brought new methods of leukocyte and tissue typing. The MLC reaction (3) allows the reproduction in vitro of the reaction of transplantation immunity in vivo (1) by the selective measurement of antigenic incompatibilities for the important transplantation antigens.

Specific tumor cell antigens are known to be weaker than transplantation antigens. We undertook a program to determine if MLC is capable of detecting these weaker "foreign" antigens by blastoid transformation of autologous lymphocytes. The observation of the simultaneous occurrence of malignant melanoma of the skin in monozygous brothers of a set of triplets who are reported elsewhere in detail (25) provided us with a unique experimental model with which to study this question.

**PATIENTS AND METHODS**

**Patients**

Patient D (RPMI No. 124-168). An infiltrative growing malignant melanoma of the left anterior chest wall was removed at age 53. Four and one-half years later, after several episodes of tumor recurrence and reoperation, the patient died with widespread metastases, predominantly pulmonary. No chemotherapy was given prior to the experiments. Afterward, chemotherapeutic trials with imidazole carboxamide dimethyltriazeno and L-asparaginase were unsuccessful.

Patient T (RPMI No. 124-183). The monozygous triplet of Patient D was found to have malignant melanoma of the same localization at the same time of life as Patient D (congruent contemporaneous concordance). An identical operation was performed by the same surgeon. On elective admission to Roswell Park Memorial Institute 4.5 years later, no evidence of tumor recurrence or metastasis was found.

Patient V. The fraternal triplet of Patients D and T was seen at Roswell Park Memorial Institute for history and physical examination, which were within normal limits.

Control Subject N. N was a 33-year-old healthy, genetically unrelated donor of control lymphocytes.

**SUMMARY**

We observed congruent contemporaneous concordance of malignant melanoma in identical brothers of a set of triplets. Four and one-half years after surgery, one twin had metastatic disease and the other was apparently free of tumor.

Blastoid transformation of lymphocytes measured by DNA synthesis and cytotoxicity of lymphocytes for melanoma cells measured by fluorescein diacetate loss in mixed lymphocyte-tumor cell cultures were both substantially greater with lymphocytes from the tumor-free twin. Since the brothers were monozygous, this cannot be attributed to transplantation-antigenic differences. Possible explanations include neoantigenization of the tumor and/or depletion or blocking of sensitized lymphocytes from the autochthonous host. The data extend the evidence for immunity against tumors in man and the techniques for demonstrating it.

**INTRODUCTION**

The existence of tumor-specific immune mechanisms in animal systems has been firmly established during the past 15 years. Numerous methods have been developed which allow the conclusion that tumor-directed immunity has 2 components, antibody-mediated and cell-bound immunity. This has been the subject of several review articles (4, 14, 15, 21).

Experimental data showing immune response to tumors in man recently have been presented for colonic carcinomas by immunological tolerance and absorption techniques (8), for Burkitt's lymphoma with membrane immunofluorescence (13), for postnasal sarcomas or carcinomas by cytotoxic assays (6), for malignant melanoma by immunofluorescence (19), for osteogenic sarcoma by immunofluorescence (18), for neuroblastomas and adenocarcinomas of colon and lung by colony inhibition assay (9, 10), and for leukemia and lymphoma by cytotoxicity, immunofluorescence, complement fixation, and immune adherence (7).

Advances in organ transplantation in man have necessarily brought new methods of leukocyte and tissue typing. The MLC reaction (3) allows the reproduction in vitro of the reaction of transplantation immunity in vivo (1) by the selective measurement of antigenic incompatibilities for the important transplantation antigens. Specific tumor cell antigens are known to be weaker than transplantation antigens. We undertook a program to determine if MLC is capable of detecting these weaker "foreign" antigens by blastoid transformation of autologous lymphocytes. The observation of the simultaneous occurrence of malignant melanoma of the skin in monozygous brothers of a set of triplets who are reported elsewhere in detail (25) provided us with a unique experimental model with which to study this question.

**REFERENCES**

1. This investigation was supported by USPHS Research Grant CA-5834 from the National Cancer Institute.
2. The abbreviations used are: MLC, mixed lymphocyte culture; TdR-3H, tritiated thymidine; PHA, phytohemagglutinin P; FDA, fluorescein diacetate; HLA, human leukocyte antigen; NLT, normal lymphocyte transfer.

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All experiments described below were carried out with sterile precautions in siliconized tubes. The culture medium throughout was RPMI 1640 (17) supplemented with fresh 20% autologous plasma. Antibiotics were omitted.

MLC

The MLC (23) was carried out to confirm the monozygosity of the identical twins among the triplets, to prove the responsiveness of lymphocytes to allogeneic cells, and to compare the in vitro lymphocyte:tumor cell interaction with the lymphocyte:lymphocyte interaction. A "one-way" modification of the MLC (2) was chosen, which allows the selective blastoid transformation of only 1 cell population of lymphocytes, measured as incorporation of TdR-3H (Schwarz BioResearch, Orangeburg, N. Y.) into DNA. Modification of the MLC (2) was chosen, which allows the selective blastoid transformation of only 1 cell population of lymphocytes, measured as incorporation of TdR-3H (Schwarz BioResearch, Orangeburg, N. Y.) into DNA. The lymphocytes were prepared from peripheral venous blood by sedimentation with heparin, 30 units/ml, at 37°. A suspension of mononuclear cells (97 to 99% purity) was recovered from the supernatant by passing it through a column of cotton wool to which the granulocytes adhered.

The stimulating cell population was treated for 20 min with mitomycin C, 50 µg/ml, and then washed twice with culture medium. As responding cells 1 X 10⁶ lymphocytes were used. Mitomycin-treated cells were added as stimulus to give a final responding:stimulating cell ratio of 2:1, 1:1, and 1:2, which in our experience reflects the optimal range of the MLC. The cells were cultured in 2.5 ml culture medium in 15/125-mm test tubes sealed with silicone rubber stoppers at 37° for 144 hr. TdR-3H (2 µCi), specific activity, 1.9 Ci/mM, was added to the cultures for the last 5 hr. For termination of the experiment, unlabeled thymidine was added in 1000 times excess in order to dilute the uptake of the isotopic compound. DNA was precipitated with trichloroacetic acid according to the method of Bach (2). The acid-insoluble material was thereafter dissolved in 0.2 ml phenethylamine and 15 ml liquid scintillation solution (POPOP, 7 g; dimethyl-POPOP, 0.3 g; naphthylene, 100 g, and dioxane to make up 1000 ml) and counted in a Packard Tri-Carb spectrometer with correction for quenching. The results are expressed in median cpm of experiments done in triplicate. The controls included lymphocytes without stimulus, lymphocytes stimulated with PHA, and tumor cells stimulated with PHA.

FDA Cytotoxicity Test

Tumor cells as target cells were exposed to autologous, syngeneic, and allogeneic lymphocytes in vitro. The cytotoxic action of lymphocytes was measured by FDA release from tumor cells. Crude suspensions of lymphocytes and tumor cells were prepared as described above. Lymphocytes were incubated in the presence of PHA for 3 days. The lymphocytes were thereafter adjusted to 2 X 10⁶/ml viable cells. To this preparation of lymphocytes, tumor cells were added in sufficient numbers to give a lymphocyte:tumor cell ratio of 10:1. The tumor cells were labeled with FDA before they were added to the lymphocyte suspension. The procedure of labeling was adapted from Rotman and Papermaster (24) and consisted of the following steps. Five mg FDA were dissolved in 1 ml of analytical acetone to give a stock solution. Five µl of the stock solution were added to 10 ml of phosphate-buffered 0.9% NaCl solution, which served as working solution, prepared fresh daily. Packed cells to be labeled were suspended in 1 ml working solution. After 15 min incubation at room temperature, the cells were washed twice with 5 ml culture medium and counted. The labeled cells appear as bright fluorescent bodies on a black background under the ultraviolet microscope. Immediately after the lymphocytes and tumor cells were mixed, they were counted in a counting chamber. The number of fluorescent cells counted in unit volume was taken as 100% viability. The cell mixtures were kept in test tubes at 37° until further counts were made after 2, 4, 8, 12, and 24 hr. As controls, FDA-stained tumor cells were kept under identical conditions but were not exposed to lymphocytes.

Leukocyte Antigen Typing

HLA typing of lymphocytes was done in another laboratory (Courtesy of Dr. P. Terasaki, Center of Health Sciences, University of California at Los Angeles, Los Angeles, Calif.). This analysis was done as an attempt to correlate both the MLC and mixed lymphocyte tumor culture with antigens known to be involved in the lymphocyte transformation process (12).

NLT Test

The NLT (5, 20) was done with 3 X 10⁶ lymphocytes of Patient T. The cells were injected intracutaneously into his identical twin Patient D on Day 1, 3, and 8 of the leukocyte
transfusions (v.i.) and, on Day 8, into the normal Control Subject N. The injection sites were observed for 72 hr.

**In Vivo Lymphocyte Transfusions**

On 8 consecutive days, Patient T underwent daily leukopheresis of 1000 ml venous blood. Blood was drawn into Fenwal PA-220 Blood-Pack units which contain 75 ml acid-citrate-dextrose as anticoagulant per 500 ml blood. The unit was spun for 15 min at 900 X g and the leukocyte-rich pellet was withdrawn together with approximately 125 ml of plasma. A small sample was separated for total and differential counts. The leukocytes were transfused immediately thereafter into Patient D. In all, 2.6 \( \times 10^7 \) cells containing 1.6 \( \times 10^6 \) lymphocytes in a total of 1980 ml plasma were transfused. The donor and recipient were monitored by daily hematological studies before and after the transfusions. Electrophoresis of Patient T’s serum was performed repeatedly.

**RESULTS**

The results of the MLC are presented in Chart 1. The mixtures DTm (D with T cells treated with mitomycin C) and TDm show some variability at the different ratios studied. This variability, however, does not reflect a response pattern, but demonstrates within what baseline limits “non-stimulation” varies. All other mixtures show increasing DNA synthesis in responding lymphocytes when increasing numbers of stimulating cells are added. It is obvious that N shows a practically identical response to D and T. In similar fashion, T and D respond equally to Nm cells. The mixture of DT cells and the mixture NmDm fall within the background of the unstimulated controls D, T, and N. The mixture NmTm indicates, however, that since the Nm cells were all obtained from 1 batch, DNA synthesis of the Tm population was not completely inhibited with the short exposure to mitomycin C. The untreated lymphocytes respond equally well to PHA.

The results of the mixed lymphocyte tumor culture are shown in Chart 2. No TdR-3H incorporation into DNA of Patient D’s lymphocytes was observed in the mixtures with his own tumor cells, except at the ratio 1:1. In that single circumstance, counts in all of the triplicate tubes lay slightly above the counts of the controls. The mixture of T lymphocytes with Patient D’s tumor cells shows counts clearly higher than background counts even at the ratio 50:1. Neither the healthy fraternal triplet, Patient V, nor the unrelated Control Subject N, shows lymphocyte transformation at this level. The latter 2 mixtures respond earliest at the concentration 25:1, and then show a nearly steady increasing incorporation with further addition of cells, whereas the T lymphocytes completely fail to respond when tumor cells over a given maximal number (ratio, 2:1) are added. All of the control counts fell within the range of the controls in Chart 1. No stimulation of tumor cells with PHA was observed.

The results of the FDA cytotoxicity test are shown in Chart 3. Although the observations were extended up to 12 and 24 hr, these data are not recorded here because after this time the FDA label “bleaches” out and exact quantitative analysis becomes impossible. Cytotoxicity is observed in all mixtures; the most rapid loss of label is found in the T tumor cell mixture. The statistical analysis of the FDA test took into account sampling and counting errors of 32 observations. The analysis of variance has shown that the cell viability curves are not likely to be straight lines. This implies that the killing rate in a given preparation was not constant over the whole duration of the experiment. To compare the effects of the different varieties of lymphocytes on the killing of tumor cells, we have used the slope of the
Immunity against Melanoma

Chart 3. FDA cytotoxicity test. Tu, tumor cells alone. D, T, and N, mixtures of lymphocytes with tumor target cells. The viability of tumor cells is measured by retention of FDA.

best-fitting straight line, so that the sum of the squared deviations of the data from the fitted line is minimal. The hypothesis of equality of all 4 averaged rates is rejected by the F-test ($p < 0.001$). Individual $t$ tests for each of the possible 6 pairs gives the following results: every pair is significantly different at the level of 1% except the pair N/tu D/tu, for which no difference could be shown.

The results of the transplantation antigen typing report are summarized in Table 1. The HLA type of Patients D and T is identical. Patient V and Control Subject N differ from Patients D and T in 1 group each.

Table 1

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<th>HLA leukocyte antigens: D and T identity</th>
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No definite information as to the efficacy of the lymphocyte transfusions could be gained. The clinical situation had been stable for 3 weeks of observation prior to the transfusions. After the 3rd transfusion, Patient D complained of increasing pain in the left shoulder, the site of subcutaneous metastases. He developed at the same time a large pleural effusion on the left side, and, the following day, a pleural effusion on the right side, requiring daily taps. The lung metastases showed rapid progression, and the patient died because of respiratory decompensation. Tumor tissue (2500 g), mostly in the lungs, was estimated at autopsy. The metastases showed central necroses with massive lymphocytic infiltrations.

The normal Patient T lymphocyte transfer onto the arm of Patient D before and after the leukocyte transfusions was negative. Control Subject N responded to Patient T's lymphocytes with an erythematosus induration of 22 mm at the injection site after 30 hr.

DISCUSSION

These results indicate that there is a very sensitive cell population among lymphocytes which is able to recognize some structure in tumor cells as foreign. This structure may be similar to but is not identical with transplantation antigens. Positive stimulation in the MLC indicates presence of foreign antigens. Lack of stimulation does not necessarily mean antigenic identity, however, however, since lack of this response has been observed in siblings (1). The monozygosity of the brothers was calculated with 99.80% probability (25).

The zero stimulation in the MLC, the identical HLA type, and the negative NLT test add further support. No stimulation of Patient D's lymphocytes by his own tumor cells was observed. Positive stimulation was obtained in all other mixtures, however. The responses of Control Subject N and Patient V are most likely a reaction to normal tissue transplantation antigen differences. The response of Patient T's lymphocytes to his identical brother's tumor cells is a unique observation which might be explained in several different ways: (a) The tumors of Patients T and D were characterized by identical new antigenic structures. Patient T was able, after successful operation, to accumulate sensitized lymphocytes which recognized Patient D's tumor cells as foreign. The presence of circulating "sensitized" lymphocytes 4.5 years after the operation could be in contrast to the finding that circulating antimelanoma antibody decreased markedly 1 year after operation (19). This case would indicate that sensitized cells circulate much longer after operation, or that Patient T is not cured of his disease but has enough immunological function to keep the disease under control. (b) The tumors of Patients T and D were characterized by different new antigenic structures. Patient T developed tolerance to his tumor neoantigens, whereas Patient T would not necessarily have done so for his own tumor, and certainly not for Patient D's. This requires the supposition that the tumor neoantigens of Patient T were different from those of Patient D, or, if the tumor-specific antigens were the same, that Patient T did not become tolerant. In this case the blastoid transformation of Patient T's lymphocytes would indicate that his immunocompetent cells were able to recognize nontransplantation foreign cell structures without previous challenge. (c) The tumors of Patients T and D were originally characterized by identical antigenic structures, but the antigenicity of Patient D's tumor was altered in its immunological specificity by some infectious agent (23). Again, it would be of interest that Patient T's lymphocytes possess enough discriminatory power to recognize such differences.

A rather strong antigen seems to be involved in these experiments. The lack of response of Patient D's lympho-
lymphocytes is of interest. Although no proper controls with cells. Other possible explanations lie in the theory that a tumor-bearing patient is exhausted of specifically sensitized cells (16) or that there is an effect of antagonizing antibodies on Patient D's lymphocytes (22).

The clinical deterioration of the patient following the lymphocyte transfusions was striking, but such variabilities may be a characteristic of the underlying disease. The possibility of tumor enhancement must nonetheless be kept in mind.

The observation of the marked cytotoxicity of Patient T's lymphocytes for Patient D's melanoma cells and some cytotoxicity of Patient D's and Control Subject N's lymphocytes is of interest. Although no proper controls with normal D tissue cells were studied, the data strongly imply enhanced capacity of T lymphocytes to kill D melanoma cells.

ACKNOWLEDGMENTS

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Cell-mediated Immunity against Malignant Melanoma in Monozygous Twins
