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# A Nude Mouse Tumor Neutralization Test Applied to Patients Suffering from Skin Melanoma. A Methodological Study (39737)

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A new mutant laboratory mouse was described by Flanagan (8). The animal was characterized by hairlessness and immune deficiency caused by the lack of thymus (20). In 1969, Rygaard and Povlsen (25) reported that the mutant mouse would accept and support transplants of human malignant and nonmalignant tissues without the immunosuppressive pretreatment otherwise necessary in heterologous transplantation experiments (1, 4). Transplanted tumor tissue would grow locally (21, 26). Metastases were reported in a few cases (10, 11).

It is generally assumed that lymphocytes derived from melanoma patients are sensitized against a common group-specific antigen. It might be expected, therefore, that such lymphocytes mixed with otherwise tumor-producing inocula of melanoma cells transplanted into nude mice would: (i) decrease the number of takes, (ii) increase the latency period of tumor takes, and possibly (iii) reduce the growth rate of tumors.

The present report describes a tumor neutralization test (TNT) utilizing the nude mouse system for the evaluation of cell-mediated immunity in patients suffering from localized skin melanoma (stages I and II).

*Material and methods. Melanoma target cells.* A mycoplasma-free established melanoma cell line RPMI 7931 was kindly provided by Dr. C. O'Toole. An inoculum of $3 \times 10^5$ of these cells produces tumors in more than 50% of nude mice with a latency period of less than 3 weeks. Invasion or distant metastases have not been observed. The tumors are amelanotic and remain localized. Caryotyping has shown human chromosomes. The melanoma cells were cultured as monolayers in roller bottles. Culture medium was Eagle's minimum essential medium with a fourfold increase of the concentrations of vitamins and glutamine, a twofold increase of the concentration of essential amino acids, and a 35% increase of the glucose concentration. Twenty percent inactivated fetal calf serum, penicillin (250 IU/ml) and streptomycin (25 μg/ml) were added.

*Nude mice.* Animals with a genetic background of BALB/c/ABOMFib-nu [1-($^1/_2$)$^{10-12}$] were used.

The mice were bred and raised under specified-pathogen-free (SPF) conditions. At the age of 3 weeks the animals were transferred to minimum disease conditions with an atmospheric humidity of 80% and a room temperature of 28°. The animals were transplanted at the age of 3-6 weeks.

*Human lymphocytes.* Lymphocytes were derived from seven patients suffering from localized skin melanoma (stages I and II) and control lymphocytes were derived from seven healthy donors. Patients and controls were not matched with respect to age and sex.

Blood was drawn preoperatively under sterile conditions and stabilized by the addition of heparin (100 units/10 ml of blood) without preservative.

Lymphocyte separation was performed by a technique slightly modified (17) after Böyum (3). Differential counting of the stained smears showed approximately 99% lymphocytes. Trypan blue exclusion test showed a viability of 98-99%.

*Tumor neutralization test.* For each of the seven patients examined, 30 nude mice, comparable with respect to sex and age, were divided into three groups. Animals in group 1 received $3 \times 10^6$ control lymphocytes plus $3 \times 10^5$ melanoma cells mixed in a 0.1-ml volume of PBS. Similarly, group 2 animals received $3 \times 10^6$ patient lympho-

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cytes plus $3 \times 10^5$ melanoma cells. In group 3, animals were inoculated with $3 \times 10^5$ melanoma cells alone. The cell mixtures were inoculated sc without preincubation in the right side of the chest wall. The animals were examined at weekly intervals for palpable tumors. The area of the individual tumors was measured in two dimensions. The volume of the tumors was calculated using the smallest diameter twice (volume = $0.5236 \times \text{dia}_A \times \text{dia}_b \times \text{dia}_b$) (24). Observation periods within each experiment ranged from 6 to 10 weeks being limited by a high rate of spontaneous mortality among nude mice under non-SPF conditions. Only mice surviving for more than 3 weeks after inoculation were included in the experiments. Thus 13, 14, and 10 animals, respectively, were excluded from the control lymphocyte group, the tumor cell group minus lymphocytes, and the patient lymphocyte group.

*Immunofluorescence.* Indirect immunofluorescence was performed with FITC-conjugated goat anti-mouse IgM, goat anti-mouse IgG (Meloy Laboratories, Springfield, Va.), or rabbit anti-human immunoglobulins (DAKO, Copenhagen).

*Results.* A reduced number of takes (Table I) was noted in the group treated with patient lymphocytes as compared to that seen in the groups inoculated either with control lymphocytes plus melanoma cells or with melanoma cells alone. This difference was found to be significant ($P < 0.005$) when the pooled results were evaluated by $\chi^2$ test.

The influence of lymphocytes on the latency period is illustrated in Fig. 1 which shows the gradual increase in number of palpable tumors during the first 10 weeks after inoculations expressed as percentage of the final total number of tumor takes. Thus animals which did not develop tumors are not included in this figure. It appears that patient lymphocytes as well as control lymphocytes delay the appearance of tumors as compared to inoculation with tumor cells alone. The increased latency caused by control lymphocytes was on the borderline of significance, whereas patient lymphocytes exerted a significant increase of the latency period at weeks 2–5 ($\chi^2$ test, $P < 0.005$–$0.0005$). It appears from Fig. 2 that the growth rate of the tumors decreased with increasing tumor age. It furthermore appears that tumor growth was not influenced by the admixture of either patient or control lymphocytes.

TABLE I

| | CONTROL LYMPHOCYTE GROUP | TUMOR CELL GROUP MINUS LYMPHOCYTE | PATIENT LYMPHOCYTE GROUP |
|---|---|---|---|
| TUMOR POSITIVE | 47 | 46 | 35 |
| TUMOR NEGATIVE | 10 | 10 | 25 |

*Circulating antibodies.* It could not be excluded *a priori* that humoral antibodies might influence the TNT. Therefore, sera from nude mice transplanted with melanoma cells plus human lymphocytes or with human lymphocytes alone were assayed in serial dilutions for the presence of human or murine antibodies (IgA, IgG, IgM) directed against cultured tumor cells or against human lymphocytes. The animals were sacrificed by exsanguination 1–10 weeks post-transplantation. In no case did indirect immunofluorescence reveal such antibodies.

*Discussion.* It is generally assumed that lymphocytes from melanoma patients are sensitized against a group-specific melanoma antigen. This has been demonstrated by means of various *in vitro* methods such as microcytotoxicity test (6, 9, 28), colony inhibition (13), lymphocyte migration inhibition (2, 5), leukocyte adherence inhibition (12), and blast transformation tests (16). In recent years, however, doubts have been raised concerning the specificity of some of these methods (7, 14, 15, 17, 19, 27).

The present work aims at the elaboration of a new test system for cell-mediated immunity (cmi). The TNT system may elucidate cmi from another and maybe a more physiological angle, and possibly may be less or otherwise influenced by sources of error afflicting traditional *in vitro* methods.

The effects of patient lymphocytes, a decreased tumor incidence and a prolonged latency period, apparently may be due to an initial killing effect of the lymphocytes reducing the effective size of the tumor cell inoculum. Such an effect would have no influence on the growth rate of tumors arising from surviving melanoma cells. Histologically, tumors from the three groups appeared identical and no lymphocytic infiltration was seen. Furthermore circulating hu-

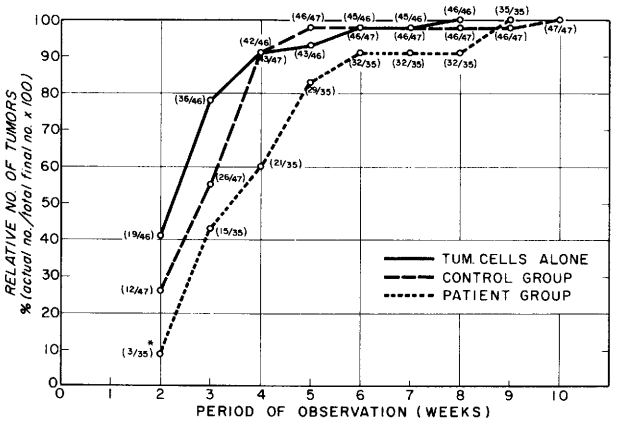


FIG. 1. The appearance of tumors in the patient lymphocyte group was delayed significantly ($\chi^2$, $P < 0.005$–0.0005) as compared to that seen in the group with tumor cells alone. (*) Actual number of tumor-bearing animals/total final number of tumor-bearing animals at the end of the experiment.

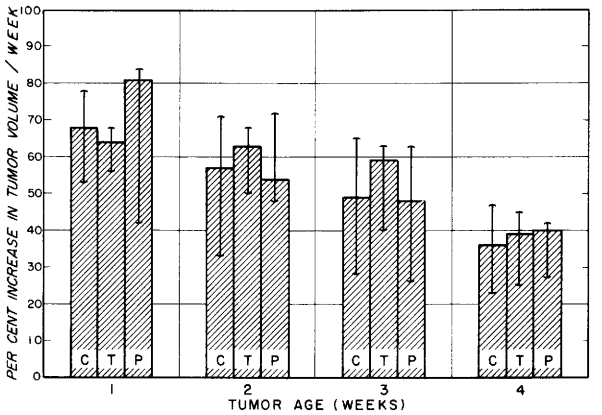


FIG. 2. The increase in tumor volume per week. Each bar represents the median for seven experiments with ranges indicated. (C) Control lymphocyte group; (P) Patient lymphocyte group; (T) Tumor cell group minus lymphocytes. Lymphocytes exert no influence on the growth rate of surviving melanoma cells.

man anti-melanoma immunoglobulins could not be demonstrated in these animals.

As a prerequisite for the present TNT the target tumor cell line must form localized tumors at the site of injection without invasion or distant spread. Under such conditions the appearance and subsequent growth of tumors may be used as a parameter for the possible influence of lymphocytes on tumor incidence and growth rate.

The immune deficiency of the nude mouse is caused mainly by the lack of thymus. However, it has also been demonstrated that the levels of IgG and IgA are decreased in these animals whereas the IgM level has been found normal (18). Transplantation of Burkitt tumor cells into nude mice has resulted in the appearance of circulating antibodies with anti-human specificities (22). This implies that a possible humoral host-immune response should be taken into consideration when results obtained by the TNT are evaluated. However, the present findings indicate that if such an immune response has occurred it has at least been unable to conceal the cytotoxic effect of patient lymphocytes. Furthermore, mouse anti-human antibodies could not be demonstrated in sera from tumor-bearing animals.

The stimulatory effect of immune lymphocytes reported by Prehn (23) was not

found in this study. However, the 10/1 lymphocyte/target cell (L/T) ratio used in the present TNT by far exceeds that found stimulatory by Prehn.

Thus, apparently, a common antigenicity in malignant skin melanoma appears to manifest itself in the TNT.

*Summary.* Lymphocytes from seven patients suffering from localized skin melanoma were mixed with melanoma cells derived from an established *in vitro* cell line, shown to be tumorigenic in nude mice but without tendency to invasive growth or formation of distant metastases. These lymphocyte–tumor cell suspensions were inoculated sc into nude mice. Comparable inoculations were performed with lymphocytes derived from seven healthy control donors. The L/T ratio was 10/1. Three parameters were measured: tumor incidence, growth rate, and latency period. It appeared that patient lymphocytes decreased the number of tumor takes and increased the latency period. The growth rate was not decreased by the patient lymphocytes. These findings support the general assumption that lymphocytes derived from melanoma patients are sensitized against a group-specific antigen.

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