Just as during investigation of the mutagenic action of CP and thiotepa, WR mice were thus relatively resistant to the immunosuppressant action of CP and highly sensitive to the action of thiotepa. It can be postulated on the basis of these results that a parallel may exist between the mutagenic and immunosuppressant action of these compounds. This view is supported also by data in the literature. In particular, it has been shown [1, 4] that DBA/2 mice are more sensitive to the immunosuppressant action of CP *in vivo* than mice of the highly resistant BALB/c line. The same relations between mice of these lines were found in a study of induction of sister chromatid exchanges *in vivo* in bone marrow cells [5] by means of CP.

The differences thus revealed in the response of WR mice to the immunosuppressant action of CP and thiotepa can thus perhaps be atributed both to differences in the pharmacodynamics of these compounds in WR mice and to the character of interaction of immunocompetent target cells with immunosuppressants.

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# EXPRESSION OF HUMORAL ANTIBODIES TO MURINE MAMMARY TUMOR VIRUS-RELATED ANTIGENS IN BREAST CARCINOMA PATIENTS AND CONTROL SUBJECTS


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Murine mammary tumor virus (MMTV) is an agent which produces a high percentage of spontaneous mammary gland carcinomas under natural conditions and in mice of laboratory strains. Expression of exogenous MMTV in mice of many strains is accompanied by the appearance of a malignant neoplasm in the mammary gland when the titer of antibodies against MMTV proteins is raised in the animal's serum [8]. Expression of antigens immunologically related to MMTV has been established in breast cancer patients [3, 5] in the tumor cells; antibodies reacting specifically with MMTV proteins have been found in the serum of various groups of normal subjects and cancer patients [1] and sequences with high homology with the genome of this virus have been found in the human genome [2]. However, the results so far are somewhat contradictory and do not reflect unambiguously a strict association between expression of MMTV-related antigens (or antibodies to them) with the appearance of a neoplastic process in the mammary gland [8].

In the investigation described below, highly sensitive immunologic methods were used to determine whether the malignant process in the mammary gland is accompanied by a humoral response to MMTV-related antigens, and to establish at what level these antigens are expressed normally and in neoplasms in other situations.


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TABLE 1. Detection of Humoral Antibodies against Antigens Related to Antigens of MMTV Structural Proteins in Donors of Different Groups (only women)

| Group of subjects tested | Total number of sera | Number of positive sera | Percent of positive sera |
|---|---|---|---|
| Untreated breast cancer patients | 92 | 5 | 5,43 |
| Clinically healthy women | 152 | 5 | 3,1 |
| Clinically healthy pregnant women | 59 | 6 | 10,1 |
| Patients with tumors in other situations | 94 | 3 | 3,2 |

## EXPERIMENTAL METHOD

The immunoperoxidase test on No. 3030 polystyrene plates (Falcon, USA) and the antigen spot test on a nitrocellulose filter (with radioisotope label) were used. A preparation of MMTV was used as the antigen. The data were analyzed and compared with case histories with particular attention to sex, the character of the course of the tumor, and its morphology. The MMTV preparation was obtained by ultracentrifugation of culture medium of mammary gland tumor cells from C3H mice, grown on roller flasks (medium RPMI 1640 with 10% bovine serum and hormones: insulin in a dose of 10 μg/ml and dexamethazone in a dose of $10^{-6}$ M), ensuring maximal virus production. After sedimentation of the MMTV it was purified by centrifugation through a 20% sucrose solution and analyzed by fractionation in a 7-15% polyacrylamide gel gradient to determine the completeness of purification.

The globulin fraction from sera against human globulins (anti-IgG, anti-IgM, and anti-IgA, produced by the N.F. Gamaleya Institute of Epidemiology and Microbiology, Academy of Medical Sciences of the USSR) was precipitated with 40% ammonium sulfate solution, reprecipitated, and the antibodies were conjugated with peroxidase [6] or labeled with $^{125}I$ by the chloramine T method [4].

To perform the indirect immunoperoxidase test, virus previously disintegrated with 0.5% NP-40 solution was added in a dose of between 1 and 10 μg to a well in 0.2 M carbonate buffer (pH 9.6) and incubated over night. Unabsorbed protein was washed off with phosphate buffer containing 0.05% Tween-20 (Schuchardt München, West Germany). The human test sera were added in dilutions of 1:2-1:10 and incubated for 2 h. The resulting conjugate was used in a dilution of 1:50. To determine the presence of antibodies binding MMTV proteins in the serum by the antigen spot test, 5 μg of virus was applied to the filter in a volume of 5 μl and dried. A spot of antigen about 4 mm in diameter was obtained on the filter. HAWP filters (Millipore, USA) were used. After incubation of the filter in 4% hemoglobin solution to inactivate free binding valencies on the filter (pH 7.2; 40°C) each filter was immersed for 6 h in a solution of serum (1:4) in phosphate buffer with 1% hemoglobin, after which the filters were washed and immersed in a solution of $^{125}I$-labeled antibodies against human immunoglobulins (label concentration $10^6$ counts/ml, specific label $10^8$ cpm/kg antibodies). After incubation with the test serum the filters were washed for 3 h in phosphate buffer with 0.05% Tween-20, and after radioisotope labeling for 3 h, in 6 changes of buffer; in the last two changes Triton X-100 was added to the solution up to 0.1%. The dried filters were exposed on ORWO HS11 film (East Germany). Test sera were obtained from cancer patients who had not received any form of treatment (Department of Breast Tumors, All-Union Oncologic Scientific Center, Academy of Medical Sciences of the USSR), from patients with other malignant diseases (Department of Clinical Biochemistry, All-Union Oncologic Scientific Center), from patients with breast cancer in late stages, receiving antitumor therapy (Department of Radiosurgery, All-Union Oncologic Scientific Center), and from healthy pregnant and nonpregnant women (from various Moscow hospitals).

TABLE 2. Distribution of Patients by Stages of Tumor (with positive and negative sera)

| Stage of breast cancer | Number of positive sera | | Number of negative sera | |
|---|---|---|---|---|
| | absolute | % | absolute | % |
| I | 7 | 13 | 0 | 0 |
| IIa | 18 | 35,3 | 2 | 5,6 |
| IIb | 16 | 31,4 | 5 | 13,8 |
| IIIa, b, c | 10 | 19,6 | 27 | 74,6 |
| IV | 0 | 0 | 2 | 5,6 |
| Total ... | 51 | | 36 | |

Legend. Number of positive or negative sera as a percentage of total number of sera for that group.

TABLE 3. Presence of Antibodies against MMTV Structural Proteins in Sera of Patients with Breast Tumors at Different Stages

| Stage of tumor | Total number of sera | Number of positive sera |
|---|---|---|
| I | 7 | All |
| IIa | 20 | 90% |
| IIb | 21 | 76% |
| IIIa, b, c | 37 | 27% |
| IV | 2 | None |
| IV Patients receiving chemotherapy | 18 | » |

## EXPERIMENTAL RESULTS

The polystyrene plate method proved to be the most suitable for mass screening of sera for the presence of specific antibodies. However, the reaction with antigen in the nitrocellulose test enabled relative activity of the test sera to be compared. Both methods are equally convenient for determiing antibodies against MMTV in human sera, for they allow simultaneous analysis of many samples; ELISA, however, is more sensitive.

Altogether 425 sera from donors of different groups were tested. Both methods were used only to test sera from breast cancer patients and the results agreed in 95% of cases. All sera investigated by radioimmunoassay reacted with MMTV in the immunoperoxidase method also. However, 5% of sera giving positive results in ELISA were not tested since they were positive when tested by the second method. The data given below represent statistical analysis of results obtained by the immunoperoxidase method.

After the sera of some donors had been shown to contain antibodies reacting with MMTV proteins, the specific character of the reaction with virus proteins was studied with the aid of the following controls: preparations of other retroviruses (Molv, Rolv), to determine absence of a nonspecific reaction with the glycoside moiety of the virus glycoprotein molecule, described for C-type retroviruses [7]; a lysate of mouse cells (1% solution of Triton X-100 in 10 mM Tris-HCl buffer, pH 7.4), NIH 3T3, and cells of murine mammary gland tumor GR (grown on hormone-free medium under conditions of sharply depressed synthesis of MMTV proteins) to prevent any possible nonspecific reaction of the test sera with mouse antigens present as impurities in the MMTV preparation.

After the appearance of a collection of sera reacting positively and negatively with MMTV, each subsequent experiment was carried out using them as controls.

Data on the presence of antibodies in sera from different groups of subjects are given in Table 1. Data on the distribution of the sera tested by stage of the tumor process and data on the presence of antibodies against MMTV antigens in patients with tumors in different stages are given in Tables 2 and 3. This material was obtained by analysis of 87 case histories of patients untreated at the time when the serum was taken. Unfortunately, no significant differences could be detected either in the character of the primary medical history or in the histologic type of tumor in patients with positive and negative (in our test) sera. However, separation of the patients' sera into groups depending on the stage of the tumor brought to light a phenomenon not previously reported.

Normally only a small proportion of physically healthy women have antibodies against antigens immunologically related to MMTV proteins. During pregnancy and as a result of intensified hormonal action on mammary epithelial cells an increase in the percentage of subjects with positive sera up to 10 was observed. The appearance of a neoplasm in another situation had no effect on the percentage of positive donors in the group.

Among patients with breast cancer more than half were found to have antibodies against MMTV antigens. This is a somewhat higher proportion than was obtained by Zotter [8], evidently because different methods were used, and they agree with data obtained by other workers [1, 3]. By separating the groups of patients into subgroups depending on the stage of the tumor, it became clear that practically all patients in the early stages of cancer have antibodies against MMTV-related antigens. It can accordingly be concluded that there is a closer connection between the appearance of breast tumors and expression of MMTV-related antigens in man than Zotter previously considered [8]. However, it must be pointed out that the possibility of more rapid tumor growth in positive donors and, as a result, their hospitalization in the earlier stages of malignant disease, cannot be ruled out. In the later stages the percentage of positive donors decreased, evidently due either to depression of the patient's immune response or to absorption of antibodies by the growing tumor and metastases. No positive antibodies were found in our test in serum from patients with tumors in stages III and IV, treated by chemotherapy (and in some cases by radiotherapy also).

The correlation that was revealed between the presence of a humoral response to antigens related to antigens of MMTV proteins and the early stages of malignant disease and also pregnancy confirmed the repeatedly stated view that expression of these antigens is similar to expression of endogenous MMTV in mice of certain strains. At the same time the results demand a study of various benign breast tumors and the sera of these patients in order to study whether the immune response to MMTV-related antigens can be used as a marker of the early stages of a tumor, and also the question of the effect of a benign proliferative process on their expression.

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