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Natural Cytotoxicity: Relationship to Tumorigenicity of Human Cell Lines in Nude Mice (40706)

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Most studies on immunity in human cancer have been carried out in allogeneic systems. These have involved the use of cultured cells as target or as a source of antigen. Culture conditions, however, may modify the characteristics of the original tumor type to such an extent that the cultured cells may become unrepresentative of the original tumor and, consequently, inferior or useless in assays of tumor-directed immunity. Although in a few cases tumor cells may be identified in culture by markers such as melanosomes or CEA, most cultures are defined only by the tumor tissue of origin.

Tumorigenicity in the nude mouse is a possible criterion of malignancy. On the other hand, lack of tumorigenicity in the nude mouse might be due to the effects of natural cytotoxicity (NC) in the nude mouse. Some malignant tumor cell lines might be more sensitive to natural cytotoxicity and, as a consequence, non-tumor producing in the nude mice.

Nonspecific natural cytotoxicity has been demonstrated *in vitro* with material of human as well as of rodent origin. The biological significance of this reactivity remains unknown. In various studies the natural cytotoxicity has been ascribed to different cell types (1–5). Blocking experiments indicate that, in a proportion of effector cells, free Fc-receptors are involved in the manifestation of natural cytotoxicity (4). Natural cytotoxicity directed against syngeneic as well as allogeneic target cells also has been demonstrated in the nude mouse (6–8). For these reasons natural cytotoxicity appears to be exerted also by non-T lymphocytes.

The purpose of the present work was to elucidate, in a nude mouse model, the relationship between tumorigenicity of established human cell lines of malignant origin and the natural cytotoxicity *in vitro* against the same cultured cells.

Materials and methods. Mice. Female 5- to 8-week-old nude mice (BALB/c/ABOM Fibnu [$1 - (\frac{1}{2})^{10-12}$]) 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°C.

Established cell lines. Melanoma lines: RPMI 7931, Mel-1 (originating from O'Toole), Hu 831, Hu 866, Hu 1130, Hu 1214. Sarcoma line: SAOS-2 (originating from J. Fogh). Bladder carcinoma line: Hu 456. Fibroblast lines: Hu 808 cut and Hu 666 cut, both derived from normal skin. All cell lines were mycoplasma-free. Culture conditions were as previously described (9).

Effector cells. Spleen cells for use in the microcytotoxicity assay were obtained from untreated animals belonging to the same litters as those used in tumorigenicity experiments. Spleen cell suspensions were washed twice in a medium consisting of equal parts of RPMI 1640 and medium 199 (Gibco Bio-Cult, Glasgow, Scotland) with 10% fetal calf serum, penicillin, and streptomycin (25 IU/ml and 25 μg/ml, respectively). After lysis of contaminating erythrocytes with NH_4Cl, the cell suspensions were washed twice as above.

Assay for natural cytotoxicity. The microcytotoxicity assay (MCA) (10) was performed as previously described in detail (11). Briefly, the target cells for MCA were harvested from confluent monolayers by trypsinization. The same batch of cells was used for MCA as well as the tumorigenicity assay as described below. Ten-microliter volumes of cell suspension were seeded into each well of the microplates (Falcon 3034) and incubated for 18 hr at 37°C in a humidified gas mixture containing 5% CO_2. The effector cells were added.

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Lymphocyte/target cell ratios (L/T) ranged from 60/1 to 8000/1. The microplates were incubated for a further 48-hr period. At the end of this period the plates were fixed and stained. The number remaining were counted under a microscope.

Evaluation of results. A cytotoxic index was calculated as follows: $CI = (1 - L/M) \times 100$, where L = mean number of residual target cells in 12 wells after incubation with spleen cells, and M = mean number of target cells in 12 wells after incubation with medium only.

Tumorigenicity. For each cell line, approximately 30 nude mice comparable with respect to sex and age were divided into three groups. Animals in group 1 received 10^5 cells in 0.1 ml phosphate-buffered saline, whereas animals in group 2 and group 3 received 5×10^5 and 2.5×10^6 cells, respectively. The cell suspensions were inoculated sc into the right side of the chest wall. The areas of the individual tumors were measured in two dimensions and the volume was calculated using the smallest diameter twice (volume = $0.5236 \times \text{diam}_A \times \text{diam}_b \times \text{diam}_b$) (12).

Indirect immunofluorescence test. Sera from tumor-bearing and non-tumor-bearing animals were examined for antibodies against the cultured cell lines used for inoculation. The tests were performed using FITC-conjugated goat anti-mouse IgG, A, M (Nordic Immunology).

Results. Five out of six melanoma lines, as well as the sarcoma line, were found to be tumor producing in nude mice, whereas one melanoma, one bladder carcinoma, and two fibroblast lines were non-tumor producing. The tumor-producing capacity showed no correlation to passage number.

Observation periods were limited by the rate of spontaneous mortality, but ranged from 3–7 weeks. The number of animals with progressive tumor growth depended upon the size of cell inoculum. At the dose level of 2.5×10^6 cells, takes ranging from 7/7 to 9/10 were registered. In animals receiving non-tumor-producing cell lines no palpable tumors were observed in any instance.

It appears from Fig. 1 that in the MCA, spleen cells derived from untreated mice exerted a stimulatory effect ($CI < 0$) upon the growth of target cells obtained from tumor-producing cell lines. This effect appeared especially pronounced at L/T ratios above 1000/1. However, with some cell lines the effect of the spleen cells upon cell growth was pronounced even at low L/T ratios (125/1–250/1). In contrast to these findings, the growth of target cells derived from the non-

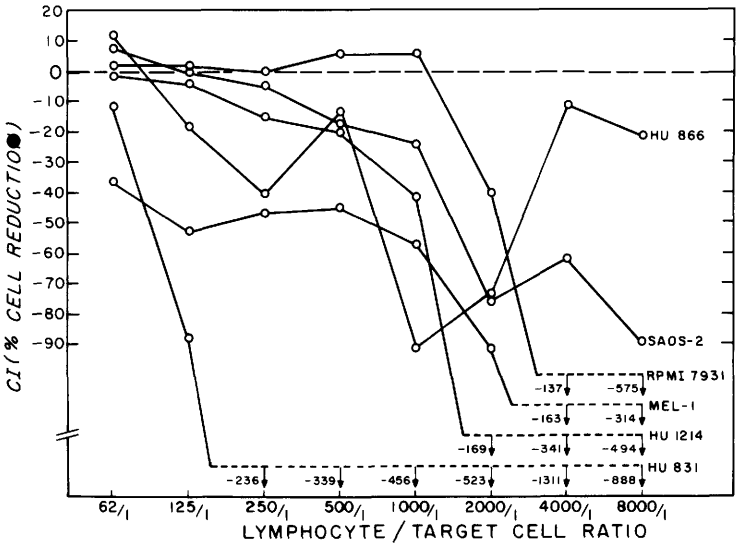

FIG. 1. Microcytotoxicity assay revealing a significant ($P \leq 0.001$) growth stimulation of tumor-producing cell lines by spleen cells derived from nontreated nude mice (L/T ≤ 2000/1). Melanomas: RPMI 7931, Mel-1, Hu 831, Hu 866, Hu 1214. Sarcoma: SAOS-2.

tumor-producing cell lines was inhibited (CI > 0) by spleen cells from untreated animals (Fig. 2).

In no case were humoral antibodies against the inoculated cells demonstrated.

Discussion. A growth-stimulatory effect of lymphocytes has previously been reported from *in vivo* as well as *in vitro* experiments. Thus, transplantation experiments (13) demonstrated that immune lymphocytes, at a low lymphocyte/target ratio, exerted a stimulatory effect upon tumor growth in a syngeneic murine model, whereas inhibition was observed at higher L/T ratios. Human lymphocytes derived from patients as well as control persons may also have a stimulatory potential as measured by the MCA (4, 11). In this system it has been observed that in some cases the number of target cells is increased as compared to that of medium control during the incubation ("feeder effect"). It should be borne in mind, however, that what is read in cytotoxicity assays are the net results of cytolysis, detachment, and cell proliferation whether spontaneous or accelerated by a "feeder effect" due to the presence of lymphocytes. In contrast to the observations with the murine models it is not known whether human lymphocytes under such experimental conditions are sensitized against the target. In spite of identical performance of the MCA, either a feeder effect or an inhibition was seen. The reason for this difference in pattern of reactivity is not known.

In the present work, using a xenogeneic model, a correlation was established between the tumorigenicity of human cell lines in nude mice and the *in vitro* stimulation exerted by nonsensitized spleen cells. The melanoma line RPMI 7931 has previously been used as the target in allogeneic MCA with lymphocytes derived from melanoma patients and control persons (4, 11). In these experiments, a few cases of stimulation were observed with patient lymphocytes as well as with control lymphocytes using L/T ratios ranging from 125/1 to 2000/1.

The finding that the *in vitro* growth rate of some cell lines (non-tumor producing) was inhibited, whereas the growth of other cell lines (tumor producing) was stimulated by the same effector cells, might indicate that tumorigenicity in the nude mouse may be determined by more than one factor.

It has generally been assumed that the immunological background which allows the support of xenogeneic tumor growth in nude mice is the lack of killer effector cells in the host. However, current experimental evidence may be interpreted to the effect that the nude mouse does possess cytotoxic effector cells different from killer T cells (6–8).

In the present work two out of four non-tumorigeneic cell lines were derived from

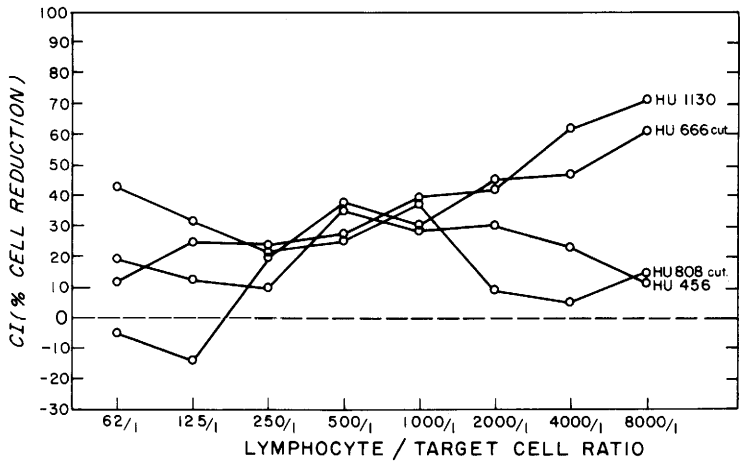

FIG. 2. Microcytotoxicity assay disclosing growth inhibition of non-tumor-producing cell lines by spleen cells derived from untreated nude mice. Inhibition at the 19.63% was significant ($P \leq 0.05$). Inhibition exceeding approximately 30% was significant at the $P < 0.001$ level. Fibroblast lines: Hu 666 cut, Hu 808 cut. Bladder carcinoma: Hu 456. Melanoma: Hu 1130.

malignant human tissues, i.e., melanoma and bladder carcinoma. The lack of tumor formation by these cells might possibly be explained as the result of selection of nonmalignant or stromal cells during cultivation. However, the lack of a tumor in the mouse does not definitely exclude malignancy, since the *in vitro* inhibition observed within such cells might reflect differential sensitivity among tumor-cell lines to the effect of natural cytotoxicity.

A stimulatory effect upon tumorigeneic cell lines was observed in the MCA with nonsensitized murine spleen cells. Provided that these *in vitro* observations reflect the existence of similar *in vivo* reactions, nonspecific cell-mediated reactions might support the early phase of tumor growth, whereas the capacity for immune-mediated inhibition of tumor growth may be acquired only at a later stage.

Summary. In the microcytotoxicity assay, a stimulatory effect exerted by nonsensitized spleen cells derived from nude mice was observed on the growth of tumorigeneic human tumor cells. In contrast, the growth of non-tumor-producing cell lines (malignant and nonmalignant) was inhibited by identical effector cells. Two out of four non-tumor-producing cell lines were of malignant origin. This lack of tumorigenicity, despite a derivation from tumor tissue, might be due to a selection of stromal cells during cultivation or might reflect a variation in the sensitivity to natural cytotoxicity independent of malignancy vs nonmalignancy. Consequently, lack of tumorigenicity does not exclude the possibility that the cells are malignant.

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