed several times with buffered physiological saline with 0.05% Tween-20 (PBS-Tween-20) to remove unbound proteins. A 10% solution of ovalbumin (from Pharmacia, Sweden) was then incubated for 24 h in the wells in 0.2 M carbonate buffer, pH 9.8 to inactivate any free valencies on the plastic, after which the plate was again washed several times with PBS-Tween-20, and antiserum against all MMTV proteins and to gp-52 was added to the wells in dilutions of 1/10 to 1/200, in parallel series with the same dilutions of normal rabbit serum. After incubation for 2 h the plate was again washed and the conjugate of antibodies against rabbit immunoglobulins with peroxidase in a dilution of 1/400 in PBS-Tween-20 was poured into the wells for 1 h. After washing, the chromogen (1% solution of para-aminosalicyclic acid, pH 6.0, with 0.05% H_2O_2) was poured into the wells. The reaction was evaluated on the basis of the intensity of staining of the contents of the well.

To investigate the material by the "dot-blotting" method 10 μl of the test extract (the same positive and negative controls were used) was applied to an HAWP nitrocellulose filter (Millipore, USA), which was then incubated in 4% hemoglobin solution (Serva, USA) in PBS, pH 7.2, at 40°C to inactivate binding valencies, after which filter was immersed in a 1/10 solution of antiserum with 1% hemoglobin for 12 h, after which it was washed for 2 h in glycine buffer, pH 5.0, and incubated with ^{125}I-labeled antibodies against rabbit globulins (10^6 cpm/ml, specific labeling not less than 10^8 cpm/μg), and again washed in PBS-Tween-20 with repeated changes of buffer. After drying, the filter was exposed on ORWOH-11 film (East Germany).

Considering the possibility that human immunoglobulins, invariably present in tumor extracts just as in extracts of healthy tissues, may be involved in the reaction, an intensity of staining corresponding to the reac-

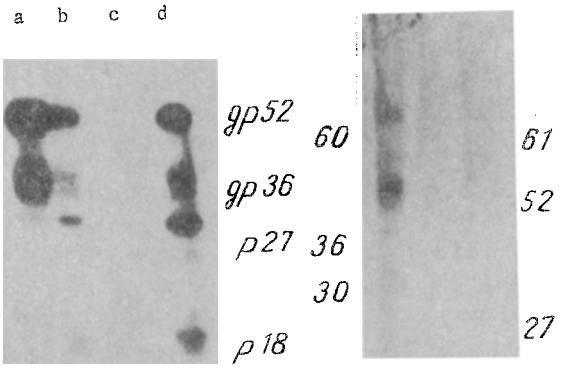

Fig. 1 Fig. 2

Fig. 1. Immunoprecipitation of proteins of ^{125}I-labeled MMTV with antisera. a) Serum against gp-52; b) serum against virus preparation; c) normal rabbit serum; d) MMTV preparation. Separation in 7-15% polyacrylamide gel.

Fig. 2. Immunoblotting with antiserum against MMTV. a) Preparation of mammary gland tumor tissue (positive), b, c) lysate of HeLa and Hep-2 cells. Besides specific bands, zones of protein corresponding to immunoglobulins can be seen on track *a*.

tion with extracts of embryonic tissue, which are known to contain more protein, including immunoglobulins, was taken as the "background" intensity of staining in both methods. The reaction was considered to be positive if significantly brighter than the "background." When the dot-blotting method was used, the filters also were incubated in embryonic tissue extract after application of the test samples to them.

The most actively reacting specimens were tested by the dot blotting method to determine molecular weights of proteins taking part in reactions with antiviral sera. After electrophoresis in 7-15% polyacrylamide gel the specimen was transferred to an HAWP nitrocellulose filter in Tris-glycine buffer, pH 8.3, with 20% methanol, in an electric field with intensity of 6 V/cm for 2 h; the filter was then incubated in 3% hemoglobin solution, after which it was treated with specific antiserum in a dilution of 1/10. After washing it was incubated with peroxidase-labeled antibodies against rabbit immunoglobulins, and after the next washing it was immersed in a solution of 5-α-chloronaphthol (2 mg to 10 ml H_2O) with 0.05% H_2O_2.

Proteins reacting with antiserum were stained.

EXPERIMENTAL RESULTS

Altogether 25 specimens of malignant HBT, 10 specimens of benign HBT, 15 specimens of normal breast tissue, and extracts from tumors in other situations were tested.

Antiserum against MMTV proteins and monospecific serum against gp-52 revealed nine positively reacting specimens of HBT. The specimens reacted with both sera. The results obtained by ELISA coincided completely with the results obtained by dot blottings. The specimens of the nine tumors studied, namely fibroadenomas, gynecomastia, and fibrocystic mastopathy, gave no reaction with the test sera, except one extract of breast tissue containing proliferating mastopathy. Sera against MMTV proteins were inactive with preparations from tumors in other situations (carcinoma of the stomach, ovaries, body and cervix of the uterus, kidney, rectum, and liver). One specimen of normal breast tissue reacted positively. When the results of the investigations were compared with the history of the disease no correlation could be found between the presence of antigens in HBT tissue cross-reacting with MMTV antigens and any particular features of the history, stage of the process, histologic type of the tumor, or degree of its malignancy. It was observed, however, that tumors carrying the test antigens are evidently more immunogenic for the host: Compared with other HBT they induced a much more intensive immune response: marked lymphoid infiltration, with hyperplasia of the regional lymph nodes.

In the dot blotting test with serum against MMTV proteins, proteins with mol. wt. of 30,000, 36,000, and 60,000 daltons, which also gave cross reactions, were discovered in positively reacting specimens. Their investigation was made more difficult because of the tendency of the proteins to undergo degradation. Instability of the extracted proteins was perhaps connected with the use of preoperative radiotherapy (Fig. 2).

The results of the present investigation confirm the existence of antigens immunologically related to structural proteins of MMTV in HBT. Nucleotide sequences homologous with the sequences of MMTV have been found in the human genome, although no complete copy of the provirus has been found [4]. Positive results of searches for antigens similar to MMTV antigens in human material support the hypothesis [2] that the expression of these sequences is similar to that of endogenous mouse provirus. The results are in agreement with those obtained in [3], which demonstrated the considerable immunogenicity of tumors containing proteins resembling virus gp-52 for man compared with HBT, which do not contain it.

The results do not evidently reflect completely expression of the test antigens in all mammary gland tumors, for the tumor node proper did not always constitute the greater part of the preparation as received. Some HBT probably contain amounts of test antigens outside the limits of sensitivity of the methods used.

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EFFECT OF METHYLCOBALAMINE ON METHOTREXATE UPTAKE BY NORMAL AND TUMOR TISSUES

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Methylcobalamine (MeCbl), a coenzyme of methionine synthetase (EC 2.1.1.13), a key component controlling the formation of reduced folates, stimulates proliferation of normal and tumor cells in vivo [1, 2]. An increase in the pool of proliferating cells in tumors with a low rate of proliferation, under the influence of MeCbl, enables the antitumor activity of cycle-dependent antimetabolites to be increased [2]. The therapeutic efficacy of the dihydrofolate reductase (EC 1.5.1.3) inhibitor, methotrexate (MTX), when used in combination with MeCbl, is increased as a result of inhibition of DNA synthesis in the greater part of the tumor cell population [3]. Meanwhile the effect of Cbl on MTX uptake by tumor cells cannot be ruled out. Cobalamines are known to facilitate uptake of methyltetrahydrofolic acid (the basic form of folic acid in the blood), which competes with MTX for cell membrane transport protein [5, 8], by hematopoietic cells. The problem of the effect of MeCbl on uptake of folate analogs, including MTX, by tumor cells has not previously been studied.

The aim of this investigation was to study the effect of MeCbl on [^{3}H]-MTX uptake by mammary gland adenocarcinoma cells and by the tissues of the small intestine and spleen of animals with transplanted tumors.

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