tae. In liver mitochondria from pallid mice, no distinct ultrastructural changes were seen (Fig. 1E).

The biochemical data presented here support earlier work reporting lowered oxidation in manganese-deficient liver mitochondria (*7*). This and the observation that the ultrastructure of mitochondria in manganese-deficient animals is altered show that manganese is required for normal mitochondrial function and structure in vivo. However, although the mutant gene *pallid* interacts with manganese in the development of otoliths, it does not appear to affect mitochondrial function or structure. This observation is consistent with earlier evidence that *pallid* genes do not act by producing a simple manganese deficiency; instead, metabolic differences between the two conditions were found (*17*).

The morphological findings reported here are consistent with the biochemical observations. In a study on the fine structure of lung tissue, Rosenbaum *et al.* (*18*) found mitochondrial transformations similar to those reported here after the great alveolar cells had been exposed to adaptive quantities of oxygen. These authors suggested that the mitochondrial changes were adaptive rather than degenerative, since they did not occur after toxic amounts of oxygen, and that they were possibly brought about through inhibition of oxidative enzymes (*19*). In manganese deficiency perhaps the activity of such enzymes is affected, causing reduced oxidation and structural change.

LUCILLE S. HURLEY
LINDA L. THERIAULT
IVOR E. DREOSTI*
Department of Nutrition,
University of California, Davis 95616

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* Present address: Department of Biochemistry, University of Natal, Pietermaritsburg, Natal South Africa.

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Microcytotoxicity Test: Detection in Sarcoma Patients of Antibody Cytotoxic to Human Sarcoma Cells

Abstract. *A microcytotoxicity test has been used to detect a factor cytotoxic for human sarcoma cells; the factor was found in serums from 70 percent of sarcoma patients, 58 percent of their family members, and 8 percent of serums from normal blood donors. This cytotoxin is an antibody against a common cell surface sarcoma antigen since it is specific for sarcoma cells, is complement dependent, and is extractable with the globulin fraction of serum.*

Tumors induced in animals by a given virus express a common antigen, regardless of their histologic type or anatomic location (*1*). This observation has led to the use of a variety of serological techniques in search of antigenic identity in human tumors, a finding which might implicate a viral agent. A common sarcoma specific antigen has been demonstrated in human sarcomas of different histologic types by immunofluorescence and complement fixation techniques (*2*). It has remained open to question whether this antigen could induce transplantation resistance or was only an intracellular antigen capable of inducing antibody formation, but failing to render the cell vulnerable to immune attack. Transplantation studies, which have resolved similar questions in animal studies, are not appropriate to human investigations. Immune cytotoxicity tests would provide the most direct method in vitro for investigating both surface antigenic expression and immune vulnerability, but classical methods of testing cytotoxicity have not been found applicable to the human sarcoma cells which were resistant to cytotoxic antibody. We now describe a microcytotoxicity test demonstrating that sarcoma antigens are located on the cell surface and render the cell susceptible to immune cytolysis.

Human sarcoma cells were grown at 37°C in 5 percent CO_2 in stationary cultures with RPMI 1640 media containing 20 percent fetal bovine serum, kanamycin, aureomycin, and fungizone (Grand Island Biological). When the cells were more than 50 percent confluent, they were dispersed with 0.25 percent trypsin in Gey's balanced salt solution. After being counted in trypan blue, the cells were diluted to give 10,000 viable cells per milliliter of tissue culture media. These cells were planted in Microtest tissue culture plates (Falcon Plastics) with 0.01 ml per well, and incubated for 3 to 18 hours. Media were then rinsed from the plates and was replaced with 0.01 ml (per well) of the test serums that had previously been inactivated at 56°C in a water bath for 20 minutes. Cells and serum were incubated at 37°C for 1 hour. The serum was removed and 0.01 ml of complement was added to each well. Pooled rabbit serum diluted 1:1 with pooled human umbilical cord serum was used as a source of complement. After incubation with complement for 2 hours, the plates were flooded with 6 ml of RPMI 1640 media and incubated overnight. In the morning each well was examined by phase microscopy, and the remaining viable cells were counted. Dead cells exhibited a retraction of the cell processes and "rounding up"—with loss of the nuclear halo and nucleolus, increased granularity of the cytoplasm, and high refractility (Fig. 1, right). No difficulty was experienced in classifying cells as viable or dead. Six replicate wells were used for each test serum, and each plate contained a positive control and 12 replicates of the negative control. Pooled umbilical cord serum was chosen as a negative control because of its low antibody content and consistent behavior from pool to pool.

The test was evaluated by averaging the six replicates of each test serum and comparing them with the average of the control serum to derive a cytotoxic index (C.I.).

$$\text{C.I.} = (C - T)/C$$

where C is the number of viable control cells and T is the number of viable test cells. Thus a C.I. of 0 represents no cytotoxicity, and 1.00 represents 100 percent cytotoxicity. All serums with a C.I. over 0.20 differed significantly ($P < .05$) from the 12 replicate controls in the same Microtest plate and were considered "positive" or cytotoxic.

Human serum was obtained from 50 sarcoma patients, 113 members of their immediate families or close associates, and 50 normal blood bank donors and stored until used at −190°C. To study the antibody nature of the cytotoxic factor, gamma globulin was extracted from selected cytotoxic and normal serums by the addition of 280 mg of ammonium sulfate to 1 ml of serum. The mixture was stirred for 1 hour at 4°C and then centrifuged at 10,000 rev/min for 10 minutes (Sorval RC2-B). The precipitated globulin was dissolved in 1 ml of normal saline and dialyzed against saline for 36 hours at 4°C. Immunoplates (Hyland) were used to quantitatively measure the immunoglobulins in these extracts. The normal and sarcoma gamma globulin extracts contained similar quantities of immunoglobulins G and M (IgG and IgM).

Two established human sarcoma cell lines were used. SA-1, derived from a liposarcoma, and SA-2, from an osteogenic sarcoma, have been described (*3*); SA-13 resulted from the ninth passage of a cell line also derived from an osteogenic sarcoma. A skin fibroblast cell line from this same patient was used as a control for testing antigenic specificity.

Absorptions were performed by incubating equal volumes of serum and culture media containing the absorbing cells (2 hours at 37°C). The cells were then removed by centrifugation at 1600 rev/min for 10 minutes (International 2-V), and the supernatant was assayed for residual cytotoxicity in the microcytotoxicity test.

The results of the assay of 213 serums for cytotoxicity are summarized in Table 1. Seventy percent of the serums from patients with a variety of different skeletal and soft tissue sarcomas were significantly cytotoxic against this sarcoma target cell line (SA-2), as contrasted with only an 8 percent incidence of cytotoxicity in serums from a normal blood bank population. This cross-reaction with a single cell line suggests a common antigen. If a common etiologic agent, such as a virus, were postulated to account for this common antigen, it would not be surprising to find evidence of exposure to such an agent among the relatives and close associates of these patients. Indeed, previous studies with immunofluorescence (*3*) and complement fixation tests (*4*) have indicated that these related individuals possess a much higher incidence of antibody to sarcoma antigen than is found in the general population. Consequently, 113 serums from family members and close associates of sarcoma patients were screened for cytotoxicity against sarcoma cells. Sixty-six of these showed significant cytotoxicity, suggesting prior exposure to the surface antigen of the sarcoma cells.

Table 1. Incidence of cytotoxic activity against SA-2 osteosarcoma cells. Numbers in parentheses show percentage.

Serum	Tested (No.)	Positive* (No.)
Sarcoma patients	50	35 (70) †
Family and associates	113	66 (58)†
Normal controls	50	4 (8)

* Cytotoxic index 0.20 or greater, varied from control with $P < .05$. † Differ significantly from controls by chi square, $P < .01$.

The disparity between the incidence of cytotoxins in sarcoma patients and their families compared with the incidence in serums from normal blood donors suggested that the antigen against which the cytotoxins are directed is a tumor-associated antigen. To further explore the tumor specificity of this antigen, 12 serums which were cytotoxic to SA-13 cells were assayed for cytotoxicity to a skin fibroblast line derived from a biopsy of normal skin from the same patient (Table 2). Autologous serum of the patient (D.A.) from whom the SA-13 sarcoma culture was derived was significantly cytotoxic to his own sarcoma cells but not to normal fibroblasts. Eight of the 12 serums showed no cytotoxicity to the skin fibroblast line, and of the four which did, two were more cytotoxic to the sarcoma cell line. It appears, therefore, that the cytotoxic activity in eight of these serums is directed toward a tumor-associated antigen. However, some of the cytotoxicity in the other four serums may also be due to isoantibodies against normal histocompatibility antigens in these cell lines.

The specificity demonstrated in our studies suggested an antibody reaction directed against an antigen expressed on the surface of the sarcoma target cells. To investigate the antibody nature of the cytotoxin, 17 serums previously found to be cytotoxic with fresh complement were tested with complement inactivated by heat. The activity

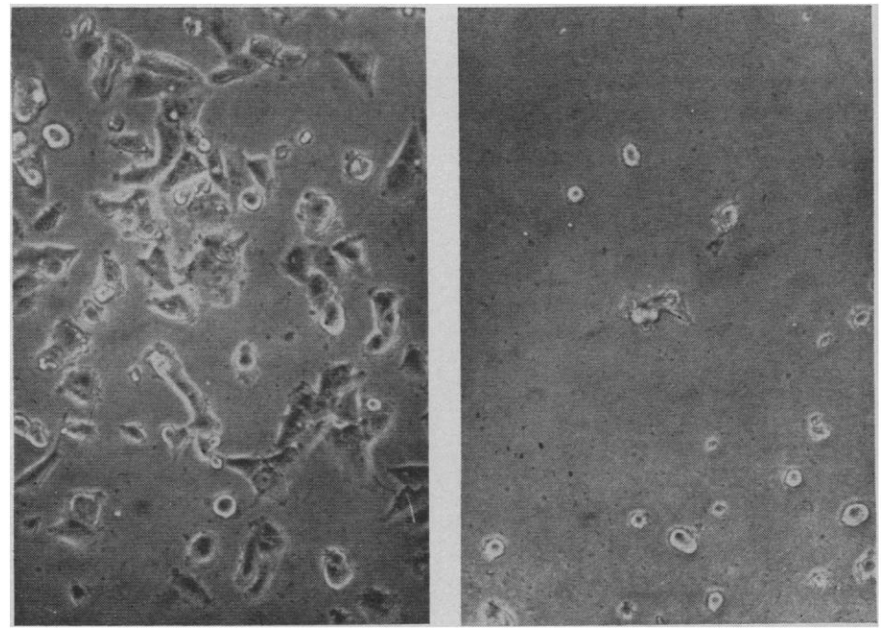

Fig. 1. Phase photomicrographs of two wells in one plate at conclusion of the microcytotoxicity test with SA-2 osteosarcoma cells. (Left) Well containing normal serum. (Right) Well with serum from a sarcoma patient with high cyotoxic index.

Table 2. Specificity of cytotoxicity for sarcoma cells in contrast to that for skin fibroblasts from the same patient. Parentheses indicate that the value is statistically significant.

Serum	Cytotoxic index	
	SA-13 (D.A. sarcoma line)	D.A. skin fibroblast line
D.A.*	.56	(.18)
T.A.	.86	(.04)
J.W.	.79	(.02)
B.S.	.34	(.18)
R.M.	.32	(0)
K.C.	.30	(.09)
N.C.	.21	(0)
J.T.	.94	.44
T.S.	.83	.26
C.H.	.44	.50
J.B.	.43	.37

* Autologous serum of patient from whom SA-13 sarcoma culture was obtained.

was found to be complement dependent in all serums except one serum with a high C.I. against SA-2 cells, which was found to be cytotoxic to a dilution of 1:32. When diluted 1 to 4, the complement dependency of this serum also became demonstrable.

Serum was fractionated to further establish the antibody character of the cytotoxic factor. The gamma globulin so obtained demonstrated complement dependent cytotoxicity in the microcytotoxicity test. Gamma globulin similarly extracted from three normal serums possessed no cytotoxic activity, indicating that the method of extraction introduced no cytotoxic substance.

Absorption studies were undertaken as another test of antibody specificity. One serum from a patient (D.A.) with osteogenic sarcoma was selected and absorbed with increasing numbers of sarcoma cells. Absorption with either 10^5 SA-1 or SA-2 cells, living or dead, removed the cytotoxicity from 0.1 ml of serum, whereas absorption of the serum with equal quantities of KB, HeLa, or Chang liver cells failed to remove the cytotoxicity for SA-2 cells.

The microcytotoxicity test as described possesses several advantages over classical techniques of cytotoxicity testing. It allows the detection of cytotoxicity directed against resistant sarcoma lines which show no reaction when tested by dye exclusion methods. By using only 100 cells per well, the test is applicable to nonestablished cell lines, such as SA-13, which are available in very limited numbers. The ratio of undiluted serum to this small number of cells is important to the sensitivity of the method, as was demonstrated by Boyse, Old, and Stockert (*5*) in a mouse system. The sensitivity of the test was further increased by incubation overnight because counts of viable cells beginning soon after the complement was added to the plates showed progressive cytotoxicity over a period of 4 to 6 hours.

Finally, the complement source was important because rabbit complement alone was frequently toxic for human sarcoma cells, producing nonspecific cytotoxicity. Human serum alone often failed to demonstrate cytotoxicity, but the combination was found to provide both specificity and cytotoxic potential.

It seems reasonable to conclude from the specificity of the cytotoxic effect that the serum factor responsible is an antibody. This conclusion is supported by the demonstration that this activity is complement dependent and that it resides in the gamma globulin fraction of the serum.

The high incidence of antibody against a single osteosarcoma target cell line found in the serum of patients with many diverse types of sarcoma indicates that the antigen against which this cytotoxic antibody is directed is shared by most sarcomas. This is consistent with our earlier studies in which other serologic methods were used. The existence of this common antigen suggests a common viral etiology for sarcomas by analogy with animal tumor systems. The existence of a sarcoma-inducing viral agent would help to explain the findings of a 58 percent incidence of antibody cytotoxic to sarcoma antigen among the relatives and close associates of sarcoma patients.

The sarcoma antigen is a tumor-specific antigen because only 8 percent of the normal blood donors, as compared with 70 percent of sarcoma patients, have cytotoxic antibody directed against it, and because the cytotoxic antibody is absorbed by sarcoma cells, but not by HeLa, KB, or Chang liver cells. Further evidence that the cytotoxic antibody is directed against a tumor-specific antigen rather than a histocompatibility antigen is provided by the observation that serum of patient D.A. was cytotoxic to his own sarcoma cells and by the failure of the majority of the serums which were cytotoxic to a sarcoma line (SA-13) to exhibit cytotoxicity to a normal cell line derived from the same patient.

Two implications of our data seem especially significant. First, the demonstration of a common antigen, specific to sarcoma cells, by this technique corroborates similar findings by immunofluorescence and complement fixation (*2*). These data suggest induction by a single viral agent. Second, this antigen is located at the cell surface and renders the tumor cell vulnerable to immune attack. This finding adds strength to the case being made for the importance of host immune defenses in human sarcomas.

WILLIAM C. WOOD
DONALD L. MORTON
Tumor Immunology Section, Surgery Branch, National Cancer Institute, Bethesda, Maryland 20014

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Marihuana: Studies on the Disposition and Metabolism of Delta-9-Tetrahydrocannabinol in Man

Abstract. *Δ^9-Tetrahydrocannabinol (the major active component of marihuana) administered intravenously to normal human volunteers persists in plasma for more than 3 days ($t_{1/2}$ = 56 hours). Its metabolites appear in plasma within 10 minutes after administration and persist along with the precursor compound. Δ^9-Tetrahydrocannabinol is completely metabolized in man, and the radioactive metabolites are excreted in urine and feces for more than 8 days.*

Marihuana and hashish are psychoactive plant materials prepared from *Cannabis sativa*. The active component of *Cannabis* in animals (*1*) and in man (*2*) has been reported to be Δ^9-tetrahydrocannabinol (Δ^9THC). Until recently there has been little information regarding the metabolism and disposition of Δ^9THC because of the difficulty in synthesizing and assaying this com-