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Osteogenic Sarcoma

IMMUNOLOGIC PARAMETERS BEFORE AND DURING IMMUNOTHERAPY WITH TUMOR-SPECIFIC TRANSFER FACTOR

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ABSTRACT 18 patients with osteogenic sarcoma were followed by serial measurements in vitro of tumor-specific cell-mediated cytotoxicity and of "active" and total rosette-forming T-cells. 13 of these patients have had or are currently receiving injections of osteogenic sarcoma-specific dialyzable transfer factor derived from healthy donors. In three patients with very small lesions, cytotoxicity was high before amputation and decreased within 2 mo after removal of tumor. Cytotoxicity was low at time of diagnosis in all patients with large tumor masses. The cytotoxicity of the patients' lymphocytes increased after administration of tumor-specific transfer factor in all patients so treated. Patients receiving nonspecific transfer factor showed evidence of declining cell-mediated cytotoxicity.

Tumor-specific transfer factor may produce an increase in cell-mediated cytotoxicity to the tumor in patients with osteogenic sarcoma. This possibility is suggested by the pain and edema that occurred in the area of the tumor in patients who had metastatic disease when therapy was started and by lymphocytic infiltrates in the tumor, as well as by the increase in cell-mediated cytotoxicity and the increase in percentage of active rosette-forming cells from subnormal to normal. Serial measurements of cell-mediated cytotoxicity are helpful in monitoring the efficacy of transfer factor and other modes of therapy in these patients, and these measurements are the best available criteria for selection of patients of tumor-specific transfer factor.

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INTRODUCTION

Dialyzable transfer factor was first described in 1955 by Lawrence (1), who subsequently conducted a series of meticulous studies of its properties (2). In 1970 our group reported the therapeutic use of transfer factor in a patient with Wiskott-Aldrich syndrome, a genetically determined cellular immune deficiency (3). Since 1970 transfer factor has been used to treat a variety of diseases (4–7); we have used it in more than 200 patients.

The possible relationship of immunologic defenses to survival of patients with malignant disease has been the subject of many reports (8). It is currently believed that antibody-mediated responses to tumors may, in some instances, enhance growth of tumors and, in others, be toxic to malignant cells; cell-mediated immunity usually inhibits growth of tumors (9, 10). Transfer factor can enhance cell-mediated immunity, but has no effect on humoral immunity (3); therefore, it appeared useful to investigate its use in the treatment of malignant neoplasms unresponsive to other measures.

In patients with osteogenic sarcoma, surgical excision, chemotherapy, and radiation therapy have produced a survival rate of only about 20%. Because some household contacts of patients with this tumor have cell-mediated immunity against the tumor (11) and can serve as a source of tumor-specific transfer factor, this investigation of the immunologic status of 18 patients with osteogenic sarcoma was undertaken. In 13 of these patients, clinical results of treatment with tumor-specific transfer factor, obtained from carefully selected donors and used as an experimental adjunct to conventional

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TABLE I

*Clinical Status of Patients with Osteogenic Sarcoma**

Patient	Age	Sex	Phase at diagnosis	Circulating antitumor antibodies	Received transfer factor	Length of follow-up	Status
						mo	
1	16	F	I	IgG-kappa	Yes	15	Treatment started with patient in phase II; now healthy and clinically tumor-free
2	18	M	I	No	Yes	11	Treatment started with patient in phase II; now healthy and clinically tumor-free
3	17	F	I	No	Yes	13	Treatment started with patient in phase II; now healthy and clinically tumor-free
4‡	17	F	II	No	Yes	27	No prophylactic immunotherapy; developed metastatic lesions unresponsive to chemotherapy and radiation; lesions resected; transfer factor, begun in phase IV, produced lymphocytic infiltrates in lesions; doing well 4 mo after surgery
5	10	F	II	No	No	6§	Disease progressed through phase IV; did not respond to chemotherapy, surgery, or radiation
6‡	15	F	III	No	Yes	23§	Inoperable primary tumor in ilium; after 9 m of immunotherapy, donor lost cytotoxicity and metastatic lesion developed; remained stable with single metastatic lesion during 14 mo of additional treatment with transfer factor; died with massive pulmonary involvement 7 wk after voluntary discontinuation of immunotherapy
7‡	15	M	III	IgG-kappa	Yes	3§	Developed lesions in lung after irradiation of primary; transfer factor resulted in pneumonitis and pleural effusion; chemotherapy ineffective
8	16	F	III	No	Yes	24	Became pregnant during treatment with transfer factor; CI and T-E_a both dropped, but recovered after elective termination of pregnancy; remains healthy and clinically free of tumor
9	12	F	III	IgM-kappa	No	11	Receiving prophylactic chemotherapy
10‡	29	F	IV	No	Yes	28	Single metastatic lesion, present at diagnosis, remained stable during 20 mo of treatment with transfer factor; second lesion resected and irradiated; patient again receiving transfer factor; now clinically stable
11	20	M	IV	No	Yes	11	No prophylactic immunotherapy; solitary lung lesion, which developed 2 mo after amputation, resected; patient now receiving transfer factor; clinically stable and free of tumor
12‡	13	F	IV	No	Yes	17	Primary tumor irradiated; did not appear to respond to transfer factor; did well for 14 mo with chemotherapy, then developed massive pulmonary metastases; patient is now terminally ill

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TABLE I—(*Continued*)

Patient	Age	Sex	Phase at diagnosis	Circulating antitumor antibodies	Received transfer factor	Length of follow-up	Status
						mo	
13‡	21	M	IV	No	Yes	12	Lung lesions appeared to be unresponsive to chemotherapy and to immunotherapy, but increase in size of lesions may have resulted from lymphocytic infiltrates
14‡	16	F	IV	IgG-kappa	Yes	14	Appeared unresponsive to transfer factor; now receiving chemotherapy, but lesions are growing and increasing in number
15	17	M	III?	No	No	6§	Lost to follow-up after amputation; not treated
16	51	F	?	No	No	34 yr	Diagnosis and amputation occurred at age 17; malignant thyroid tumor removed at age 31; now clinically healthy with low CI to osteogenic sarcoma
17	17	M	II	IgG-kappa and IgM-kappa	Yes	7	Receiving prophylactic immunotherapy; presently clinically tumor-free
18	14	F	II	IgG-kappa	No	8	Receiving prophylactic chemotherapy; presently clinically tumor-free

* A detailed report on each patient is provided in the Appendix. Unless otherwise stated, each patient's primary tumor was removed by amputation.
‡ Clinically obvious primary or metastatic tumor was present when transfer factor was begun.
§ Deceased.

therapy, are correlated with the changing state of cell-mediated immunity.

METHODS

Clinical studies. Clinical histories of 14 patients and notes on 4 more are given in the Appendix. After informed consent was obtained, specimens were taken at the time of biopsy or amputation from patients with osteogenic sarcoma, and both benign (skin or muscle) and tumor tissues were placed in long-term culture.

Before immunotherapy was initiated, the tumor mass was reduced as much as possible by conventional therapy. The primary tumor was removed by amputation whenever feasible, and metastatic lesions were either removed by segmental resection or, if not resectable, treated by chemotherapy or localized radiotherapy of affected areas. Immunotherapy was then started, with transfer factor injected subcutaneously into the deltoid region until tumor-specific cell-mediated cytotoxicity (12) increased to 25 and active rosette-forming cells ($T\text{-}E_a$)[1] increased to more than 20%. Transfer factor was then administered at 2–4-wk intervals. All patients were monitored for cell-mediated cytotoxicity and $T\text{-}E_a$, and by monthly roentgenograms of the chest and semiannual tomograms.

[1] *Abbreviations used in this paper:* CI, cytotoxicity index; DTIC, 5-(3,3-dimethyl-1-triazeno) imidazole-4-carboxamide; PPD, purified protein derivative; T lymphocyte, thymus-derived lymphocyte; $T\text{-}E_a$, active rosette-forming cells; $T\text{-}E_t$, total rosette-forming cells.

Healthy potential donors of transfer factor (usually adult members of the patients' families) were screened for tumor-specific cytotoxicity; only those with cytotoxicity indices (CI) greater than 50 (range 50–90) on osteogenic sarcoma lines and less than 19 on control cell lines (carcinoma and fibroblast) were used as donors. Transfer factor was prepared by one of us (A. S. L.) by a modification of the method of Lawrence (13). Briefly, white blood cells of the donor were collected by leukapheresis. The cells were rinsed from the collection bag with normal saline and counted before being frozen and lyophilized. The lyophilized material was reconstituted in 10 ml of distilled water and dialyzed against two changes of 500 ml of pyrogen-free distilled water. The dialysis bag was then discarded and the water and dialysate were lyophilized. The lyophilized material was then reconstituted in water and divided into doses equivalent to 10^9 leukocytes per dose.

Tumor cell lines. Osteogenic sarcoma cell lines TE-85 (passages 17–19), and TE-418 (passages 10–12), and TE 415, the matching fibroblast line to TE 418, were provided by Dr. R. McAllister. Control cell lines included those from mammary carcinoma, hypernephroma, rhabdomyosarcoma, and matching fibroblast cell lines (12). Additional osteogenic sarcoma cell lines derived from tumors and skin of 11 of our patients were isolated and purified by the differential trypsinization technique of Owens (14). For the studies reported here, requiring a simple assay highly reproducible over a long period of time, TE 85 was chosen as a marker line for osteogenic sarcoma because donors of transfer factor and patients whose cells reacted to autologous

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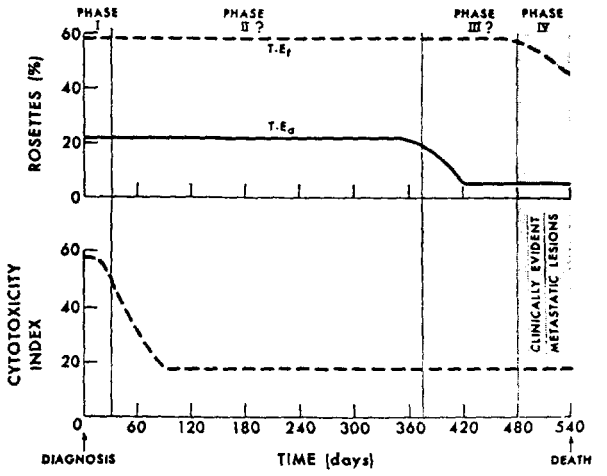

FIGURE 1 Postulated natural history of osteogenic sarcoma. Although the elapsed time varied, the pattern suggested has been observed by us in all patients reported here who received conventional therapy (amputation, resection, radiation, chemotherapy) and who died with metastatic lesions. Transfer factor appeared to alter the natural course of the disease.

and other osteogenic sarcoma cell lines also reacted to TE 85. The hypernephroma line A 498, passages 23–26, and breast carcinoma lines ALAB 496 and BT 20, together with a rhabdomyosarcoma line and hypernephroma line A49B, were used as specificity controls (12). All lines were stored in dimethyl sulfoxide, the cells were maintained in antibiotic-free medium, and limited passage levels were used to prevent genetic drift. Lines were harvested just at confluence for use in the assay.

Tumor-specific cell-mediated cytotoxicity of the patients' cells was assayed against cultured autologous tumor cell lines when available and against homologous osteogenic sarcoma cell lines, as well as against control cell lines as described above from mammary carcinoma, hypernephroma, rhabdomyosarcoma, and matching fibroblast lines.

Rosette-forming cells. Circulating thymus-derived (T)-lymphocytes were evaluated for their capacity to form rosettes with sheep erythrocytes. The numbers of T-cells capable of rosette formation were quantitated by the technique of Wybran, Levin, Spitler, and Fudenberg (15, 16). The lower limits of normal (mean±2 SD) are 16% for T-E_a and 55% for T-E_t (total rosette-forming cells).

Fluorescence microscopy. Antitumor antibodies were classified by immunoglobulin class and light-chain types by direct and indirect immunofluorescence. Indirect immunofluorescence studies were conducted with the serum obtained from each patient before amputation and during treatment; autologous tumor cells were used to characterize the immunoglobulin class and light-chain type of all antitumor antibodies (17).

Skin tests. Skin-test reactivity to six marker antigens, *Candida, Coccidioides,* mumps, purified protein derivative of tubercle bacillus (PPD), Varidase (streptokinase/streptodornase, Lederle Laboratories, Div. of American Cyanamid Co., Pearl River, N. J.), and trichophytin, was also followed.

RESULTS

13 patients with osteogenic sarcoma were treated [illegible] tumor-specific transfer factor derived from house[illegible] contacts who had specific cell-mediated cytotox[illegible] against the tumor. Their case histories are described [illegible] the Appendix and summarized in Table I.

Serial assays of cell-mediated cytotoxicity, T-E_a [illegible] T-E_t, and serial assessments of clinical states sug[illegible] that the natural history of osteogenic sarcoma can [illegible] described in four phases (Fig. 1).

During the first phase of the disease (patients 1[illegible] when first seen) primary tumors were small, and w[illegible] usually detected on roentgenograms taken for incide[illegible] trauma. Metastatic lesions were not present. Bef[illegible] amputation, these patients had high cytotoxicity ([illegible] greater than 30) and normal T-E_a and T-E_t.

Patients first seen during the second phase of the di[illegible]ease (patients 4 and 5) had large primary tumors, b[illegible] no metastatic lesions could be located. These patients ha[illegible]

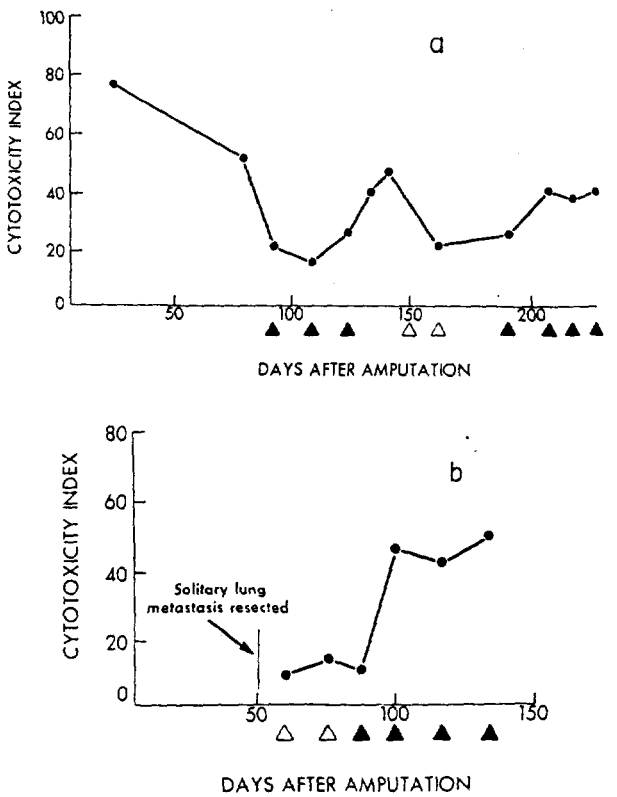

FIGURE 2 Tumor-specific cytotoxicity in patients with osteogenic sarcoma before and after administration of transfer factor. ▲, one dose of tumor-specific transfer factor; △, one dose of nonspecific transfer factor. *a*, patient 1; *b*, patient 11. These results are representative of those observed in every patient who received both specific and nonspecific transfer factor.

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normal T-E_a but low cell-mediated cytotoxicity (CI less than 30).

During the third phase (patients 6–9) primary lesions were large, but metastatic lesions were still not demonstrable. These patients uniformly had low T-E_a and low cell-mediated cytotoxicity, but T-E_t were within normal limits.

During the fourth phase (patients 9–14), patients had large primary tumors and metastatic lesions, low T-E_a, and low cell-mediated cytotoxicity. As metastatic lesions grew, T-E_t declined. Reactivity to skin tests also disappeared during the terminal phase. For convenience in presenting our results, patients are discussed in order of the apparent phase of the disease at diagnosis.

Tumor-specific cell-mediated cytotoxicity. The three patients who had small primary tumors and no detectable metastatic lesions when first seen (patients 1, 2, and 3) had high cytotoxicity against osteogenic sarcoma. When CI showed a significant decrease (usually within 3 mo after amputation), each patient began receiving biweekly injections of 1 U of tumor-specific transfer factor (1 U is the amount prepared from 10^9 leukocytes and contains 9–12 mg of RNA and 1 μM of protein). Cytotoxicity increased dramatically in all three patients (Fig. 2*a*). The same rise in cytotoxicity occurred after injection of tumor-specific transfer factor in patients who had low cytotoxicity when first seen (Fig. 2*b*). These patients were either clinically tumor-free after amputation of a large primary tumor (patients 8 and 17) or after removal of a primary lesion and surgical or radiologic treatment of small isolated metastasis (patients 10 and 11).

Administration of nonspecific transfer factor was followed by a drop in cell-mediated cytotoxicity to tumor in the patients to whom it was given (patients 1, 2, 10, 11). In patient 10, a sudden drop in cytotoxicity occurred 90 days after the administration of nonspecific transfer factor was begun; about 90 days thereafter, a new metastatic lesion was noted (Fig. 3). Although therapy with tumor-specific transfer factor was begun immediately, cytotoxicity did not increase until the lesion was surgically removed. The increased cytotoxicity to osteogenic sarcoma seen after resection was not accompanied by increased cytotoxicity to the control carcinoma cell lines.

Rosette-forming cells. The percentage of T-E_t is considered to represent the percentage of T lymphocytes among the whole population of lymphocytes found in peripheral blood. T-E_a appear to be a subpopulation of T lymphocytes closely linked to the capability of the host to mount an immune response to antigen. This population is seen to drop approximately 6 wk before clinical evidence of metastatic lesions in patients with cancer.

Patients who, when osteogenic sarcoma was diagnosed, had high cell-mediated cytotoxicity against their tumor (CI greater than 30) also had normal T-E_a. These patients usually had small primary tumors and no metastatic lesions (patients 1, 2, and 3; phase I). After amputation, tumor-specific cytotoxicity decreased, but T-E_a remained within normal limits. In patients in whom T-E_a was low when they were first seen, or in whom it dropped later in the disease, tumor-specific transfer factor increased T-E_a to normal levels. Patients not treated prophylactically after amputation of the primary tumor (patients 4 and 5) lost tumor-specific cell-mediated cytotoxicity before T-E_a began to decline. There was usually an abrupt drop in T-E_a approximately 6 wk before a metastatic event occurred.

Humoral immunity. Concentrations of immunoglobulins IgG, IgA, and IgM were within normal limits in all patients before and during therapy. Antitumor antibodies were detected before therapy in 5 of 18 patients (patients 1, 7, 14, 17, and 18) by indirect immunofluorescence; four had IgG-kappa circulating antibodies; the other had plasma cells and lymphoid cells containing both IgG and IgM within the tumor mass, but no circulating antibodies. All of the immunoglobulin-containing cells stained with anti-kappa antiserum, but not anti-lambda. Serial samples taken during immunotherapy

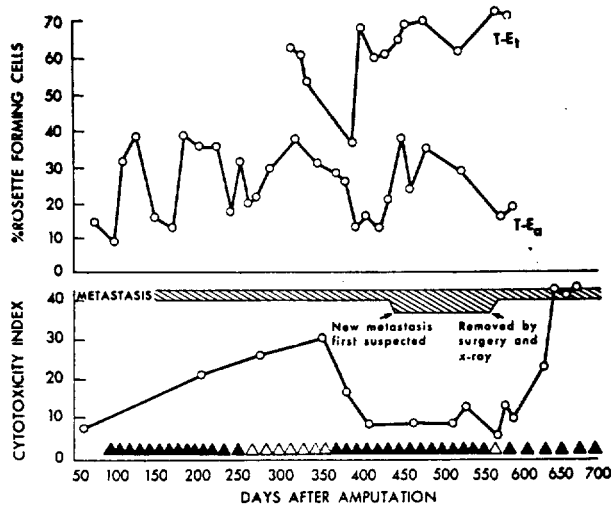

FIGURE 3 Serial measurements of the percentage of "active" and total rosette-forming cells and of osteogenic sarcoma-specific cell-mediated cytotoxicity in the peripheral blood lymphocytes of patient 10. The patient remained clinically stable and symptom-free for almost 1 yr after amputation, despite the presence of an inoperable metastatic lesion in her spine. Loss of cell-mediated cytotoxicity in the donor of transfer factor was followed by a drop in the patient's cell-mediated cytotoxicity; shortly thereafter, metastatic lesions were found in the clavicular region. ▲, tumor-specific transfer factor; △, nonspecific transfer factor.

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showed no change in antibody class or titer attributable to tumor-specific transfer factor, and the presence or absence of antibody did not appear to correlate with clinical status.

Skin test reactivity. All patients, even those with clinically significant tumors, had positive skin-test reactions (5 mm of induration) to at least one of six skin test antigens. Patients 4 and 6, however, lost all reactivity to skin tests during the terminal phase of the disease. Transfer factor was capable of transferring skin test reactivity to marker antigens to all patients for whom such reactivity was present in donors. Because skin tests remained consistently positive until the terminal phase of the disease, such tests were not performed serially in all patients.

Evidence in vivo for passive transfer of tumor immunity. In patient 6 a large primary tumor remained static for 22 mo, and a single metastatic lesion in the lung did not increase in size for 14 mo during treatment with tumor-specific transfer factor (and, later, BCG). Pain and edema, presumably caused by cellular immune reactions at the primary tumor site, were exacerbated by each injection of transfer factor. Immunotherapy was discontinued because the primary tumor did not regress. Pain and edema then subsided and new metastatic lesions appeared; the patient died 7 wk later with massive lung involvement. Patient 7, who had X rays suggesting many pulmonary lesions, developed chest pain, shortness of breath, fevers, and pleural effusions after the institution of transfer factor therapy. The pleural effusions were sterile but contained copious numbers of reactive leukocytes, primarily lymphocytes, and monocytes; no tumor cells could be detected. Patient 13, shortly after the institution of immunotherapy, developed shortness of breath and chest pains. After one dose of methotrexate and leucovorin (18), his pulmonary status improved dramatically and his lung shadows appeared to diminish in size. Despite further doses of methotrexate, the shadows did not further diminish in size. Other lesions grew and the patient died with massive lung involvement. Excisional

FIGURE 4*a* Biopsy specimen of tumor tissue from patient 4 before tumor-specific transfer factor (hematoxylin and eosin; original magnification, × 250).

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FIGURE 4*b* Biopsy specimen of tumor tissue from patient 4, 3 days after administration of transfer factor (×250). Lymphocytic infiltrate and perivascular cuffing are clearly visible.

biopsies of the lesions of patients 4 and 10 were performed 3 days after administration of tumor-specific transfer factor. Each biopsy specimen contained massive lymphocytic infiltrates and also showed perivascular cuffing of lymphoid cells in their tumors. Biopsy specimens of both patients taken before therapy with tumor-specific transfer factor contained no lymphocytic reaction (Fig. 4).

DISCUSSION

Osteogenic sarcoma in children and young adults has a dismal prognosis: amputation of the primary tumor and resection, conventional chemotherapy, and radiation therapy of metastatic lesions offers a 2-yr survival rate of about 20%. In more than 50% of patients, metastastic lesions become clinically evident within 6 mo of diagnosis. The survival of patients with metastases is counted in weeks (19), and the incidence of spontaneous remissions is less than 1% (20). For these reasons, this tumor was chosen as the first to be treated with tumor-specific transfer factor. In the study of our first patient (patient 6) treated with tumor-specific transfer factor, we found that two household contacts had specific cell-mediated cytotoxicity against this tumor; thus, one of these became our first donor (11). We have since shown that approximately 26% of household contacts of patients with osteogenic sarcoma have levels of cytotoxicity adequate for the preparation of transfer factor (12, 21).

Our treatment program attempted to retain the benefits of conventional therapy, while simultaneously providing specific immunotherapy to supplement residual latent host defenses. Before immunotherapy was initiated, T-E_a, T-E_t, cell-mediated cytotoxicity against the tumor, and antitumor antibodies were measured in each patient. Cytotoxicity was also measured in household contacts of the patients, and tumor-specific transfer factor was prepared from those who had CI greater than 50%. Subcutaneous injections of transfer factor were begun when either T-E_a or cytotoxicity dropped below normal levels. Patients were serially monitored for cell-mediated cytotoxicity to tumor, T-E_a, and antibodies to tumor during immunotherapy.

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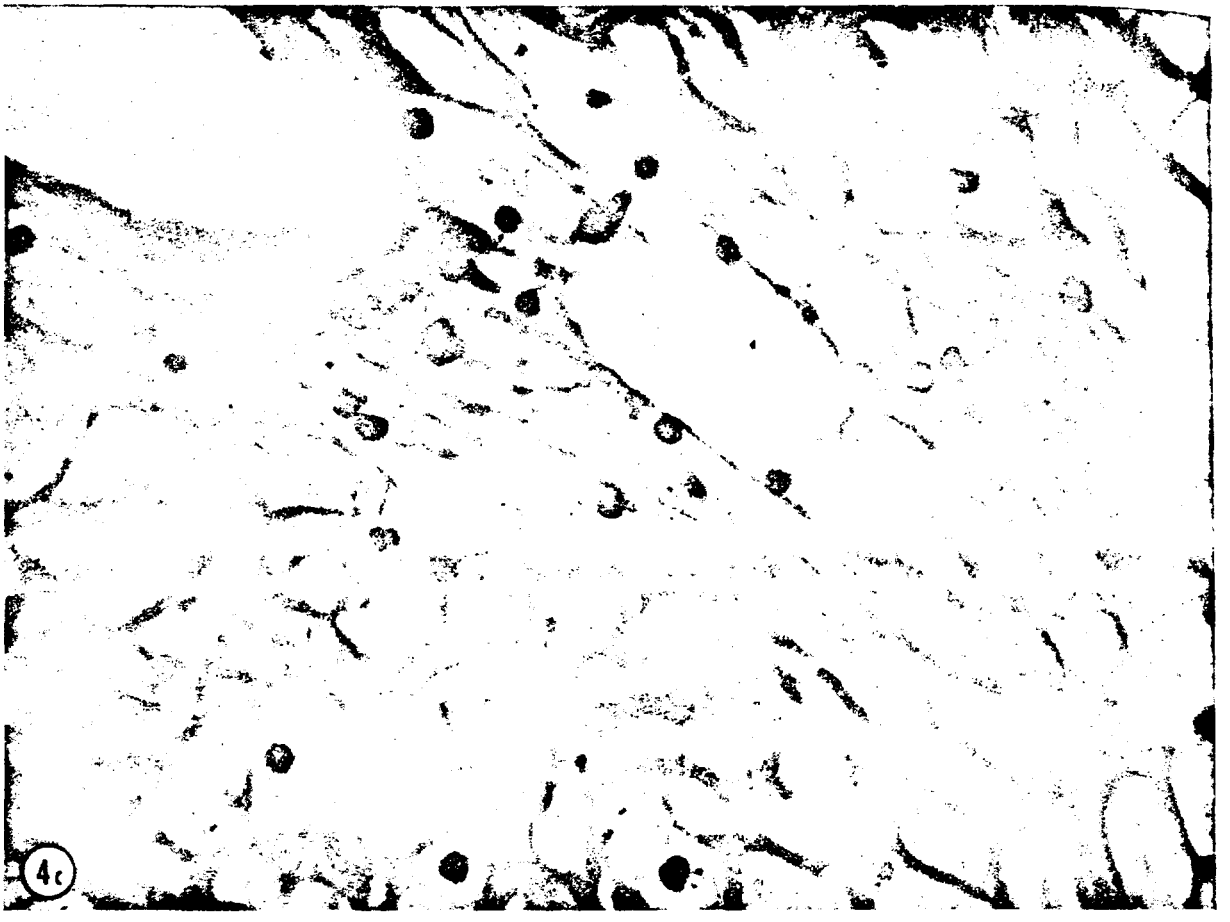

FIGURE 4*c* Biopsy specimen of tumor tissue from patient 4, 3 days after administration of transfer factor (× 500).

A suggested natural course of immunologic events in patients with osteogenic sarcoma, based on our studies of 18 patients, is depicted in Fig. 1. When the tumor was quite small, patients usually had high cytotoxicity, and T-E_a and T-E_t appeared to be within normal limits. Cell-mediated tumor-specific cytotoxicity decreased rapidly within 3 mo after amputation (we believe that it may decrease more gradually in nonamputee patients). The rest of the patients had low cytotoxicity at the time of discovery of tumor. About 90 days after the fall of celluiar immunity, T-E_a declined, followed in 6 wk by clinical evidence of metastatic lesions, a fall in T-E_t, and death.

Administration of tumor-specific transfer factor appears to alter this course of events. In five patients who had inoperable primary or metastatic lesions at the time transfer factor was injected, pain and local inflammation in the area of the lesions, accompanied by transient febrile episodes, were noted 18–36 h after subcutaneous injection of tumor-specific transfer factor into the deltoid region. In three patients, specimens obtained by biopsy or surgical removal of tumors during and after such an episode revealed substantial new infiltration of lymphocytes, evidenced by perivascular cuffing of lymphocytes and monocytes in the tumor tissue, not seen in the biopsy specimens taken before administration of transfer factor. Although these infiltrates suggest, they by no means prove passive transfer of tumor-specific immunity.

Suggestive evidence in vitro of the capability of transfer factor to enhance immunity is reflected by the following findings: an increase in T-E_a is a consistent indicator of clinical response in immunotherapy of nonmalignant states (16); a decrease in T-E_a appears to be a harbinger of clinically or radiologically evident metastatic events (22). Therefore, active rosettes appear to measure the nonspecific capability of the patient to respond to antigen. T-E_a rose in response to injections of tumor-specific transfer factor and fell after injections of nonspecific transfer factor in the patients studied. Cell-

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[illegible] cytotoxicity (a measure of the patient's specific [illegible] to respond against tumors) increased in all [illegible] after injections of tumor-specific transfer factor [illegible] indication of increased cell-mediated cytotoxicity [illegible] control tumor cell lines. Non-tumor-specific [illegible] factor, administered on several occasions to [illegible] patients, was uniformly associated with a de[illegible] in cell-mediated cytotoxicity in the patient; rein[illegible] of therapy with tumor-specific transfer factor [illegible] associated with a concomitant rise in cell-mediated [illegible] toxicity.

Any mode of therapy for malignant neoplasms in hu[illegible] must be considered in the light of its differential [illegible]city, i.e., the "tumor-cell lytic" effect, as opposed to [illegible] "host-cell lytic" effect. A good example of this is [illegible] therapy. Perlmann, Perlmann, and Biberfeld have [illegible]nstrated that cell-mediated cytotoxicity against the [illegible] is transiently eliminated during radiotherapy of [illegible]der carcinoma (23). We noted the same phenome[illegible] in one of our patients with osteogenic sarcoma. It [illegible] highly unlikely that radiotherapy of a "radioresistant" [illegible]or is advantageous for all patients, because radio[illegible]erapy, by virtue of its lympholytic effect, may reduce [illegible] defenses and lead to more rapid growth of tumor. The same is true of most lympholytic chemotherapeutic [illegible]gents. Immunotherapy, although boosting the endoge[illegible] host responses against the tumor, is beneficial only [illegible] the host has the resources to respond.

In two patients, BCG was also used in an attempt to [illegible]gment the T-cell immune response. This practice was [illegible]andoned because BCG appeared to decrease cellular [illegible]mmunity (as measured by T-E_a) in one patient. Our [illegible]idence suggests that the effect of BCG on T lympho[illegible]tes differs from that of tumor-specific transfer factor [illegible]24).

Active transfer of immunity in solid tumors by vac[illegible]nation is currently under study; data are too scant to [illegible]ow conclusions at present, but there may be a slight [illegible]rend toward prolongation of the disease-free interval [illegible] treated patients (25). More recently, passive trans[illegible]er of cell-mediated immunity has been attempted with [illegible]ransfer factor (11). Lobuglio, Neidhart, Hilberg, Metz, and Balcerzak have also reported a patient with an al[illegible]eolar soft part sarcoma treated similarly (26), with [illegible]migration inhibitory factor as the laboratory parameter [illegible]f change in cellular events. "The tumor neither re[illegible]gressed nor progressed during the 6-mo period follow[illegible]ng transfer factor therapy."

Three major drawbacks limit the efficacy of tumor-[illegible]pecific dialyzable transfer factor in patients with cancer. First, a large tumor mass may contain more cells than can be destroyed by the entire lymphocyte population of the host. Second, if a vital organ (such as the lungs), which could be significantly compromised by an inflammatory reaction, contains large amounts of tumor (and hence tumor antigen), resection, chemotherapy, or radiotherapy is indicated to reduce the tumor mass before immunotherapy is initiated. Third, tumor-specific transfer factor treatment requires careful selection and constant monitoring of the donors and tedious monitoring of the recipient. At this time tumor-specific transfer factor treatment is far too arduous and expensive to be feasible in other than major research centers.

The results reported here indicate that patients with large inoperable tumors are poor candidates for immunotherapy; therefore, only patients who are clinically tumor-free should be considered for therapy with tumor-specific transfer factor. Our clinical goals are presently directed toward eliminating occult metastases, increasing the time interval between amputation and development of clinically evident metastases, and ultimately increasing the 5-yr survival rate.

The five patients in our study who were clinically tumor-free at the initiation of immunotherapy have all remained so to date (from 12 to 24 mo after diagnosis). Of the seven patients entering our program with clinically evident metastastic lesions, one died 7 wk after therapy with transfer factor was halted. She had survived for 23 mo with a solitary metastasis and without removal of the primary tumor. Two patients are presently stable with metastasis 27 and 28 mo after diagnosis. Transfer factor was halted in four patients after lung masses appeared to increase in size. Because there was no way to differentiate between tumor growth and lymphocytic infiltrate, all of these patients were started on chemotherapy.

APPENDIX

The clinical courses of 14 patients are described below. Patients are presented in order of phase of disease at the time they were first seen. Brief clinical notes are included on four additional patients.

Phase I

Patient 1. A roentgenogram obtained because of minor trauma revealed a 1-cm single tumor in the distal femur of this 16-yr-old girl. A biopsy specimen showed sclerosing osteogenic sarcoma. IgG-kappa antibodies directed against this tumor were found, and she had strong cytotoxicity against the homologous tumor line (CI of 77.7). Amputation was postponed by the patient because she had no symptoms. A second biopsy at the same site 2 mo later showed a friable tumor to which her original antibody did not react; however, she had strong T-cell-mediated cytotoxicity against this second autologous tumor along with the same homologous line. Amputation was performed, and the CI gradually dropped to 13.2 within 2 mo; T-E_t and T-E_a remained within normal limits (64% and 19%). Treatment with tumor-specific transfer factor induced a rise in CI to 50.3. The CI fell to 24.4 after two doses of nonspecific transfer factor, and rose to 46 after administra-

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tion of tumor-specific transfer factor. Tumor-specific transfer factor has been given every 2 wk since, and the CI remains between 40 and 50. This patient is currently free of detectable tumor 15 mo after amputation.

Patient 2. This 18-yr-old boy developed osteogenic sarcoma in the proximal tibia. At the time of amputation, tumor-specific CI was 48, and T-E_a and T-E_t were within normal limits. Within 2 mo after amputation, CI fell to 16.2; T-E_t remained within normal limits (58%), but T-E_a were low. After two doses of tumor-specific transfer factor, the CI rose to 67.7; 1 mo after administration of nonspecific transfer factor, the CI fell to 25.7. Subsequent treatment with tumor-specific transfer factor resulted in a rise in CI to 54; it has remained at this level. This patient is tumor-free 11 mo after amputation.

Patient 3. Osteogenic sarcoma was discovered in the proximal tibia of this 17-yr-old girl during inspection of an X ray taken as a result of incidental trauma. The leg was amputated. Cell-mediated cytotoxicity was high (CI of 46), and T-E_a and T-E_t were within normal limits. Within 3 wk of amputation, although T-E_a and T-E_t remained normal, the CI had fallen to 11.3. After two doses of tumor-specific transfer factor, CI rose to 31.2; however, T-E_a had decreased to 12%. Subsequent therapy with tumor-specific transfer factor was associated with a rise in CI to 43 and of T-E_a to 18. She is currently tumor-free 13 mo after amputation, and CI remains more than 40.

Phase II

Patient 4. A 17-yr-old girl had osteogenic sarcoma in the proximal left tibia. There were no discernible metastatic lesions. An above-knee amputation was performed; immunologic parameters were measured serially but no prophylactic therapy was administered. 7 mo after surgery she had normal T-E_a (23%) and T-E_t (67%), with moderate cell-mediated cytotoxicity (CI of 28.6).

4 mo later, CI had declined to 5.1 and, although T-E_a remained normal, she had developed metastatic lesions in the femur, tibia, and scapula. The patient received adriamycin and 5-(3,3-dimethyl-1-triazeno)imidazole-4-carboxamide (DTIC) (27) and tolerated therapy well for a brief period before the metastatic lesions began to grow. Local excision of several lesions was performed; histologic examination revealed classic osteogenic sarcoma with no evidence of lymphocytic infiltrates. Tumor-specific transfer factor was administered; 3 days after the third injection, all lesions were resected. The tumor specimens showed marked lymphocytic infiltration and perivascular cuffing of lymphocytes. 5 mo later she is receiving tumor-specific transfer factor; she is clinically stable and is attending a special school.

Patient 5. A 10-yr-old girl had osteogenic sarcoma in the proximal humerus. T-E_a and T-E_t were within normal limits (25% and 68%), but cell-mediated cytotoxicity was low (CI of 5). Forequarter amputation was performed. T-E_a fell below normal limits (14%); cytotoxicity remained low (CI of 9). No prophylactic therapy was administered. 4 mo after amputation, approximately 6 wk after T-E_a had fallen, five tumor nodules were observed in the lungs. At this time CI was 12. Actinomycin and radiation therapy were administered and the patient responded, but the lesions recurred within 1 mo after termination of radiotherapy. She died shortly thereafter with massive pulmonary metastases. Cell-mediated cytotoxicity, measured on three occasions during radiotherapy and chemotherapy, ranged between 8 and 11. T-E_a measured on those occasions remained below 14%.

Phase III

Patient 6. The first patient treated with tumor-specific transfer factor was a 15-yr-old girl who had had a bony protuberance in her right lower quadrant for 1 yr. Biopsy showed osteogenic sarcoma. Roentgenograms revealed an ellipsoid tumor mass with a 17-cm axis and a 12-cm diameter, protruding from the posterior lip of the right ilium. A bone survey revealed no other tumor masses, and no pulmonary metastases were seen on chest roentgenograms. The patient refused the only surgical procedure that offered a chance for cure, hemicorporectomy. She received 5,500 R directed to the abdomen, but this radiation produced an estimated reduction in tumor mass of only 15%.

Transfer factor was prepared from the father and sister, both of whom had strong cell-mediated cytotoxicity against osteogenic sarcoma. Neither patient nor family members had antibodies against this tumor detectable either by immunofluorescence or by complement fixation.

Skin tests with six antigens (*Candida, Coccidioides,* mumps, PPD, streptokinase-streptodornase, and trichophytin) showed that one donor had a positive response to trichophytin; before immunotherapy, the patient had a negative response.

One dose of tumor-specific transfer factor was administered subcutaneously in the deltoid region each month. Trichophytin skin tests became positive after administration of transfer factor, suggesting that transfer factor of specific cell-mediated immunity had occurred. No growth of the primary tumor occurred for 7 mo. T-E_a were approximately 30% at the initiation of therapy with transfer factor. (In this patient, T-E_a and T-E_t were not measured before immunotherapy.) After 7 mo of therapy, the patient had a severe viral flulike illness, and began to show signs of deterioration. She had an immediate decrease in T-E_a, and 3 wk later skin reactivity decreased, followed by a depression of hematocrit and a loss of weight. 6 wk after the decrease in T-E_a was seen, a single metastatic lesion was detected in the lung. A second series of tests for tumor-specific cytotoxicity at this time revealed that, concomitant with clinical deterioration, her tumor-specific cellular immunity had disappeared. More importantly, her donor had lost cell-mediated cytotoxicity to the tumor after repeated leukaphereses.

The patient was given one dose of BCG by scarification, followed by eight doses of tumor-specific transfer factor (derived from another donor with high cytotoxicity for osteogenic sarcoma) in 1 day. She then received alternating doses of transfer factor and BCG at biweekly intervals. T-E_a again increased, skin-test reactivity became more intense, and clinical well-being returned. Measurements of T-E_a were discontinued after the 12th mo because of logistic and psychological problems of the patient and family. After approximately 7 mo of treatment with both transfer factor and BCG (14 mo after the institution of transfer factor therapy), the child first noted edema in the right leg and pain in the area of the tumor; this was so exacerbated by injections of transfer factor that morphine was required for analgesia. Injections of BCG did not appear to produce the pain or edema. The patient was taking 45 mg of morphine sulfate intramuscularly daily together with large doses of other pain medications. 21 mo after the beginning of immunologic therapy, a chordotomy was performed to reduce pain. Immediately after surgery, the pain medica-

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tion requirement was zero. 2 wk later, pain and edema returned within 24 h after an injection of transfer factor, and the patient again required medication for pain. After two injections of transfer factor (and one of BCG), the local physician decided to discontinue immunotherapy and to administer cyclophosphamide in low doses (50 mg orally each day). The pain and edema disappeared within 36 h. However, new lung metastases appeared soon after, and the patient died 7 wk after immunotherapy was stopped.

Patient 7. A 15-yr-old boy had painful swelling of the distal femur. Roentgenograms and biopsy established the diagnosis of osteogenic sarcoma. Chest roentgenograms revealed a single metastatic lesion in the lung. The first set of immunologic tests were done just before radiation was administered. His CI was high (88), although his T-E_a were low (7%). After approximately 5,000 R had been directed to the leg, his CI was 10.5, and his T-E_a remained low (6%).

The patient developed massive pulmonary metastases during radiotherapy. After radiotherapy was completed, palliative immunotherapy was administered, although there was no expectation of prolonging his life. After three weekly doses of tumor-specific transfer factor, the patient gradually developed severe chest pains with dyspnea. Roentgenograms revealed that the densities in the lungs were larger than before treatment. He then developed bilateral pleural effusions. The pleural aspirate was sterile fluid with copious numbers of leukocytes. The differential count was approximately 40% small mature lymphocytes, 25% monocytes, 20% polymorphonuclear leukocytes, and 15% pleural mesothelial cells that in gross appearance were benign. No tumor cells could be detected in the pleural effusion. Routine cultures for bacteria and fungi were negative, as were smears for acid-fast bodies and guinea pig cultures for tuberculosis.

The patient became moribund; his course was marked by prolonged fevers despite the fact that multiple blood cultures revealed no organisms. He was given 125 mg of hydrocortisone (Solu-Cortef, The Upjohn Co., Kalamazoo, Mich.) intravenously twice daily, in hope that its lympholytic effect would reduce the sterile pulmonary inflammation apparently caused by transfer factor. The patient responded dramatically within 24 h and became ambulatory. Adriamycin and DTIC (27) were started; soon afterward the chest lesions grew, and the patient died in respiratory failure.

Patient 8. A 16-yr-old girl had osteogenic sarcoma of the metaphyseal region of the left proximal tibia. An above-knee amputation was performed; T-E_a were 10% at that time. Tumor-specific transfer factor was initiated. T-E_a increased to 22%, and cell-mediated cytotoxicity gradually rose. She became pregnant, and both cytotoxicity and T-E_a declined; the pregnancy was electively terminated. She is well and clinically tumor-free 24 mo after amputation. T-E_a and cell-mediated cytotoxicity have risen again to prepregnancy levels.

Patient 9. This 12-yr-old girl had complained of pain in the upper third of the left tibia for approximately 2 yr after minor trauma. Roentgenograms revealed a large osteolytic lesion; biopsy revealed osteogenic sarcoma with much osteoblastic activity. At the time of amputation, T-E_a were below normal (12%), and CI was 6.7. No metastatic lesions have been seen. The patient is now receiving prophylactic chemotherapy with methotrexate and leucovorin (23); immunotherapy has not been started. Follow-up assays are not available; however, the patient is maintaining normal activities 11 mo after amputation.

Phase IV

Patient 10. A 29-yr-old woman had a pathologic fracture; osteogenic sarcoma was found in the proximal metaphyseal region of the right femur. Bone survey revealed a metastatic lesion at T-7. A roentgenogram of the hip taken 1 yr earlier for an unrelated problem was reexamined; the tumor had been present at that time. Hip disarticulation was performed. The metastatic lesion was treated with radiation therapy (3,500 R). Tumor-specific transfer factor (derived from her husband, who had high cell-mediated cytotoxicity to the tumor) and BCG were administered. During 14 mo of immunotherapy there was no evidence of new metastatic lesions, and no change in the lesion at T-7.

The percentage of T-E_t remained stable at 60% or higher, except on one occasion, when it dropped to 37% concomitant with a severe virallike syndrome; T-E_a were also subnormal at this time (13%). Cell-mediated cytotoxicity and T-E_a gradually increased during therapy with tumor-specific transfer factor. A later drop in T-E_a, 1 yr after amputation, coincided with loss of cell-mediated cytotoxicity by the donor of transfer factor after repeated leukaphereses. T-E_a increased after administration of several doses of transfer factor and one of BCG. (It was noted retrospectively that the patient had had several significant decreases in T-E_a and cell-mediated cytotoxicity after BCG. By then, based on observations in other patients, we decided that BCG may be detrimental as often as it is advantageous, and its use was discontinued in patients with osteogenic sarcoma.)

Approximately 18 mo after amputation, cell-mediated cytotoxicity began to decline; 3 mo later a significant decrease in T-E_a was noted. 30 days later the patient complained of pain in the left clavicular region; after 2 mo several calcified lesions in the clavicular region became obvious by X ray. The patient received several doses of tumor-specific transfer factor from a new donor with high cell-mediated cytotoxicity and, although T-E_a rose to the low normal region, cytotoxicity remained low. The metastatic lesions were surgically removed. Sections through the lesions showed necrotic tumor cells with extensive infiltration of lymphocytes. Radiation (5,000 R) was administered to the tumor region, and therapy with tumor-specific transfer factor was resumed. CI is 46 and T-E_a is 22% 28 mo after amputation.

Patient 11. A 20-yr-old man developed osteogenic sarcoma in the proximal tibia; amputation was performed. 2 mo later a solitary metastatic lesion in the lung was resected. At that time, T-E_a and T-E_t were normal, but CI was low (13). CI remained low during the subsequent 8-wk period, and two doses of nonspecific transfer factor at 2-wk intervals did not change it. However, the first injection of tumor-specific transfer factor was followed 2 wk later by an increase in CI to 46. With injections of tumor-specific transfer factor every 2 wk, the CI remains over 40. The patient is clinically tumor-free 9 mo after resection of the lung lesion (11 mo after amputation).

Patient 12. A 13-yr-old girl had osteogenic sarcoma in the distal femur and many small metastatic lesions in the lungs. The primary tumor was irradiated. T-E_a were subnormal (10%) and CI was 40. Two doses of tumor-specific transfer factor increased the CI to 82 and T-E_a to 22%. 4.5 mo after diagnosis CI dropped to 23, T-E_a dropped to 8%, and the lung masses became more dense on X-ray. The films could not differentiate between tumor growth and lymphocytic infiltrates around the pulmonary lesions, and the patient was not subjected to lung biopsy to define the

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nature of the lesions. The decision was made to discontinue transfer factor and start chemotherapy. She was started on methotrexate and leucovorin. 16 mo after diagnosis she was noted to have recurrent metastatic lesions; she now has massive lung metastasis and pneumothorax 17 mo after diagnosis.

Patient 13. A 21-yr-old man developed osteogenic sarcoma of the distal femur, and his leg was amputated 1 yr before referral. He developed three large, rapidly growing lung lesions that were unresponsive to adriamycin and radiation. After administration of tumor-specific transfer factor, he developed chest pains and shortness of breath. Immunotherapy was stopped, and he received high doses of methotrexate and leucovorin. The pulmonary symptoms cleared after the first dose of methotrexate. Roentgenograms of the chest revealed that the lung densities were diminishing in size, but new ones were appearing. These increased in size during 1 mo despite the chemotherapeutic regimen.

Serial assessments of lymphocyte function during chemotherapy showed normal T-E_a and normal responsiveness to phytohemagglutinin despite a drop in total lymphocyte count. The patient died with massive pulmonary involvement. The original response to chemotherapy (rapid diminution in size of the lung densities) may have been a result of the lympholytic action of methotrexate, although it is possible that lysis of more susceptible tumor cells occurred, leaving resistant tumor cells to grow and cause death.

Patient 14. This 16-yr-old girl had had a progressively growing osteogenic sarcoma in the proximal tibia for 8 mo before amputation; there were two metastatic nodules in the lungs. After amputation, tumor-specific transfer factor was administered. There was no significant increase in cell-mediated cytotoxicity, which was low and continued to decline slowly. The patient was considered as not helped by transfer factor, and she is on a regimen of adriamycin and DTIC 14 mo after diagnosis; the lung lesions are growing and increasing in number.

Other patients

Patient 15. A 17-yr-old man had a large osteogenic sarcoma in the proximal femur. T-E_a were 7% at time of diagnosis. This patient was lost to follow-up; he died of metastatic disease 6 mo after amputation with no prophylactic therapy. He appeared to be in phase III of the disease at the time of diagnosis.

Patient 16. This patient is a 51-yr-old woman who had an amputation of the right leg for osteogenic sarcoma at the age of 17. At the age of 31, she developed carcinoma of the thyroid, which was surgically removed. She is now well and asymptomatic. Her CI is 20, T-E_a are 19%, and T-E_t are 62%.

Patient 17. This 17-yr-old man recently entered the protocol in phase II. He is receiving tumor-specific transfer factor and is clinically tumor-free 7 mo after amputation.

Patient 18. This 14-yr-old girl entered the protocol in phase II. She is doing well with a regimen of prophylactic chemotherapy 8 mo after amputation.

Note added in proof. All patients who began treatment with tumor-specific transfer factor when they had no clinically evident lesions remain healthy and tumor-free: patients 1 (19 mo), 2 (13 mo), 3 (15 mo), 8 (26 mo), 11 (13 mo), and 17 (9 mo). Patients 4 and 10, who had metastases, remain clinically stable 29 and 30 mo after diagnosis. Patient 18 is doing well on prophylactic chemotherapy 10 mo after diagnosis. Patients 13 and 14 have died, and patient 12 is terminally ill; current follow-up is not available on the other patients.

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Tumor-Specific Cell-Mediated Immunity in Household Contacts of Cancer Patients

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ABSTRACT Patients with osteogenic sarcoma (and related tumors), hypernephroma, and breast carcinoma, and their household contacts were tested for tumor-specific cell-mediated immunity against these tumors with the use of a short-term chromium-51 release assay. This assay, reproducible over many months and well-correlated with the clinical course of the patients, was used to demonstrate that household contacts of patients with osteogenic sarcoma and breast carcinoma have specific immunity against the tumor type with which they have been in contact. In both types of tumors, the range of cytotoxicity values produced by lymphocytes from the household contacts was significantly higher than that of the normal population. The incidence of immunity was much higher in household contacts of patients with breast carcinoma than in those of patients with osteogenic sarcoma. Immunity was found with equal frequency in men and women, as well as in genetically and nongenetically related household contacts (guardians, adopted children, spouses). Immunity against hypernephroma was not demonstrated in either patients with hypernephroma or their household contacts.

INTRODUCTION

The occurrence of tumor-specific immunity in normal humans who had been in contact with persons with cancers has been alluded to in several reports. Hellström, Hellström, Bill, Pierce, and Yang described cell mediated-immunity to neuroblastoma in mothers of infants with that tumor (1). Morton and Malmgren demonstrated a high incidence of tumor-specific antibodies in family members of osteogenic sarcoma patients (2). This, in conjunction with their other studies, was interpreted as evidence for a possible viral etiology in some human cancers (3–5).

We selected three histologically different tumor types, osteogenic sarcoma, hypernephroma, and breast carcinoma, and performed a series of experiments to determine if household contacts of individuals with these tumors demonstrated higher than normal levels of tumor-specific cell-mediated cytotoxicity against cell lines derived from these tumors.

Individuals with osteogenic sarcoma or breast carcinoma who are immune to their own tumor will also demonstrate immunity against the same histological type of tumor from another individual (6). Therefore, representative tumor cell lines derived from the three tumor types were used as target cells in a 3-h chromium-51 release assay. A random sample of normal individuals who had no known contact with carcinoma was used as a control population for carcinoma immunity; a random sample of normal individuals who had no known contact with sarcoma was used as the control population for sarcoma immunity. The reactions of the control group against these tumor cells were compared with those of household contacts of cancer patients with one of three tumor types. We found that the household contacts of patients with osteogenic sarcoma and with breast carcinoma had a significantly higher level of tumor-specific immunity directed against the histologic type of cancer to which they were exposed. We found no such incidence of

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TABLE I

Characteristics of Cell Lines Used in the Cytotoxicity Assay

Cell line	Donor sex	Origin	Isolated by	Passage used	Media lysis Mean	Media lysis SD	Media lysis SE	Water lysis Mean	Water lysis SD	Water lysis SE	Tumorogenicity	Reference
					% of total			% of total				
BT-20	F	Breast carcinoma	Lasfargues	33–35	28.6	8	3	44.8	10.5	4	ATS mice,* slow-growing tumors	7–10
ALAb 496	F,	Breast carcinoma	Sykes	34–36	24	9	3.4	37	11.8	4.5		9, 10, 11
Hs 736T	M	Fibrosarcoma	NBRL‡	4	20	id§	id	35	id	id		
Hs 736sk	M	Skin	NBRL	4	27	id	id	17	id	id		
A 498	F	Hypernephroma	Giard	26–28	14.6	6	0.9	41.5	12.6	1.8	ATS mice, carcinoma in young mice	12
Hs 706T	F	Giant cell sarcoma	NBRL	7	16.8	3.3	1.7	26	2	1		
Hs 706sk	F	Skin	NBRL	7	31.7	id	id	41.6	id	id		
Te 85	F	Osteosarcoma	MacAllister	17–19	22	7.1	1.0	40.4	9.4	1.4		8, 13
Hs 699T	M	Hypernephroma	NBRL	10	30	id	id	40	id	id		
Hs 699sk	M	Skin	NBRL	6	18	id	id	35	id	id		
Hs 715T	M	Hypernephroma	NBRL	8	29	id	id	39	id	id		
Hs 715sk	M	Kidney	NBRL	10	13	6.2	4.4	28	7	5		

* Mice treated with antithymocyte serum.
‡ Naval Biomedical Research Laboratories, Oakland, Calif.
§ Insufficient data.

elevated immunity in either the patients with hypernephroma or their household contacts.

METHODS

Cell lines. The human cell lines used in this study are listed in Table I (7–13). All Hs-prefixed cell lines were isolated by the Owens differential trypsinization technique (14). All cell lines were propagated in antibiotic-free medium, either Dulbecco modified medium (Grand Island Biological Co., Grand Island, N. Y.) with 10% fetal calf serum (DEM-FCS),[1] or minimal Eagle's medium with 10% FCS (MEM-FCS). Cells were subcultured at confluence with trypsin-EDTA (Gibco) and were frozen for storage in 10% dimethyl sulfoxide (DMSO) in DEM-FCS. One ampule of each frozen passage level was tested for mycoplasma contamination by Dr. L. Hayflick, Stanford University, using electron microscopy, and found negative. Other ampules from the same lot were thawed and used in the cytotoxicity assay. All cell lines were shown to be free of HeLa tumor cells by chromosome analysis and isoenzyme mobility patterns (8). Some of the cell lines were tested for ability to produce tumors in immunosuppressed mice. Karyology was performed on the tumor cells, usually within three passage levels of use; these studies verified in most cases that cell lines isolated in other laboratories retained their abnormal karotypes.

Although it is not possible to state that a culture is composed of pure tumor cells and therefore completely free of fibroblasts or other cellular contaminents, one can state that the vast majority of cells in a specific population are homogeneous tumor cells with abnormal karyotype, glucose-6-phosphate dehydrogenase patterns, and/or sex chromosomes matching those of the donor (8). This was the case with the cell lines used in this study.

To avoid genetic drift, only the three passage levels listed in Table I were used in the assay. This increased the probability of homogeneity in the target cell line preparations. That these cells were homogeneous is shown by the fact that lymphocytes tested on the same day from the same donor reacted the same on all three passage levels of each tumor cell line.

Freezing and storage of cells. Both target cells and lymphocytes (separated on Ficoll-Hypaque) were suspended in a final concentration of approximately 1×10^7 cells/ml in a prechilled solution of MEM-FCS with 10% DMSO. 1-ml aliquots were added to ampules, which were then flame-sealed and placed overnight in a −70°C freezer in a pipette can completely wrapped with 2-in-thick foam rubber to allow slow cooling. The ampules were then transferred to a liquid nitrogen container. When needed, the cells were rapidly thawed by dropping the ampule in ethanol, 37°C. The ampules were opened just as the last ice crystal was dissolving. The contents were then slowly diluted with MEM-FCS at 37°C and washed twice with Hanks' balanced salt solution (HBSS, Gibco). The viability of the tumor cells ranged between 85–98% by trypan blue exclusion test. Lymphocytes were less stable to freezing, and their viability varied greatly upon thawing. They were used in the assay only if they were over 96% viable by trypan blue exclusion.

Target cells. Tumor cells were trypsinized just at confluence. They were then washed once in HBSS, and each target cell suspension was incubated in a total volume of 1–2 ml of RPMI 1640 (Gibco) with 0.1 ml [^{51}Cr]sodium chromate (New England Nuclear, Boston, Mass., sp act 1 mCi/ml, used through one half-life only) at 37°C for 30 min. They were washed by layering them over and

[1] *Abbreviations used in this paper:* CI, cytotoxicity index; MEM-FCS, Dulbecco modified medium with 10% FCS; DMSO, dimethyl sulfoxide; FCS, fetal calf serum; Fx, fluorescein-labeled; HBSS, Hanks' balanced salt solution; HH, household contact; HHC_{br}, HHC of breast carcinoma patient; HHC_{hn}, HHC of hypernephroma patients; HHC_{os}, HHC of osteogenic sarcoma patients; MEM-FCS, minimal Eagle's medium with 10% FCS; RPMI-FCS, RPMI 1640 with 10% FCS and antibiotics; SRBC, sheep red blood cells.

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TABLE II

Raw Data from Replicate Samples in a Representative Experiment with Te 85, Osteogenic Sarcoma

Treatment of target cells*	Replicate samples	Mean of replicates	SD	CI‡ Individual replicates	CI‡ Average
Water lysis	3,574	3,590	56		
	3,557				
	3,556				
	3,674				
Spontaneous lysis	1,306	1,343	27		
	1,342				
	1,370				
	1,354				
Lymphocyte lysis	2,017	2,042	36.4	30	31.0
	2,084			33	
	2,026			30	

* Total cpm incorporated into 5×10^4 target cells = 8,342.

‡ $CI = \frac{cpm_{(lymphocyte\ lysis)} - cpm_{(spontaneous\ lysis)}}{cpm_{(water\ lysis)} - cpm_{(spontaneous\ lysis)}}$.

centrifuging them through a 15-ml FCS (Gibco) gradient; the cells were resuspended in HBSS and immediately recentrifuged. They were resuspended to a final concentration of 5×10^4 cells/ml in RPMI 1640 with antibiotics and 10% FCS (RPMI-FCS). 1 ml of this cell suspension was placed in small Petri dishes (Falcon 3001, Falcon Plastics, Div. of BioQuest, Oxnard, Calif.) and either preincubated for 3 h before the addition of lymphocytes (BT-20 and ALAb cell lines) or mixed with lymphocytes immediately.

Lymphocyte separation. Ficoll-Hypaque: Peripheral blood was drawn into a syringe filled with sodium heparin (3M Co., Medical Products Div., St. Paul, Minn., final concentration 50 U/ml) and layered on a Ficoll-Hypaque gradient. The lymphocytes were separated by centrifugation, and washed two times in HBSS.

Column: 40 ml of peripheral blood was layered over 4-mm glass beads (VWR Scientific Div., UNIVAR, San Francisco, Calif.) that covered the bottom of a sterile 500-ml Erlenmeyer flask and were gently swirled by hand for 15–30 min until the blood was defibrinated. The blood was lightly centrifuged and the buffy coat was separated from the serum and red cells. The buffy coat was diluted 1:1 in HBSS, and poured over a sterile column formed by packing a 12-ml syringe with 1 g of nylon wool (Fenwal Inc., Ashland, Mass.) presoaked with HBSS. The column and cells were incubated at 37°C for 30 min and gently washed through with 30 ml HBSS. The eluate was centrifuged and resuspended in HBSS, to the original volume added to the column. The suspension was then layered on Ficoll-Hypaque and the lymphocytes were separated by centrifugation. They were then washed twice in HBSS and resuspended to the desired concentration. These lymphocytes are depleted of a population of cells with surface immunoglobulin ("B" cells), consistent with the finding of Julius, Simpson, and Herzenberg (15).

Assay. The lymphocytes were adjusted to a final concentration ranging from 1×10^5 to 4×10^6 cells/ml in RPMI-FCS. When 1 ml of each suspension was mixed with 5×10^4 tumor cells, lymphocyte: target cell ratios of 2:1 through 80:1 were obtained. This mixture was incubated for 3 h at 37°C in moist 5% CO_2, on a rocking platform. The supernatant fluid was then aspirated from the dishes into small test tubes and the cell debris removed by centrifugation. The resulting supernatant fluid was decanted and counted in a gamma counter (Packard autogamma spectrometer, Packard Instrument Co., Inc., Downers Grove, Ill.). A cytotoxicity index (CI) was obtained with the use of the formula shown in Table II. In this formula, "water lysis" refers to the counts per minute in the supernatant fraction of dishes of target cells that contain 1 ml water rather than 1 ml lymphocyte suspension (CI = 100), and "media control" refers to radioactivity in the supernatant fluid of similar dishes that contain 1 ml media without lymphocytes (CI = 0). The water lysis was more constant from test to test than the "freeze-thaw" value, even though the total number of counts releasible was much lower. This was therefore routinely used as an approximation of the maximal amount of chromium-51 releasible from the target cells. The water lysis value, however, measures only the amount of chromium-51 incorporated into soluble proteins and liberated by osmotic lysis of the cells. In contrast, lymphocyte-mediated lysis causes both osmotic lysis and enzymatic degradation of the cell membranes (16–17). Thus lymphocyte-mediated lysis was often higher than the water lysis value. We therefore refer to CI in units rather than as a percent.

Several preliminary experiments established that there was little difference between CI values obtained after 3 or 6 h incubation. However, after a 24-h incubation all parameters were increased (media control, water lysis, and lymphocyte lysis values) and, in many cases, specificity was decreased. That is, individuals who did not lyse control cell lines in the 3 h incubation did so after 24 h. We therefore used a standard 3-h incubation.

Enumeration of B-cells. Separated lymphocytes were adjusted to 2×6 cells/ml in phosphate-buffered saline (PBS). Fluorescein-labeled (Fx) goat anti-human immunoglobulin (FxIgG, FxIgM, FxIgA, purchased from Bur-

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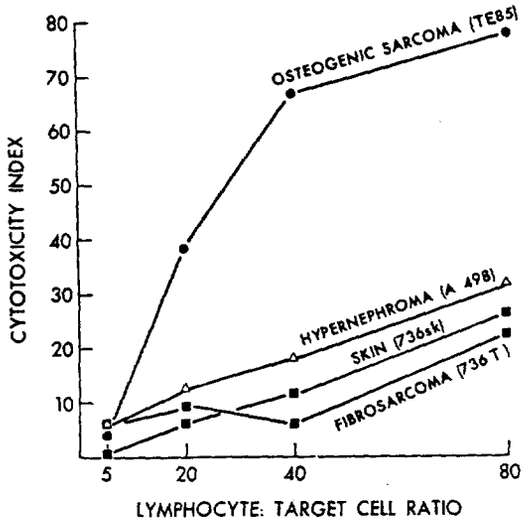

FIGURE 1 CI as a function of increasing lymphocyte: target cell ratios. Lymphocytes taken from an osteogenic sarcoma patient shortly before amputation of a small, sclerosing tumor were tested against osteosarcoma cell line (Te 85), hypernephroma cell line (A 498), fibrosarcoma cell line (736T), and the fibroblast cell line from the same patient (736sk). These data were selected as typical of the reaction. The 80:1 lymphocyte:target cell ratio is on the plateau region of the curve, where minimal variation is encountered. All subsequent values are expressed at the 80:1 ratio. Additional data concerning the clinical correlates of reactivity with the Te 85 cell line are published in the accompanying paper (21).

roughs-Wellcome & Co., Inc., Tuckahoe, N. Y.) was added to the lymphocytes at a final dilution of 1:10, and incubated at 4°C for 30 min. The cells were then washed twice in cold PBS and scored for surface immunoglobulins with the use of phase and fluorescent microscopy (Leitz fluorescent microscope; E. Leitz, Inc., Rockleigh, N. J.).

Rosette-forming cells. After the incubation period of the assay, excess lymphocytes were removed from the Petri dishes by washing gently once with HBSS. 2×10^7 sheep red blood cells (SRBC) in 1 ml RPMI 1640 were added to the dishes. After an incubation period of 30 min, the supernatant fluid was gently aspirated and the dish was gently washed once with RPMI 1640.

Staining of target cells: lymphocytes. A small sterile circular glass cover slip was fixed to the bottom of the Petri dish by heating the tip of a needle and melting four dots of plastic at equal intervals on the circumference of the glass slip. Lymphocytes and target cells were added to these dishes and the assay was carried out as usual. The cover slips were removed, washed once in HBSS, fixed, and stained according to one of the following methods: fixed in a 9:1 mixture of ethanol and methanol for 5 min at 4°C, damped dry, and either stained with Wright-Giemsa stain, or incubated with fluorescein-conjugated anti-human polyvalent immunoglobulins; or incubated unfixed with the same fluorescein-conjugated antisera at 4°C for 30 min, washed twice, and examined on a Leitz fluorescent phase microscope. Controls included lymphocytes from the same individual, incubated in Petri dishes in the absence of tumor cells for 3 h, aspirated into test tubes, and centrifuged in a table-top Sero-fuge (Clay-Adams Inc., Parsippany, N. J.) (2,000 rpm) for 10 min. The cell button was resuspended in PBS and treated with a 1:10 dilution of FxIgG and FxIgM at 4°C for 30 min. The cells were washed twice in cold PBS and placed on microscope slides.

Household contacts. An individual was classified as a household contact of a patient with hypernephroma or osteogenic sarcoma if he or she had been in close contact with a patient with an active tumor for at least 6 mo before being tested. Household contacts of patients with breast carcinoma ranged in age from 5 to 50 yr and had spent the past 5 yr in contact with the patients (all of whom either had active metastatic disease or had had a mastectomy within the past 3 yr). Household contacts of osteogenic sarcoma and hypernephroma ranged in age between 18 and 52 yr.

Normal population. Individuals classified as normal for this study had had no known contact with a patient with carcinoma (if included with the control group for breast carcinoma or hypernephroma) or with a patient with a sarcoma (if included in the control group for osteogenic sarcoma). The normal population was matched to the

FIGURE 2 Wright-Giemsa stain of the cytotoxicity reaction at the end of the 3-h incubation period; osteogenic sarcoma-derived tumor cells (Te 85) and lymphocytes from an immune donor were used. The lymphocytes can be seen fused to the target cell membrane of the lysed cell, and an adjacent tumor cell without lymphocytes adherent is not lysed. Fluorescence microscopy of similar preparations reacted with fluorescein anti-human immunoglobulins demonstrated that these cells lack surface immunoglobulin receptors. Monocytes were occasionally but not often seen adhering to the target cells. They were neither necessary nor sufficient for tumor cell lysis in this assay.

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TABLE III

Comparison of One Person's Lymphocytes Separated on Ficoll-Hypaque with those Separated on Nylon Wool Columns

Lymphocyte:target cell ratio*	Ficoll-Hypaque‡	Nylon wool column§
	CI	*CI*
80:1	34.8	33.9
40:1	20.2	20.8

* Assayed on Te 85, osteogenic sarcoma.

‡ Lymphocytes are 15–21% surface immunoglobulin-bearing cells after separation.

§ Lymphocytes are 1–2% surface immunoglobulin-bearing cells after separation.

household contact population by sex and age range. These individuals had no known disease.

Statistics. For the studies on normal individuals and household contacts of osteogenic sarcoma and hypernephroma patients, the number of individuals tested was sufficient to justify application of standard statistical tests, such as the Student *t* test and the Welch test for comparison of two means. For the breast carcinoma series, it was felt that the Student *t* test would be subject to severe criticism if applied to these data. We therefore used the Fisher-Irwin test for two by two tables, an exact test that does not depend on distribution assumptions.

RESULTS

The assay

Selection of cell lines. More than 25 cell lines were screened for suitability for this study. The first two criteria for selection were that they be tumor cells as measured by all available criteria (see Methods) and that their characteristics match those of their purported donor. The next criterion for selection was their suitability to the chromium-51 release assay. It was necessary that the total chromium-51 incorporation be high enough (usually over 3,000 cpm) and that the amount of chromium-51 released upon water lysis of the cell be sufficiently more than the spontaneous release level to give a measureable spread between the two values. In addition, the cell lines had to be stable enough in growth pattern to produce reliable values with all of

TABLE IV

Intrinsic Error of the Cytotoxicity Assay

Variation of Lymphocytes from the Same Individual, Drawn in Separate Syringes and Isolated and Assayed Individually

Cell line	Subject	Lymphocyte: target cell ratio	Replicate samples: Aliquot 1		Aliquot 2		Aliquot 3		Averages	SD
			CI	*Average*	*CI*	*Average*	*CI*	*Average*		
Te 85	1	80:1	26.2	27	16.2	19.3	27.6	28.1	24.8	7.8
			27.9		22.5		28.6			
		40:1	19.0	15.9	13	12.6	21.9	24.4	17.6	9.5‡
			12.8		12.3		26.9			
	2	80:1	10.3	9.7	16.6	16	ND	ND	12.8	6.3
			9.2		15.4					
		40:1	4.7	5.1	11	9.1	ND	ND	7.1	2.0
			5.5		7.3					
A 498	3	80:1	50.8	49.1	45.3	50	49.9	47.8	49.0	1.6
			47.5		54.8		45.7			

Daily Reproducibility: Comparison of CI Values between Lymphocytes Assayed Immediately after Separation and Those from the same Lot Assayed on a Different Day, and Passage Level of Target Cells either Stored at 4°C for 24 h, or Frozen in DMSO-Containing Medium and Used from 1 wk to 1 mo Later

Cell line	Subject	Lymphocyte: target cell ratio	CI, assayed immediately	CI after storage at 4°C, 24 h	Storage frozen	Average	SD*
Te 85	4	80:1	35.8	43.4	ND	39.6	3.8
	5	80:1	83.2	ND	92.4	87.8	4.6
	6	80:1	34.1	ND	32.3	33.2	0.9
	7	80:1	41.4	ND	34.8	38.1	3.3

‡ The CI value at the 40:1 ratio, near the beginning of the plateau portion of the dose-response curve, demonstrates greater variation than the CI value at the 80:1 ratio.

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TABLE V

Long-Term Reproducibility of CI Values

Cell line	Lymphocyte donor	Diagnosis	Treatment	Month 1	2	3	4	4	5	6	7
							CI				
Te 85	1	Normal	None	76	70	—	66	68	—	74	72
	2	Normal	None	—	41	—	—	—	48	—	40
	3	Osteosarcoma	Chemotherapy*	50	—	65	63	51	65	—	—
A 498	3	Osteosarcoma	Chemotherapy	20	—	—	10	23	23	—	—
BT 20	4	HHC_{br}	None	—	—	—	25	24	36	29	—
ALAb	4	HHC_{br}	None	110	126	94	96	96	—	—	—
BT 20	5	Normal	None	—	—	15	—	—	24	—	—
ALAb	5	Normal	None	—	—	76	—	—	72	—	—

* Adriamycin and 5-(3,3-dimethyl-1-triazeno) imidazole-4-carboxamide (DTIC).

the passage levels used from day to day. 10 of the 25 tumor cell lines screened were found suitable and the 4 tumor lines used most extensively in these studies had previously been especially well characterized (Table I).

Tumor-specific immunity was studied primarily against two of these cell lines, ALAb (breast carcinoma) and Te 85 (osteogenic sarcoma). Added precautions were taken to insure that these lines were not contaminated with mycoplasma. In addition to the routine screening procedure for mycoplasma, the entire lymphocyte-tumor cell preparations were randomly examined after incubation (on five separate occasions for each cell line) by scanning and transmission electron microscopy and were found in every case to be negative for mycoplasma.

A dose-response effect was noted with all target cell lines used in this assay (Fig. 1). The cytotoxicity index (CI) increased to a lymphocyte: target cell ratio of 40:1 and reached a plateau at higher ratios. The variation between replicate samples was minimal at the 80:1 ratio. Both the 40:1 and 80:1 ratios were tested at all times to ensure that the latter values were at the plateau portion of the curve and all CI values were reported at the 80:1 ratio.

Nature of the effector cell. To ascertain the nature of the cytotoxic effector cell, those cells that adhered to the tumor after the 3-h incubation period were examined as to their histology, their ability to form rosettes with SRBC, the presence of surface immunoglobulins, and their ability to be depleted by nylon wool columns. Examination of cover slip preparations showed that target cells with lymphocytes bound to their membranes could be found in various stages of destruction, and those with no bound lymphocytes looked relatively [illegible] (Fig. 2). SRBC, added to the dishes after the incubation period, were found to bind to the adherent lymphocytes and thus formed T-cell-type rosettes. Observations by immunofluorescence on the target cells and adherent lymphocytes revealed that none of the lymphocytes that adhered to the target cells had membrane Ig, although roughly 20% of the cells added to the original incubation mixture contained such cells. The Ig-bearing cells seen adhered to areas of the cover slip and Petri dish that were free of tumor cells. Ig-bearing cells were also found in the supernatant (non-adherent) cells and in preparations of lymphocytes incubated in the absence of target cells.

Peripheral blood lymphocytes from three different individuals were depleted of their surface Ig-bearing cells by passage through nylon wool columns (eluate contains 1–2% Ig-bearing cells). The CI of these lymphocytes was compared to that of lymphocytes from the same individual, separated on Ficoll-Hypaque (15–21% Ig-bearing cells). The CI values were not significantly different (Table III).

Our results demonstrate that the cells that mediate tumor cell lysis in this assay are immunoglobulin-free, rosette-forming lymphocytes that do not adhere to nylon wool columns. We therefore consider that we are measuring T-cell-mediated cytotoxicity (18, 19).

Reproducibility. A series of experiments was designed to define the variation of this cytotoxicity assay and to locate the principal source of variability (Table IV).

Peripheral blood from one person was drawn into three separate syringes. The lymphocytes from each syringe were isolated separately on Ficoll-Hypaque, and then assayed. The resultant CI values, at ratios of 80:1 and 40:1, were compared. The broadest range ever obtained in a large series of experiments was 16 CI units, with an SD of 8 CI units.

Lymphocytes from one person were separated on Ficoll-Hypaque and divided into three aliquots; the

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TABLE VI

Range of CI Values Produced by Lymphocytes from a Variety of Subjects against the Same Batch of Chromated Target Cells on the Same Day

Lymphocyte donor	Lymphocyte target cell ratio	TE 85		A 498	
		cpm*	CI	cpm*	CI
Patient with metastatic	80:1	3,330	13.1	2,899	5.3
osteogenic sarcoma	40:1	2,962	1.8	2,902	5.4
Patient with chemotheraputically	80:1	5,338	74.9	4,237	23.3
controlled osteogenic sarcoma	40:1	5,036	65.5	3,874	18.4
Patient with surgically	80:1	7,941	154.9	3,890	18.7
controlled giant cell	40:1	5,859	90.0	3,539	13.9
sarcoma of bone					
Normal household contact of	80:1	4,482	48.5	4,168	22.4
patient with osteogenic sarcoma	40:1	3,984	33.2	3,625	15.1
Normal individual with no	80:1	3,141	7.3	3,514	8.1
known contact with	40:1	3,122	6.7	3,103	8.1
cancer patients					
Media lysis		2,902		2,800	
Water lysis		6,154		9,929	

* Mean of replicate samples.

first was assayed immediately, the second was placed at 4°C overnight and reassayed the next day, and the third was frozen in 10% DMSO-MEM-FCS, thawed several days or weeks later, and assayed. Thus CI values from the same pool of lymphocytes assayed on different days on different passage levels of target cells were compared. This procedure was repeated on four different subjects and the range of variation was no greater than ±5 CI units.

Daily reproducibility was assessed by selecting either normal healthy individuals with high or low levels of cytotoxicity or patients with one of the tumor types studied here whose condition was stable. Their CI values were tested over several-month intervals (Table V) and the variation in these subjects' CI's was less than the variation produced as a result of the Ficoll-Hypaque separations. We therefore conclude that the greatest source of variation is not in daily fluctuations of the assay itself or in the different passage levels of the target cells, but rather in the lymphocyte separation procedure. The maximal intrinsic variation of the technique is ±8 CI units.

Table VI illustrates the complete range of CI values that can be seen from the cytotoxity assay in 1 day. On this day, subjects included one normal individual with consistently low CI, one patient with metastatic osteogenic sarcoma, one osteogenic sarcoma household contact with consistently high CI, and one patient with resected giant cell sarcoma of bone, a tumor that cross-reacts with osteogenic sarcoma on a humoral level (20). It can be seen from the above results that on any given day individuals score high or low CI's depending on their immune specificities.

Patients and household contacts

Detection of tumor-specific immunity. The interaction between lymphocytes from normal individuals and all cell lines (tumor or normal) produces a certain level of chromium-51 release. This represents a background release level, the nature of which is unclear. This chromium release may be mediated by a variety of nonimmune mechanisms such as mechanical interactions, and subtle pH or ionic strength changes in the media, etc. Therefore "normal" ranges were established for each cell line. These ranges represent CI values falling within two SD of the mean of CI values produced by lymphocytes from normal persons with no known contact with the type of cancer (carcinoma vs. sarcoma) against which they were tested. Any value falling above the normal range was considered to be immune lysis. Populations were selected for *tumor*-specific immune lysis by removing all individuals who scored in the immune range on any control cell line. In this manner we select out individuals with non-tumor-specific (histocompatibility) immunity. These are often multiparous women.

Osteogenic sarcoma. Of the several available tumor cell lines that had been isolated from osteogenic sarcoma tumors, Te 85 was selected for use in this assay. It has been characterized as a tumor cell line, it was well suited for use in the chromium-51 release assay on the basis of range and reproducibility of lytic

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parameters, and the lymphocyte-mediated lysis correlated well with the clinical course of patients with osteogenic sarcoma. Individuals with tumor-specific immunity to Te 85 also showed similar levels of immunity to a variety of other osteogenic sarcoma cell lines.

Normal range. 61 normal individuals with no known contact with sarcoma patients were tested on the cell line Te 85 (osteogenic sarcoma) and on one or more of the three control cell lines, A 498 (hypernephroma), BT-20, (breast carcinoma), and ALAb (breast carcinoma). The mean CI of these individuals was 23.3 with an SD of 10.8 CI units. Any value above 44 CI units on this cell line (2 SD above the mean) was considered significantly higher than normal and indicative of immunity against this cell line.

Household contacts, osteogenic sarcoma (HHC_{os}). 111 normal household contacts of patients with osteogenic sarcoma were tested on the osteogenic sarcoma line, and one or more of the three control cell lines, A 498, BT-20, and ALAb. The range of CI values produced by these individuals on the osteogenic sarcoma line had a mean CI of 33.4 U, with a standard deviation of 22 CI U (Table VII). Statistical comparison of the CI values of this population of individuals with those of the normal individuals demonstrated a significant difference between them ($P < 0.01$) with the Student t test. By the Welch approximation, an estimate of the difference of the means was 10.1, SD 2.53, and $P < 0.001$.

The control and the household contact populations were then adjusted to remove values provided by individuals who may have had non-tumor-specific immunity. This was done by removing the values from individuals who scored higher than 2 SD from the mean of the normal range on any of the other control tumor cell lines (all individuals were tested on the A 498 line and often on one other cell line, BT-20 or

TABLE VII

Normal Individuals and Household Contacts of Cancer Patients Tested on Cell Lines Derived from Osteogenic Sarcoma, Hypernephroma, and Breast Carcinoma

	Total population		Corrected for specificity*	
	Normal	HHC	Normal	HHC
Tested on osteogenic sarcoma, TE 85				
Number tested	61	111	45	103
Mean CI	23.3	33.4	21	32.1
SD	10.8	22	11.2	22
CI 2 SD above the mean	44			
P value	<0.01§		<0.001	
Tested on hypernephroma, A 498				
Number tested	117	18	100	17
Mean CI	22.5	21.3	21.6	19.5
SD	15.6	15.5	17.0	12.7
CI 2 SD above the mean	53			
P value	0.5 NS		0.5 NS	
Tested on breast carcinoma, ALAb 496				
Number tested	14	13	14	13
Mean CI	72.9	117.2	72.9	117.2
SD	24.2	49.8	24.2	49.8
CI 2 SD above the mean	120.3		120.3	
P value	<0.01		<0.01	
Tested on breast carcinoma, BT 20				
Number tested	13	13	13	13
Mean CI	25	14	25	14
SD	28			
CI 2 SD above the mean	81		81	
P value	0.5 NS			

* All values from individuals scoring more than 2 SD above the mean on the control cell lines were removed.

‡ These data were also analyzed by the Fisher-Irwin test; see text.

§ These data were also analyzed by the Welch test; see text.

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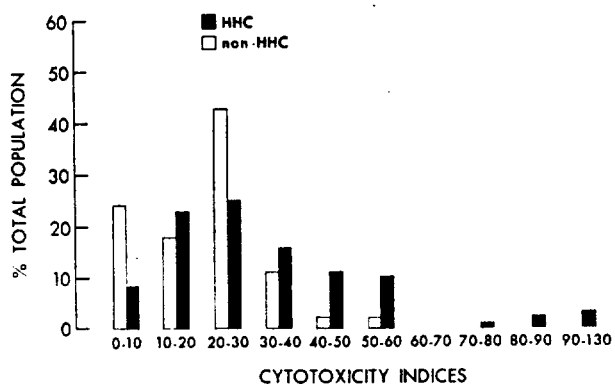

FIGURE 3 The reactions of lymphocytes from 45 normal individuals with no known contact with patients with sarcomas compared with 99 household contacts of patients with osteogenic sarcoma. Lymphocytes were assayed for CI on an osteogenic sarcoma-derived cell line (Te 85) and a hypernephroma-derived cell line (A 498). The lymphocytes from individuals included on this bar graph were corrected for specificity; only those scoring in the normal range on the hypernephroma-derived cell line (2 SD above the mean of the normal population) are included. The difference between the two populations, household contacts and normals, is statistically significant to a P value of 0.001.

ALAb). These corrected populations were found to be significantly different by the Student t test ($P < 0.001$) (Fig. 3).

26% (29/111) of the HHC_{os} scored above 44 CI units on Te 85 (osteogenic sarcoma), while scoring within the normal range on control cell lines. 3% (2/61) of the normal individuals had this type of immunity. One of these two was a pathologist.

Although most of the HHC_{os} who demonstrated tumor-specific immunity on the Te 85 cell line were genetically related to the patients (24/94), tumor-specific immunity was also found in 5 of 17 genetically unrelated household contacts, including household servants, guardians, and spouses. The incidence of immunity in both populations is the same, 25% and 29%, respectively. Although the number of genetically unrelated household contacts is small, comparison with the control (normal) group by use of the Fisher Irwin test demonstrated that the difference between the two groups is statistically significant (to the $P = 0.014$ level).

Patients—osteogenic sarcoma. Only 3 of 12 patients with osteogenic sarcoma demonstrated positive cell-mediated immunity against osteogenic sarcoma before immunotherapy. This immunity was seen just before or just after amputation, when the primary tumor was quite small, and disappeared some time after amputation. The reaction of one such patient is shown in Fig. 1. Similar specificity was seen in the other two patients tested. These reactions are discussed in another paper (21).

Two patients with metastases had very low [illegible] against osteogenic sarcoma before resection of metastatic lesions. These CI's increased sharply after surgery. Four patients with giant cell sarcoma of bone also demonstrated positive specific immunity against the osteogenic sarcoma tumor line.

Hypernephroma—normal range. 117 normal individuals with no known contact with carcinoma patients were tested on the hypernephroma line A 498 and the other control cell lines (primarily Te 85, osteogenic sarcoma). The mean CI value on the hypernephroma cell line (A 498) for the normal population was 2[illegible] (SD 15.6). Therefore, any CI on this cell line above 53% (2 SD above the mean) was considered above the normal range and indicative of tumor-specific cellular immunity against this cell line.

Hypernephroma household contacts (HCC_{hn}). [illegible] HHC_{hn} were tested on these lines. The CI values from these individuals were not significantly different from the normals. Correcting these populations by eliminating individuals with "nonspecific" immunity did not produce any significant change. (Fig. 4).

Hypernephroma patients. Nine patients with hypernephroma were tested and none were found to have CI values above the normal range.

The A 498 cell line appears to be excellent for detecting non-tumor-specific T-cell immunity. Individuals with high CI's on this line were also found to have high CI's on most of the other tumor lines tested. A-

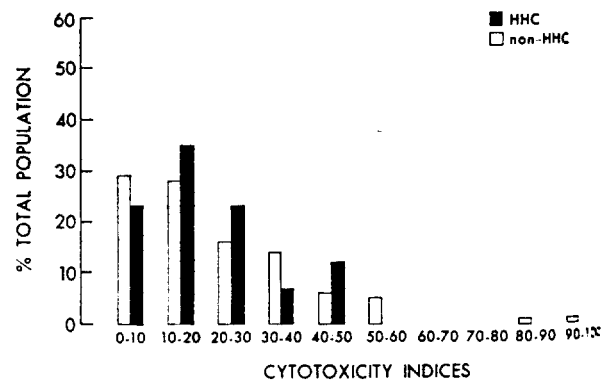

FIGURE 4 Comparison of the cytotoxicity indices of 1[illegible] household contacts of patients with hypernephroma with those of 100 normal individuals with no known history of contact with carcinoma patients. The lymphocytes were assayed against a hypernephroma-derived cell line (A 498) and a cell line derived from an osteogenic sarcoma. Only the reactions of those individuals scoring within the normal range on the osteogenic sarcoma-derived cell line are included in this bar graph. The two populations, household contacts and normals, are not statistically significantly different.

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example of one such individual was a 50-yr-old woman who scored 160 CI on A 498, 39 CI on BT-20, 183 CI on ALAb 496, and 52 on Te 85. All of her maternal and paternal relatives had died of carcinomas, and her husband had active carcinoma.

That this line (A 498) is capable of being lysed by lymphocytes, but does not detect tumor-specific immunity in patients with hypernephroma nor in HHC_{hn} raised the question as to whether it had lost its T-cell-defined tumor-specific antigens. Therefore, four of the hypernephroma patients and five of their HHC were tested on two additional hypernephroma lines, Hs 715T, and Hs 699T, and their matching fibroblast lines (derived from skin or kidney). In no case did the lymphocytes produce higher CI values on the tumor cell lines than on the genetically matched benign cell lines.

Breast carcinoma—normal range. Six cell lines derived from breast carcinomas were tested for possible use in this assay. Two lines, BT-20 and ALAb 496, were found suitable for use in this assay. Both are secretory epithelial cell lines (10). The other four were unsatisfactory for the following reasons: HBT 3 (22) and HBT 39B (23) were found to be HeLa cells (8). 734B (24) had growth characteristics that produced unreproducible day-to-day results. MDA-MB 157 (25) had a spontaneous release chromium-51 value too high for use (too close to the water lysis value).

Normal individuals, both men and women, with no known contact with patients with carcinoma were tested on BT20 and ALAb 496 and on the control tumor cell lines, A 498 and Te 85. The mean CI of this population was 25 on BT-20, and 73 on ALAb 496. Therefore the immune range was above 81 CI on BT-20 and 120 CI on ALAb. All of these individuals had CI values in the normal range on the control cell lines.

Breast carcinoma—household contacts (HHC_{br}). 13 household contacts of breast carcinoma patients who had had mastectomies within the past 5 yr were assayed against the two breast carcinoma lines (Fig. 5) and the control tumor cell lines. All were in the normal range on the control tumor cell lines. The two populations, normals and household contacts, were not significantly different in the range of CI values produced on the BT-20 line but were markedly different on the ALAb line.

7 of 13 household contacts tested (54%) produced CI values greater than 2 SD above the mean of the normal range on the ALAb line. The two populations were analyzed by the Student t test and found to be statistically significantly different ($P = 0.001$, Table VII). However the validity of this test as used on these values is questionable, since the populations being compared do not form normal bell-shaped curves. The CI values of the HHC_{br} clearly fell into two distinct

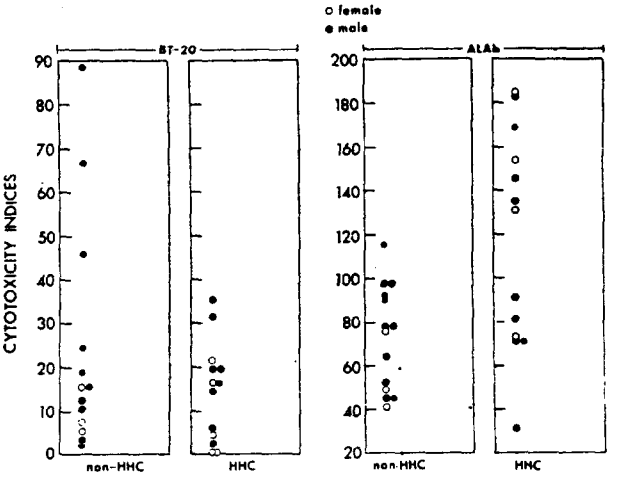

FIGURE 5 Comparison of cytotoxicity indices of 13 household contacts of patients who had active breast carcinoma either at the time of testing or within the past three years, to those of 14 normal individuals with no known exposure to patients with carcinoma. Lymphocytes were assayed on two cell lines derived from breast carcinoma, BT-20, and ALAb, and on A 498 (hypernephroma), and Te 85 (osteogenic sarcoma). Lymphocytes from all individuals whose reactions are shown in these scatter graphs showed CI in the normal range (2 SD above the mean of the normal range) on the latter two lines. Comparison of these two populations, household contacts and normals, demonstrated no statistically significant differences when assayed against BT-20. However, the reactions on ALAb were significantly different to a P value of 0.01 as compared by the Student t test, or to a level of $P = 0.002$ compared by the Fisher-Irwin test.

ranges, neither bell-shaped. They were therefore compared by the Fisher-Irwin test, in which the number proportion of normal individuals whose CI levels were greater than 120 (2 SD above the mean of the normal population) were compared with those with CI below 120, without regard to exact values. When compared in this fashion, the two populations, normal and HHC_{br}, were again found to be significantly different ($P < 0.002$).

The immunity to breast carcinoma, as measured on the ALAb line, was equally divided between men and women and, again, there was an equal percentage of genetically related household contacts and genetically unrelated household contacts (husbands, adopted children) demonstrating tumor-specific immunity (3/7 and 4/7). These populations were too small to be statistically analysed. Since the data were analyzed and the graphs made, 14 more individuals have been tested in this system and their CI results are concordant with the ranges described here.

Breast carcinoma—patients. Four patients with breast carcinoma were tested on four cell lines: ALAb, BT-20, and the two specificity control lines, Te 85 and

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A 498. All four patients had had mastectomies 2–5 yr previously. The two patients clinically disease-free at the time of testing had CI's above the normal range on the ALAb line and within the normal range on the other cell lines. The other two patients, both with large liver metastases at the time of testing, were within the normal "nonimmune" range.

DISCUSSION

Experiments were performed to determine if individuals who are exposed to patients with cancer develop tumor-specific, cell-mediated immunity. The assay used, a short term chromium-51 release assay, is highly reproducible and measures cytolysis of tumor cells mediated by peripheral blood lymphocytes, which by presently accepted parameters are T lymphocytes.

Lymphocytes from normal individuals (with no known contact with the tumors against which they were tested) were assayed for cytolytic activity against osteogenic sarcoma, hypernephroma, and breast carcinoma cells in tissue culture. Normal ranges of CI values were established for each cell line. The nonimmune range was considered to include CI values .2 SD from the mean.

Healthy household contacts of patients with osteogenic sarcoma, hypernephroma, or breast carcinoma were also tested against the cell lines. Household contacts of osteogenic sarcoma patients were found to produce a range of CI values against osteogenic sarcoma, the mean of which was significantly higher than that of the normal population ($P < 0.001$). This difference was more pronounced when the populations were corrected for tumor specificity.

Household contacts of, and patients with, breast carcinoma were tested against two cell lines derived from breast carcinoma and against two other lines (A 498, hypernephroma, and Te 85, osteogenic sarcoma) used as specificity controls. One line, ALAb, proved to be an excellent indicator for tumor-specific immunity. Almost 50% of these patients with breast carcinoma and their household contacts demonstrated CI values above the normal range. Simultaneous tests on the other line, BT-20 (equally well documented as a breast carcinoma line), demonstrated no CI values in the immune range from either group.

The values of a normal population and of the household contacts of breast carcinoma patients were found to be significantly different when analyzed with the Student t test; however, the non-normal distribution of the values of household contacts of these patients raised a question as to the value of this test. Therefore the Fisher-Irwin test for two by two tables was used to analyze these data. Again the values proved significantly different ($P < 0.002$).

Tumor-specific immunity both to breast carcinoma and osteogenic sarcoma was evenly divided between men and women. This is especially interesting in the case of breast carcinoma, in which the incidence in women is 87 per 100,000 population (in the San Francisco Bay area) and in men is 0.8 per 100,000 population, same area.[2] However, the incidence of tumor-specific immunity among the household contacts of breast carcinoma patients was much higher than that of osteogenic sarcoma patients (almost 50% as opposed to 26%), and the CI values fell in a much higher range. This difference may be methodological and may reflect the indicator cell line or it may be related to a higher incidence of breast cancer than of osteogenic sarcoma (0.4/100,000 population, San Francisco Bay area).

Tumor-specific immunity tended to cluster in households; a disproportionately high number of individuals with immunity was found in some households and none in others. The immunity was noted both in genetically related household contacts of individuals with the tumor and in unrelated household contacts (husbands, guardians, adopted children). With both tumor types, the percent of immune household contacts was almost exactly the same, whether genetically related or not.

The higher incidence of immunity clustering among individual families is probably not based on immunity against histocompatibility antigens on the test target cell, since the immunity is found in unrelated household contacts with a frequency equal to that of related household contacts. Also, no more than one member of a family ever reacted against tumors to which they were not exposed, and that was usually a multiparous mother.

This immunity is not directed against nonspecific factors such as mycoplasma, fetal calf serum antigens, or other non-tumor-associated antigens, since that type of immunity would be randomly distributed in all populations. This immunity is not found randomly in normals exposed to cancer of any type since many of the "non-household contact" controls for the carcinoma lines were household contacts of osteogenic sarcoma patients and vice versa. On no occasion was a household contact of one tumor type found to have tumor specific immunity to a different tumor type.

The primary evidence that this assay, as described, actually measured tumor-specific cell-mediated immunity to either osteogenic sarcoma or breast carcinoma rests on the clinical correlates of the assay. Breast carcinoma provides the more obvious case; of the four patients tested with breast carcinoma who had had mastectomies within the past 3 yr, two demonstrated

[2] California State Department of Public Health, California Tumor Registry, Berkeley, Calif.

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specific immunity to breast carcinoma. The remaining two patients had CI values in the low normal range and both of these individuals had extensive metastatic disease.

Osteogenic sarcoma patients demonstrated a complex pattern of immunity more fully described in another paper (21). Tumor-specific immunity was found in patients with small tumors immediately before and after amputation of the tumors and it was gradually lost after the antigenic mass was removed. Two patients demonstrated sharp increases in tumor-specific immunity after their metastatic tumors were resected, possibly because the antigen mass was serving as a "sink" for immune lymphocytes.

The hypernephroma cell line proved to be a sensitive indicator line for T-cell immunity against cellular antigens (Fig. 4), but there was no evidence of tumor immunity found with this line in patients with hypernephroma or in their household contacts. Either there is, in fact, no detectable T-cell-defined tumor-specific immunity in these individuals or, if present, it is a low incidence (since 18 household contacts and 4 patients were found negative), or such antigens on the hypernephroma tumors from different patients do not cross-react. Alternatively, the hypernephroma line A 498 may have lost the antigens responsible for tumor-specific T-cell-mediated lysis. Levy (26) reported recently that cultured tumor cells occasionally lose their cellular antigens with increasing passage levels. We have reported a case in which the antigenic density markedly decreased from the primary tumor through the different passage levels of the tumor cell line.[3] Other such cases have also been reported (27). However, patients and their household contacts were also tested with two other hypernephroma lines with the matching fibroblast cell lines and there was no difference between cytolysis on the tumor cell lines and the benign cell lines. Baldwin, Embleton, Jones, and Langman reported results of studies of tumor-specific cell-mediated immunity on patients with carcinomas of colon, rectum, breast, kidney, and lung, and a melanoma. They found that autochthonous lymphocytes from patients with the above tumors were cytotoxic, with the exception of bronchogenic carcinoma and hypernephroma. These investigators also found that 50% of breast carcinoma patients have tumor-specific cell-mediated immunity against this tumor (28).

The second case of negative reactivity was the breast carcinoma cell lines, one of which, ALAb, proved an excellent indicator for tumor-specific immunity, but the other, BT-20, did not produce CI values above the normal range with either patients or their household contacts. Sinkovics has used the BT-20 line in studies on breast carcinoma patients. He used a modification of the colony inhibition assay, which involves a 3–4 day incubation of the lymphocytes and target cells. We used a 3-h incubation with the chromium-51 release assay. He reports that this cell line was a sensitive indicator for tumor-specific immunity (29). Colony inhibition has been reported to be more sensitive than the chromium-51 release assay (detecting immunity under circumstances in which the latter is negative); but there is some question as to its specificity (30).

The reason for the discrepancies found between the two assays may be that there are two types of antigen-specific T lymphocytes. In mice these are called T-1 and T-2. The short-term chromium-51 release assay detects the activity of the most mature population, T-2 (31). The longer incubation time may allow the immature T-1 population to differentiate into mature T-2 cells. The colony inhibition assay may, therefore, be measuring both T-1 and T-2 lymphocytes.

It is interesting that transfer factor made from leukocytes that demonstrate activity by the chromium-51 release assay is capable of transferring cell-mediated immunity from donor to patient (21). This suggests that this factor may be found in the T-2 population.

The results of this study correlate with findings of other investigators. Morton and Malmgren reported that 80–90% of relatives of osteogenic sarcoma patients had antisarcoma antibodies as measured by immunofluorescence, and that 29% of the normal population also had this immunity (2). In a later report, Eilber and Morton also demonstrated antisarcoma immunity in 60–100% of relatives of patients with synovial cell sarcoma, rhabdomyosarcoma, or fibrosarcoma (4). Priori, Wilbur, and Dmochowski (32) confirmed the increased incidence of antibody to osteogenic sarcoma in relatives of these patients. Their figures, however, demonstrated an incidence of immunity in 30% of relatives and 5% in normal individuals. They also used immunofluorescence. Hellström and Hellström reported cellular immunity directed against neuroblastoma in mothers of children with this tumor (6). Since all of these studies were performed on relatives of cancer patients, they have provided indirect evidence for a transmissible agent, either horizontal or vertical, in these tumors.

This report confirms the increased incidence of immunity among contacts of patients with breast carcinoma and osteogenic sarcoma. Although this incidence is much lower than that reported by Morton and Malgren, it is almost exactly that reported by Priori et al. This may reflect either greater specificity or decreased

[3] ...rs, V. S., A. S. Levin, J. O. Johnston, A. J. Hackett, ... Fudenberg. 1975. Quantitative immunofluorescent ... of the tumor-antigen-bearing cell in giant cell sar... bone. *Cancer Res.* (revised ms submitted Jan. 1975).

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sensitivity in the various assays used. It does seem clear, however, that genetic relationship to a cancer patient is not necessary for immunity. This suggests that the immunogen is transmitted horizontally. We cannot speculate on vertical transmission from these data.

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