In Vitro Lymphocyte Responses to Malignant, Benign Neoplastic and Normal Tissue Extracts[1,2] (36481)

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Tumor specific transplantation antigens (TSTA) have been recognized in spontaneous (1), chemical-induced (2–4), and virus-induced (5–7) tumors in animal systems. There is also evidence of the existence of tumor specific antigens (TSA) in human tumors (8, 9) as well as evidence of spontaneous or induced tumor specific immune responses in acute leukemia (10), malignant melanoma (11–14), osteogenic sarcoma (15, 16), gastrointestinal neoplasms (9), neuroblastoma (17, 18), breast and lung cancer (19), cancer of the cervix (8), and cancer of the ovary (8).

The TSTA, other tumor antigens, and the immunity to them have been identified by a variety of methods, including immunofluorescence (14, 20), complement fixation (21), immunodiffusion (8), colony inhibition (18, 19), delayed hypersensitivity (11, 22) and lymphocyte blastogenesis (23–27).

To further identify tumor antigens and tumor immunity in man, we have investigated the use of the lymphocyte blastogenesis system and measured the responses of patients' and normal subjects' lymphocytes to extracts of various autochthonous and allogenic tumors and normal tissues.

Materials and Methods. Study subjects. Sixty patients with a variety of malignant tumors were studied. One was under 20 years of age. Fifteen were between 20 and 39, 33 were between 40 and 59 and 11 were over 60. Fifty-seven patients had metastatic disease. Thirty patients had received chemotherapy or radiotherapy previously but none were on therapy when studied. Fifty-one patients (85%) were tested within 1 to 2 years of diagnosis.

Preparation of extracts. Extracts were prepared from 39 malignant melanomas, 8 sarcomas, 7 squamous cell carcinomas, 3 carcinomas of the ovary, one Hodgkin's disease lymph node, one carcinoma of the breast and one Wilm's tumor. Extracts were also obtained from the benign cutaneous moles of 5 of the patients with malignant melanoma and from a benign neurofibroma of the patient with neurofibrosarcoma. Normal tissue extracts were obtained from 22 of these patients as controls for the tumor extracts. They included 20 skin samples, one tongue and one muscle biopsy.

The tissue specimens were minced finely under sterile conditions and homogenized with 5–10 cc of Eagle's Spinner Modified Minimum Essential Medium (MEM)[3] for 10 min in an ice bath in a Sorvall mixer at medium speed. After removal of debris by centrifugation at 500*g* for 15 min, the clarified supernatant was either used fresh or dialyzed against isotonic phosphate-buffered saline at pH 7.3 and stored in 1–2 ml aliquots at −70°. They were frozen and thawed only once before use. The protein concentration of these extracts was determined by the Lowry method.

[1] This research was supported by American Cancer Society Grant No. ACS CI 22 and Grants CA 05831 and CA 11520 from the National Cancer Institute, U.S. Public Health Service, Bethesda, MD.

[2] The authors thank Mrs. Elizabeth Sweeney, Miss Ginger Johnson and Miss Sarah Dyre for their capable technical assistance and Mrs. Judith Owens for the preparation of the manuscript.

[3] Grand Island Biological Company, Grand Island, NY.

TABLE I. Autochthonous Lymphocyte Response to Tissue Extracts.

Type of preparation	Patients No. pos.[a]/No. tested	% Positive patients	Tests No. pos./Total tests	% Positive tests
Malignant Tumor Extracts	32/57	56	35/82	43
Benign Tumor Extracts	3/5	60	4/7	57
Normal Tissue Extracts	11/22	50	11/25	44

[a] Positive test: stimulated cpm at least 3X control cpm.

Preparation and stimulation of lymphocytes. Peripheral venous blood was collected in plastic syringes without anticoagulant, transferred to sterile Erlenmeyer flasks, defibrinated by swirling with glass beads and the red cells were sedimented by adding 1 ml of 4% dextran[4] (MW 250,000) to each 10 ml of blood. The resultant leukocyte-rich serum was collected and its total cell and differential counts were determined. The lymphocytes were cultured at 1×10^6 cells in 2 ml of MEM and 1 ml of serum and were stimulated with phytohemagglutinin (PHA)[5], Streptolysin-O (SLO)[5], and extracts in a dose range of 1 to over 4,000 μg per tube. Control cultures containing only lymphocytes and no stimulants and cultures containing extracts were incubated for 6 days in 5% CO_2 in air at 37°. PHA-stimulated cultures were incubated 3–5 days and SLO-stimulated cultures were incubated 5 days before harvesting.

After the appropriate days in culture, 2 μCi of tritiated thymidine[6] (sp act 1.9 Ci/m*M*) were added to each tube and the cultures were incubated for an additional 3 hr. The cells were then washed twice with ice-cold isotonic saline, the acid-insoluble fraction was precipitated twice with 5% trichloroacetic acid, and the specimens were processed for liquid scintillation counting in a standard manner (28). Blastogenic lymphocyte responses were measured by ^{3}H-thymidine incorporation and recorded as counts per minute per 10^6 lymphocytes (cpm) and as an index of response. The index of response was the cpm of the stimulated culture divided by the cpm of the unstimulated control culture. Stimulation was highly significant when the counts per minute of the stimulated culture were three times that of the unstimulated culture ($p < .01$), and this was taken as the minimal positive response.

Selected samples of extracts and lymphocyte cultures were examined for bacterial contamination by Gram stain and culture; all were sterile.

Results. The patients' responses to their own extracts are shown in Table I. Fifty-six percent (32/57) of patients with metastatic tumors showed significant responses to the extracts of their malignant tumor while 60% (3/5) also responded to extracts of their benign tumors. While not all patients were tested with extracts of their normal tissues, 50% (11/22) who were tested with these extracts responded significantly. The dose–response curves to the benign and malignant tissue extracts were similar and similar doses induced similar degrees of response. Also the mean protein concentrations of the normal and malignant tissue extracts were comparable (6.8 and 7.6 mg/ml).

Figure 1 relates the protein concentration of the autochthonous extracts used in the lymphocyte cultures to the degree of stimulation. The amount of extract used to stimulate individual cultures varied from 1 to over 4,000 μg per culture. Because of the variable protein concentration of the extracts, not every set of cultures was stimulated with the

[4] Pharmachem Corporation, Bethlehem, PA.
[5] Difco Laboratories, Detroit, MI.
[6] Schwartz BioResearch, Orangeburg, NY.

TABLE II. Response of Patients' Lymphocytes to Stimulation with Autochthonous Tissues and Mitogens.

Stimulator	Lymphocyte Blastogenesis		
	Mean	SD	Range
PHA[b]	47.5[a]	36.7	0–197.7
SLO[c]	15.5	19.0	0– 83.9
Normal Tissue Extract	4.2	10.9	0– 52.9
Benign Tumor Extract	6.8	9.4	0– 22.8
Malignant Tumor Extract	4.1	10.4	0– 60.9

[a] Counts/min/10^6 lymphocytes $\times$ 10^3.
[b] Phytohemagglutinin.
[c] Streptolysin-0.

full dose range of antigen. A definite dose response effect was noted in each responding subject; the degree of blastogenesis increased as the concentration of protein was increased.

A comparison of the stimulation induced by autochthonous tissue extracts with that induced among these patients' lymphocytes by standard mitogens or antigen such as PHA or SLO is shown in Table II. PHA and SLO induced the greatest degree of response. The extracts of malignant tissue induced a degree of response similar to that of the extracts of benign tumor and normal tissue.

The responses of the normal subjects and the tumor bearing patients to allogenic malignant, benign and normal tissue extracts are shown in Table III. Thirty-seven percent (22/59) of normal subjects and 62% (8/13) of patients with similar tumors responded to the extracts of malignant tissue. Twenty-three percent (5/22) of normal subjects responded to allogenic extracts of skin while 60% (3/5) responded to extracts of benign tumors. It is of interest that not all of the allogenic subjects responded to either the benign or malignant tissue preparations.

The responses of lymphocytes from the normal subjects and patients with similar tumors to the allogenic extracts were compared with their responses to PHA and SLO. Again, PHA and SLO induced significantly greater responses than the tissue extracts (60,900 and 25,100 cpm, respectively). Normal tissue induced slightly less but the difference was

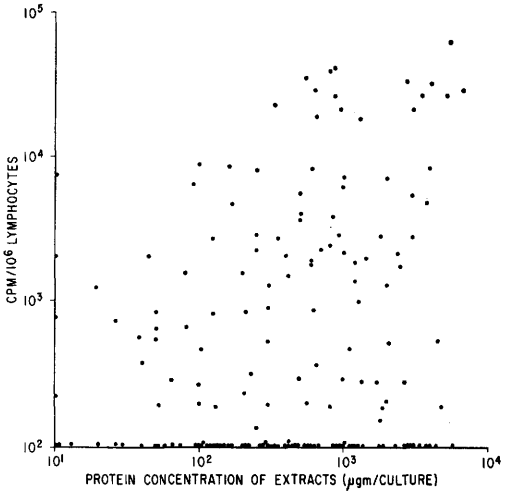

FIG. 1. Dose–response data for stimulation of patients' lymphocytes with extracts of their own tumors. Includes all data including values on patients who did not respond. Lymphocytes (10^6 cells) were cultured with extracts for 7 days before harvesting.

TABLE III. Allogenic Lymphocyte Response to Tissue Extracts.

Responder	Type preparation	Subjects No. pos.[a]/No. tested	% Positive subjects	Tests No. pos./Total tests	% Positive tests
Normal	Malignant tumor extracts	22/59	37	25/82	31
Patient with same tumor	Malignant tumor extracts	8/13	62	11/28	39
Normal	Benign tumor tumor extracts	3/5	60	3/6	50
Normal	Normal tissue extracts	5/22	23	8/25	32

[a] Positive test: stimulated cpm at least 3X control cpm.

not significant (1,600 cpm).

The ability of lymphocytes to respond to autochthonous extracts of malignant tissue seemed to be unrelated to age, sex, duration of disease, previous therapy or to extent of disease. Fifty-four percent (7/13) of patients with regional metastases and 57% (25/44) of patients with distant metastases responded.

Discussion. These studies indicated that malignant, benign, and (surprisingly) normal tissues contain mitogens which stimulated significant blastogenesis among autochthonous and allogenic lymphocytes. The ability of malignant tumor extracts to induce blastogenesis among autochthonous lymphocytes initially suggested that these extracts contained tumor specific or distinctive antigens and that the patients had specific tumor immunity even in the presence of advanced metastatic disease. Furthermore, the finding that 62% (8/13) of the patients with malignant melanoma responded to allogenic extracts of melanoma tumors might be interpreted as showing that these tumors contain a common antigen. The ability of extracts of benign moles to stimulate autochthonous lymphocytes of patients with malignant melanoma and of an extract of a neurofibroma to stimulate autochthonous lymphocytes of a patient with neurofibrosarcoma also suggested that neoantigens, perhaps tumor antigens, might be present in benign or premalignant lesions. This has been observed by Lappé (29) in mouse papillomas and skin cancers.

However, stimulation of lymphocyte blastogenesis by extracts of autochthonous skin casts severe doubt on the specificity of the response to extracts of neoplastic tissue. This observation suggested several other alternate explanations. First, nonspecific mitogens might be released during the extraction process. These would be nonantigenic and might have a mechanism of action similar to the plant mitogens. Second, tumor antigens may also be present in the skin. This is possible on the basis of an oncogenic-virus genome expressed in nontumor cells (30). Third, usually concealed autoantigens might be revealed or modified by the extraction process. This seemed to be the most likely possibility. Many cell or tissue-specific antigens are normally concealed and would be released during the cell disrupting process. Failure of response of some normals to extracts of benign and malignant tissues suggested masking or deletion of HLA antigens or damage to them by the extraction process or the simultaneous release of an inhibitor.

The findings described in Fig. 1 indicate that there are antigens in solid tumors that some patients' lymphocytes can recognize. With more sophisticated techniques tumor and nontumor antigens might be separable from these extracts permitting the study of specificity. The fact that some patients did not respond to their own tumor extract also suggests blocking serum factors or immunological deficiency.

Several authors have found evidence for the existence of cell-mediated immunity in animals and man. Hughes and Lytton (31) and Stewart (22) have shown that about 25% of patients with a variety of malignant diseases have delayed hypersensitivity reactions to autochthonous tumor extracts while patients with metastatic disease gave negative reactions.

Using the colony inhibition technique, Hellstrom *et al.* (17, 18) have found that the lymphocytes from patients with neuroblastoma but not from unrelated normal subjects could inhibit colony formation of not only the patient's own tumor cells but also the tumor cells of other patients with similar disease. Serum from patients with active disease but not from patients free of disease blocked the inhibition.

Oppenheim *et al.* (32) found that cells from the peritoneal exudate of guinea pigs immunized to a specific tumor inhibited tritiated thymidine uptake by tumor target cells to a significantly greater degree than the cells of unimmunized animals.

Stjernsward *et al.* (23, 27, 33) studied the mixed lymphocyte–tumor interaction and found that a significant number of patients responded to autochthonous tumor cell suspensions.

A number of investigators have used lym-

phocyte stimulation by tumor extracts to study tumor immunity in man. Savel (25) found that the lymphocytes of 7/56 patients responded significantly to crude saline extracts of autochthonous tumors. In no instance did normal tissue induce blastogenesis. The facts that Savel froze his biopsy material immediately and made the extracts later and that he did not use over 0.1 cc of extract as a stimulant may explain the difference between his results and ours. Other findings also contradict those of Savel.

Hsu and Cooperband (26) were unable to demonstrate a lymphocyte response to tumor extracts in 10 patients, even though their lymphocytes responded to PHA. They also froze their biopsies immediately and prepared the extracts 2-21 days following receipt of the tissue. Also, they used a maximum extract dose of only 300 μg for lymphocyte stimulation compared to our use of up to 4000 μg.

Jehn *et al.* (24), using lymphocyte blastogenesis, found evidence that suggests that patients with malignant melanoma can respond to their own tumor extracts and also that malignant melanomas share a common antigen. The fact that our results do not suggest a similar hypothesis might be explained in part by patient selection. First, the mean survival of patients in their study was 4.5 years, while the mean survival of our patients was 6–18 months. This would indicate that either the tumors of their patients were less aggressive or that the host defenses of their patients were more active. We found that 4 of 4 patients tested more than 5 years after diagnosis responded significantly to their own malignant tumor extracts. Also, Jehn *et al.* did not use extracts of normal tissues from the patients to stimulate either autochthonous or allogenic lymphocytes.

The results of the current investigation strongly suggest that simple tissue extracts will not be useful in the investigation of cell-mediated tumor immunity in man. Although they certainly do stimulate lymphocyte blastogenesis, these reactions cannot be distinguished from nonspecific responses. The reports by Stjernsward (23, 27, 33) and recent findings in our own laboratory (34) suggest that the lymphocyte blastogenesis response can reveal specific tumor immunity if intact tumor cell suspensions are used for lymphocyte stimulation.

Summary. To identify tumor antigens and tumor immunity in man, we have investigated the use of the blastogenic response of lymphocytes to tissue extracts. Extracts of malignant, benign, and normal tissues of 60 patients with cancer were used to stimulate their own lymphocytes, the lymphocytes of other patients with the same tumor and those of normal subjects. Autochthonous extracts of the neoplastic tissue stimulated blastogenesis in 56% of patients and a linear dose-response curve was observed. This suggested the presence of TSA and specific tumor immunity, even in patients with advanced metastatic disease. However, stimulation of autochthonous lymphocytes by extracts of skin in 50% of patients cast doubt on the tumor specificity of the response to extracts. The release of nonspecific mitogens, or of usually concealed autoantigens, or the presence of tumor antigens in the skin of patients with malignant disease were hypothesized as the possible cause. Because of this lack of specificity it seemed that tumor extracts, used alone, will have limited value in the investigation of tumor immunity in man using the blastogenesis test.

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Received Oct. 11, 1971. P.S.E.B.M., 1972, Vol. 140.