

**Stimulation of Murine Lymphocytes by a Syngeneic,
Methylcholanthrene-Induced Mammary
Carcinoma¹ (38004)**

GARY S. COLGROVE AND MOSHE SHIFRINE
(Introduced by C. Stormont)

Radiobiology Laboratory, University of California, Davis, California 95616

When cultured in the presence of specific antigen, lymphocytes from sensitized animals may be transformed into large, pyroninophilic cells which undergo mitosis (1, 2). Transformation (or stimulation) also results, in the absence of preexisting immunity, when lymphocytes from two unrelated individuals are cultured together (3). The degree of stimulation observed in mixed lymphocyte cultures is related to the genetic disparity of the two cell populations (3-5), and the reaction has therefore been used extensively as a measure of histocompatibility in man.

A modification of the mixed lymphocyte test has recently been applied to the detection of tumor-specific antigens. Kanner *et al.* (6) utilized a mixed lymphocyte-tumor reaction (MLTR) to demonstrate the antigenicity of two murine neoplasms, a melanoma syngeneic to the C57BL/6 mouse, and a lymphoma syngeneic to the DBA/2 mouse. Both tumors were stimulatory to syngeneic lymphocytes from animals with no prior exposure to tumor cells. Other reports (7-9) indicate that lymphocytes of tumor-bearing individuals may be stimulated by autologous neoplasms when tumor and lymphoid cells are mixed in culture.

We report here the application of the MLTR to studies on the antigenicity of a

methylcholanthrene (MC)-induced mammary carcinoma, syngeneic to BALB/C mice.

Materials and Methods. Tumor cells. Cell suspensions of an MC-induced mammary carcinoma, maintained by passage in BALB/C mice for 60-65 transplant generations, were prepared by finely mincing the tumors in MEM (Eagles Minimum Essential Medium for Suspension Cultures, Grand Island Biological Co.). After coarse material and larger clumps had settled out, the supernatant cell suspension was broken up by repeated gentle expulsion from a pasteur pipet. Cell counts were performed, and the suspension was adjusted to a concentration of approximately 10^7 cells/ml.

Mitomycin-C treatment. Mitomycin C was used in a modification of the method of Bach and Voynow (10) to prevent tumor cells from incorporating ¹⁴C-thymidine in the MLTR. Crystalline mitomycin C (Sigma) was dissolved in MEM at a concentration of 1 mg/ml. Mitomycin C was added to tumor cell suspensions at a level of 50 μ g/ 10^7 cells (0.05 ml mitomycin C/ml cell suspension) and the mixture was incubated for 30 min at 37°. The cells were then washed three times, by centrifugation and resuspension, with 10-ml volumes of MEM. After the final wash, cells were resuspended in RPMI culture medium (described below) and counted.

Spleen cells. Spleen cells were obtained from female BALB/C mice, aged 6-8 weeks, which had never been treated with tumor cells. A suspension of spleen cells

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was prepared and washed three times in MEM. Cells were then resuspended in RPMI culture medium, and nucleated and viable cell counts were obtained.

Cell cultures. The cell culture technique essentially followed that of Adler *et al.* (11). RPMI-1640 culture medium (Grand Island Biological Co.), containing heat-inactivated human serum (10%) and supplemented with 200 U penicillin, 200 μ g streptomycin, and 0.1 ml L-glutamine per ml, was used.

Responding (spleen) cells were suspended at a concentration of 1×10^6 viable nucleated cells/ml, and stimulating (tumor) cells were suspended at a concentration of 2×10^6 cells/ml. Cultures were prepared in 16- \times 125-mm glass tubes with polypropylene caps (Kimble). MLTR cultures contained 1 ml spleen cells and 1 ml mitomycin C-treated tumor cells. The ratio of stimulating:responding cells in mixed cultures was 2:1, determined by preliminary experiments to give maximum stimulation. Control cultures consisted of spleen cells (1 ml) cultured alone, and mitomycin C-treated tumor cells (1 ml) cultured alone. The expected activity of mixed cultures, without stimulation due to tumor-specific antigens, was considered to be the sum of the activity of the cells cultured separately.

MLTR and control cultures were incubated at 37° under an atmosphere of humidified 5% CO₂ in air. Cultures were harvested in triplicate on the 5th, 6th, and 7th day of incubation.

Labeling and harvest procedure. The activity of cultures in terms of DNA synthesis was determined by measuring the incorporation of ¹⁴C-thymidine into the acid-insoluble cell fraction.

¹⁴C-Methyl thymidine (Schwarz; sp act 33 mCi/mmole) was diluted to a concentration of 1 μ Ci/ml in RPMI culture medium. Six hours before termination of cultures, 0.1 μ Ci (0.1 ml) of the label was added to each tube. After the final 6 hr of incubation, culture tubes were centrifuged for 15 min at 1500 rpm and the supernate was decanted. The cell pellet was precipitated with 5 ml cold (5°), 10% trichloro-

acetic acid (TCA), and the acid-insoluble cell fraction was separated by vacuum filtration.

Filtration was done with a Millipore vacuum filtration apparatus. Cell precipitates were transferred to 2.4-cm glass fiber paper circles (Whatman GF/C), held within the filtration apparatus, and washed four times with 5-ml aliquots of cold, 5% TCA. The precipitate was finally washed with 5 ml absolute alcohol. A vacuum was retained for several minutes to remove excess moisture, then the filter was transferred to a petri dish and dried in a 37° oven for 1-2 hr.

After drying, filters were transferred to scintillation vials, and 10 ml scintillation fluid, prepared by dissolving 4 g of Omnifluor (New England Nuclear) in 1 liter of toluene, was added to each vial. Counts were performed with a Packard Model 3320 Tri-Carb Liquid Scintillation Spectrometer. Each sample was counted for a minimum of 10 min, and results were recorded as counts per minute (cpm), after subtraction of background.

Results and Discussion. In each experiment, MLTR and control cultures were harvested in triplicate on three consecutive days (5th, 6th, and 7th day of incubation). Maximal incorporation of ¹⁴C-thymidine in MLTR cultures did not always occur on the same day; the results shown in Table I are from the day of peak stimulation in each experiment.

Mitomycin C-treated tumor cells were stimulatory to syngeneic spleen cells from mice with no prior exposure to the tumor. In 5 experiments, stimulation indices (cpm MLTR/(cpm spleen + cpm tumor)) varied from 2.35 to 3.10, with a mean of 2.75. Such a level of stimulation is considered marginal; however, these results are consistent with those of Kanner *et al.* (6) on the stimulation of murine lymphocytes by syngeneic tumor cells, and are interpreted as reflecting the antigenic difference between normal and neoplastic cells in an MC-induced mammary carcinoma system.

In mixed lymphocyte tests, the degree of stimulation observed in culture is related to

TABLE I. Incorporation of ^{14}C -thymidine in MLTR (Mixed Spleen and Tumor Cell) Cultures and in Control Cultures. Results Are Given for the Day of Maximum DNA Synthetic Activity in Mixed Cell Cultures.

Expt No.	Day of maximum activity ^a	CPM observed ^b	CPM expected ^c	S.I. ^d
1	5	547	212	2.58
2	6	1978	642	3.08
3	6	482	205	2.35
4	7	866	338	2.56
5	7	2253	727	3.10

^a Cultures were harvested on the 5th, 6th, and 7th day of incubation.

^b CPM observed = mean counts per minute of triplicate MLTR cultures.

^c CPM expected = mean cpm of triplicate spleen control cultures + mean cpm of triplicate tumor control cultures.

^d Stimulation index = cpm observed/cpm expected.

the degree of antigenic disparity between the two cell populations (3-5). It may therefore be concluded that the tumor under test was of relatively low antigenicity. The antigenicity of MC-induced tumors has been shown to diminish on passage *in vivo* (12), and tumors used in these experiments were from the 60th through the 65th animal passages. This may account, in part, for the relatively low degree of stimulation observed in MLTR cultures.

The MLTR has been used principally to demonstrate antigenic differences between normal and neoplastic cells, but several authors have suggested that the technique may have potential application in evaluating host reactivity to tumor. In a study reported by Savel (13), peripheral lymphocytes from 7 of 44 cancer patients were stimulated by saline extracts of autologous tumor. All 7 patients had tumors with relatively favorable prognoses; one had a malignant melanoma that was undergoing spontaneous regression. The author suggested that the determination of lymphocyte hypersensitivity to autologous tumor may have prognostic significance. Powles *et al.* (9) reported that remission lymphocytes of patients with acute leukemia were stimulated by autologous leukemia cells in culture. Autoimmunization with irradiated leukemia cells resulted in increased MLTR reactivity in nine individuals studied, and the authors offered the

test as a basis for evaluating immunotherapeutic regimens.

Summary. The antigenicity of an MC-induced murine mammary carcinoma was demonstrated by means of the MLTR. Spleen cells from BALB/C mice with no prior exposure to the tumor were stimulated to incorporate increased amounts of ^{14}C -thymidine, when cultured with syngeneic tumor cells.

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