0022-1767/83/1316-3085$02.00/0
THE JOURNAL OF IMMUNOLOGY

Vol. 131, No. 6, December 1983
Printed in U.S.A.

SUPPRESSION OF THE RESPONSIVENESS OF LYMPHOCYTES FROM CANCER PATIENTS TRIGGERED BY CO-CULTURE WITH AUTOLOGOUS TUMOR-DERIVED CELLS[1]

MITOSHI AKIYAMA,[2] MICHAEL A. BEAN,[3] KENICHIRO SADAMOTO, YUZO TAKAHASHI,[4] AND VERA BRANKOVAN[5]

From the Viriginia Mason Research Center, Seattle, WA 98101

The question as to whether or not cancer patients have "tumor antigen"-induced suppressor T cells is of considerable interest and importance. As an approach to that question, the effect of addition of autologous irradiated tumor-derived cells (TDC) on the mixed lymphocyte response (MLR) of patients' lymphocytes (Ly) and of healthy donor Ly was tested. The rationale for these experiments was based on the fact that circulating antigen-responsive blood lymphocytes can be reactivated *in vitro* by exposure to the appropriate antigen. Thus, if there are circulating tumor "antigen"-reactive suppressor Ly, exposure to TDC as a source of the antigen should reactivate those cells. Reactivation of suppressor cells might result in diminished responsiveness to other stimuli such as alloantigens in the mixed leukocyte culture.

We found that the addition of TDC to Ly cultures produced four distinct patterns of reaction. In 26 of the 74 different patient-tumor assays, the addition of autologous TDC to the patient cultures inhibited MLR, but the addition of the same TDC to cultures of Ly from healthy donors had no effect or increased their responsiveness (*Specific Suppression*). In 21 cases, the addition of autologous TDC to the patient cultures suppressed the MLR and the addition of the same TDC to control cultures suppressed the response of some but not all the healthy donors (*Selective Suppression*). In four cases, the addition of TDC to the cultures suppressed the MLR of the patients and all of the control donors (*Nonspecific Suppression*). In 23 cases, the addition of autologous TDC resulted in no suppression of the patient MLR or of any of the simultaneously tested normal donors (*No Suppression*). When TDC of patients with noninvasive bladder cancer were added to their own Ly cultures, only four of 11 produced specific or selective suppression compared to 11 of 12 when TDC came from patients with superficially invasive cancer. These data provide indirect evidence to support the hypothesis that human tumors induce circulating suppressor cells that may be reactivated *in vitro* by co-culture with TDC.

Received for publication March 21, 1983.
Accepted for publication September 6, 1983.
The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
[1] These studies were supported by Grant CA-19165 from the National Bladder Cancer Task Force, Biomedical Research Support Grant, NIH, ROP-5588, and Grant CA-18029 from the National Cancer Institute.
[2] Present address: Radiation Effects Research Foundation, Hiroshima, Japan.
[3] Reprint requests and correspondence should be directed to Michael A. Bean, M.D., Virginia Mason Research Center, 1000 Seneca Street, Seattle, WA 98101. Dr. Bean is the recipient of a Research Faculty Award-169 from the American Cancer Society.
[4] Present address: Tokyo Medical and Dental University, Tokyo, Japan.
[5] Supported by a postdoctoral fellowship provided by Virginia Mason Research Center from a gift from the Fisher Trust. Present address: Division of Tumor Immunology, Fred Hutchinson Cancer Research Center, 1124 Columbia Street, Seattle, WA 98104.

Synthetic (1), naturally occurring (2–4), and tumor antigens (5–11) can induce antigen-specific and/or genetically restricted suppressor T cells (Ts)[6] (6) in murine models. Such Ts appear to have an important physiologic role because they induce anergy to the appropriate antigen and can facilitate tumor growth when passively transferred to naive recipients (1, 5, 10).

Although there have been many reports on the demonstration of suppressor cells of unknown specificity in humans with cancer (Ca) (12–16), relatively little is known about Ts of restricted specificity such as seen in the murine models. Recently, such Ts triggered by alloantigens or bacterial antigens were described in humans (17–23). Many of these Ts require exposure to the relevant antigen *in vitro* in order to reactivate them to suppress the lymphocyte (Ly) response of the donor to alloantigens or mitogens (20–23). Thus, we were prompted to investigate whether or not lymphocytes from Ca patients would exhibit decreased *in vitro* immune responsiveness when their Ly were co-cultured with autologous tumor-derived cells (TDC) as a source of putative tumor antigen. In this report we present evidence that Ly from some Ca patients exhibited significantly diminished MLR when their irradiated TDC were added to their cultures. By contrast, in many cases the addition of the same TDC to Ly cultures from normals or other Ca patients had no effect or enhanced their responses.

MATERIALS AND METHODS

Responder Ly. Glass defibrinated blood samples were diluted with equal volumes of phosphate-buffered saline (PBS) and centrifuged over a cushion of Ficoll-Isopaque (F-I) (specific gravity 1.076) (24). The interface was collected and washed three times before the cells were used for the assay or cryopreserved.

Preparation of stimulator cells. F-I-purified peripheral blood lymphocytes (PBL) from four healthy blood donors were cryopreserved as a standard source of stimulator cells for the one-way mixed lymphocyte culture (MLC) assay. These cells were irradiated to 2250 R before use in the assays.

Patient selection. Patients with bladder, lung, and renal Ca were included in the study. This report does not include data from experiments in which patients were treated as follows: prior irradiation or chemotherapy within 2 mo; minor surgery or anesthesia within 3 wk; or major surgery within 2 mo before the blood sample. Blood samples were drawn from the patients before preoperative medication and surgery except in a few instances in which they were drawn postoperatively after they had returned to a baseline state (3 wk in the case of minor transurethral resection of tumor, and 2 mo in the case of major surgery).

Preparation of TDC suspensions. Fresh surgical specimens were obtained under sterile conditions and immediately transported to the lab in cold PBS. After removal of any necrosis, fat, and capsule, the tumors were minced into small pieces approximately 2 mm in diameter. The medium containing spilled cells was removed and the pieces were rinsed with PBS to collect additional spilled cells. The pieces were then treated with either 0.25% trypsin (Difco, Detroit, MI) dissolved in Puck's Saline A (GIBCO, Grand Island, NY) or 0.14%

[6] Abbreviations used in this paper: Ts, suppressor T cells; Ca, cancer; Ly, lymphocytes; TC, tumor cells; TDC, tumor-derived cells; F-I, Ficoll-Isopaque; PBL, peripheral blood lymphocytes; CM, complete medium; TIL, tumor-infiltrating lymphocytes.

collagenase (Sigma, St. Louis, MO) dissolved in Hanks' balanced salt solution (GIBCO). Hard/sclerotic tumor pieces were suspended in collagenase and soft fleshy tumor pieces were suspended in trypsin solution. The pieces in enzyme solution were usually held overnight at 4°C to allow diffusion of enzyme into the pieces. Pieces were then serially digested by incubation at 37°C in enzyme solution with removal of the supernatant containing single cells followed by the addition of fresh enzyme every 30 min. This procedure was repeated until the pieces had disappeared. Dissociated TDC were washed extensively with complete medium (CM) (Eagle's minimal essential medium supplemented with 10% fetal calf serum (FCS), 100 IU penicillin, and 100 μg streptomycin/ml, 2 mM L-glutamine, and 1% nonessential amino acids). TDC were then either cryopreserved in 10% DMSO and 75% FCS in CM, or were resuspended in CM containing 10% fresh heat-inactivated normal male AB^+ human serum for the assay. Before use in the assay, the TDC were irradiated to 4500 or 6000 R by using a ^{137}cesium source. Either freshly prepared or reconstituted cryopreserved TC were used in assays. When the viability of the tumor cells in the suspension was less than 50%, an attempt was made to increase the viability by centrifugation over F-I. Experiments were not included in the data analysis presented here if the tumor cell viability was less than 50%. The mean percent viability of the tumor cells in the suspensions used in the experiments presented here was 73% for renal Ca, 85% for bladder Ca, and 77% for lung Ca. The percent (± 1 SD) of non-tumor cells in the TDC suspension was 31% ± 15 for renal Ca, 11% ± 12 for bladder Ca, and 49% ± 21 for lung Ca.

Assay for tumor-triggered suppression of the MLR. MLR assays were performed in triplicate by using 62×10^3 responder Ly and 62×10^3 pooled irradiated stimulator Ly in round-bottomed microtest plates or 125×10^3 responder and 125×10^3 pooled x-rayed stimulator Ly in microtest plates with flat-bottomed wells. Usually that number of irradiated TDC containing 62×10^3 TC were added to the round-bottomed wells and 125×10^3 TC were added to the flat-bottomed microwells yielding a 1:1 responder to tumor cell ratio. The final culture volume was 0.2 ml of CM containing 10% AB^+ serum. Cultures were incubated 6 days before labeling with 0.5 μCi of [^{3}H]thymidine/well during the terminal 18 hr. Cultures were harvested with a Skatron automatic harvester (Flow Laboratories, Rockville, MD) onto glass fiber filters, dried, and counted in a Packard Tricarb scintillation counter in PPO-POPOP toluene-based liquid scintillation fluid. Responder Ly were incubated alone, with pooled stimulator cells, with pooled stimulator cells and TDC, and with TDC only. TDC were added to the patient's own responder Ly and usually to Ly from four healthy blood donors of different HLA-DR types. In all assays, appropriate technical controls were included to rule out uptake of [^{3}H] thymidine by irradiated Ly or TC. The [^{3}H]thymidine incorporated by responder Ly stimulated with the pooled allogeneic normal Ly was the baseline positive MLR. The MLR of responder Ly stimulated with the pooled stimulators in the presence of the x-rayed TDC was the experimental response. Percent change in MLR was calculated as follows:

$$\% \text{ Change} = \frac{\text{MLR in the presence of TDC} - \text{MLR in the absence of TDC}}{\text{MLR in the absence of TDC}} \times 100$$

Within each experiment, the effect of addition of autologous TDC to the patient's MLR was compared to that of the addition of the same TDC to the MLR of each of the four normal donors tested simultaneously. Statistical significance of the percent reduction in MLR was calculated by using the Student *t*-test. A percent increase or decrease in MLR was considered real if the two-tailed test p value was ≤ 0.05.

Assay for tumor-induced suppression of the generation of cytotoxic T cells. To generate allocytotoxic lymphocytes, 62×10^3 responder Ly were incubated in triplicate for 6 days with 62×10^3 pooled allogeneic stimulator cells in the microwells as described for the one-way MLR assay. At the end of the incubation, allocytotoxicity was assessed as described elsewhere (18). Briefly, 10^4 ^{51}Cr-labeled target cells were added, followed by 4 hr of incubation and centrifugation at 1000 rpm; the culture supernatants were then harvested for determination of ^{51}Cr release. Target cells were prepared by PHA stimulation for 3 days of a pool of the same pooled Ly used as stimulator cells in the MLR. Autologous respnoder Ly stimulated with PHA in similar fashion served as control target cells. Target cells were prepared by mixing 0.1 ml of lymphoblasts in CM and 0.1 ml solution containing 2 mCi/ml ^{51}Cr (specific activity from 211 to 611 mCi/mg, New England Nuclear, Boston, MA) followed by 1-hr incubation at 37°C with occasional gentle mixing. The cells were then washed four times with CM containing 10% FCS and were resuspended to the appropriate concentration. Then, 0.05 ml of supernatant was removed from the cultures and 0.05 ml containing 10^4 labeled target cells were added to each well. Maximal ^{51}Cr release was determined by adding 0.01 ml Triton X-100 detergent to produce a final concentration of 0.5%. Percent cytotoxicity was calculated as:

$$\text{Cytotoxicity} = \frac{\text{test release} - \text{spontaneous release}}{\text{Maximal release} - \text{spontaneous release}} \times 100$$

The effect of the addition of TDC on generation of specific allocytotoxicity was assessed in the same fashion as described for measurement of the effect of addition of TDC on the MLR blastogenesis.

RESULTS

The basic experimental design used in the studies is illustrated in Table I. In addition to those combinations shown, controls demonstrating that irradiation had eliminated the [^{3}H]thymidine incorporation of the stimulator Ly and TDC were performed (data not shown). As shown in Table I, Ly from bladder Ca patient 125 exhibited a significantly depressed MLR when the patient's own irradiated TDC were added to the culture. The addition of those same TDC to each of the cultures of Ly from the four normals resulted in slight (9%) to significant (54%) enhancement of their MLR.

This specific pattern and three other patterns of suppression that were found are shown in Figure 1. The percent change in MLR was calculated as shown in Table I and as described in *Materials and Methods.* The four patterns observed were:

a. Specific suppression. Addition of autologous TDC to the cultures suppressed the MLR of the patient, but the addition of the same TDC to the cultures of the normal donors had no suppressive effect, or produced enhancement.

b. Selective suppression. Addition of autologous TDC to the patient's culture produced significant suppression of the MLR and the same TDC suppressed the response of some, but not all, of the normal control Ly responders.

c. Nonspecific suppression. Addition of TDC suppressed the response of the patient and all of the control donors.

d. No suppression. Addition of TDC to the cultures produced no suppression of either the patient's MLR or the MLR of the normal donors.

Suppression of the MLR of Ly from normal donors was never seen when the addition of the same TDC to the patient's cells did not produce suppression. When TDC suspensions were prepared with DNAase or were of poor viability (less than 50%) these patterns were not reproducible (data not shown). The number of different patient-TDC combinations exhibiting each of these patterns of suppression is summarized in Table II. Data

TABLE I

Effect of addition of TDC on MLR

Responder Lymphocytes/ Diagnosis[a]	Pooled Stimulator Lympho-cytes	#125 Tu-mor-de-rived Cells	^{3}H-TdR cpm at Day 6 of MLR[b]	% Change in Response
#125, bladder Ca	–	–	137 ± 29	
	+	–	23,931 ± 4,072	
	+	+	10,990 ± 1,483	−54[c]
	–	+	634 ± 200	
J.G., normal	–	–	1,210 ± 129	
	+	–	17,895 ± 1,139	
	+	+	19,478 ± 1,762	+9
	–	+	16,620 ± 1,153	
P.K., normal	–	–	362 ± 248	
	+	–	14,633 ± 2,632	
	+	+	16,639 ± 1,726	+14
	–	+	10,208 ± 376	
L.H., normal	–	–	170 ± 85	
	+	–	11,266 ± 2,245	
	+	+	17,349 ± 2,601	+54[c]
	–	+	10,350 ± 1,296	
M.C., normal	–	–	441 ± 353	
	+	–	12,154 ± 1,125	
	+	+	14,538 ± 93	+20[c]
	–	+	8,801 ± 1,302	

[a] 62.5×10^3 responder Ly and 62.5×10^3 pooled irradiated (2250 R) stimulator Ly were incubated with or without irradiated (4500 R) TDC containing 62.5×10^3 TC in triplicate for 6 days.

[b] Mean cpm ± 1 SD. ^{3}H-TdR.

[c] $p < 0.05$.

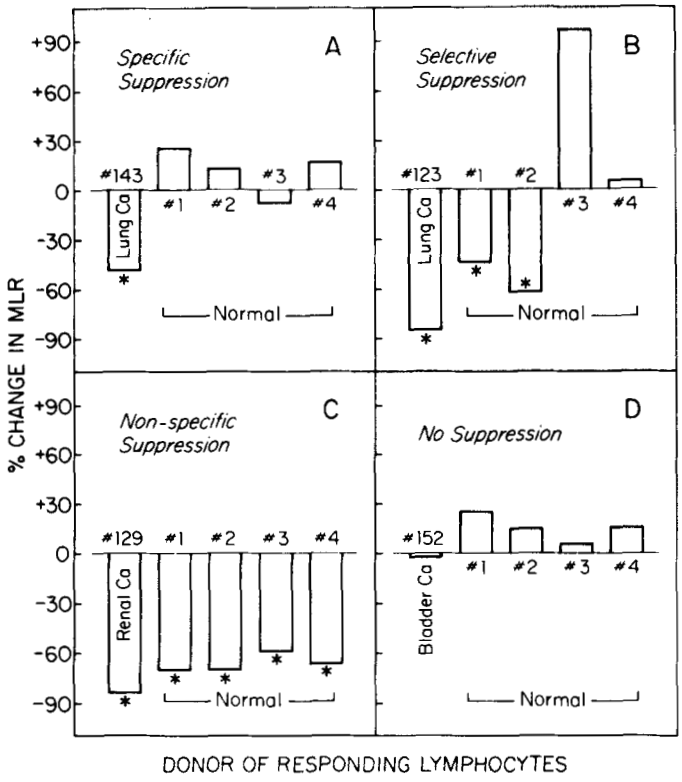

Figure 1. Four patterns of suppression of the MLR induced by addition of autologous TDC to the patient's Ly and to the Ly of four normal donors. Responder to stimulator cell to TC ratio was 1:1:1. * = $p < 0.05$.

TABLE II

Effect of addition of TDC on MLR

Type	Number of Patients	Pattern Resulting from Addition of TDC to MLR Cultures			
		Specific suppression	Selective suppression	Nonspecific suppression	No suppression
Transitional cell Ca[a]	29	12	6	0	11
Renal Ca	25	8	8	3	6
Lung Ca	20	6	7	1	6
Total (%)	74 (100)	26 (35)	21 (28)	4 (5)	23 (32)

[a] Includes three patients with transitional Ca of renal pelvis. Remainder are bladder Ca patients.

are presented here for 74 patients. These 74 were included in the analysis as the data are derived from comparable sets of experiments; i.e., a) assays were performed at similar responder to TC ratios; b) TDC were prepared by trypsin or collagenase dissociation; c) TC viability was always greater than 50%; and d) the patient blood samples were drawn preoperatively or during a baseline state when the patients were receiving no therapy (*see Materials and Methods*). Of 74 patient-TDC combinations tested in this way, 26 (35%) showed *Specific suppression*, 21 (28%) *Selective suppression*, 4 (5%) *Nonspecific suppression*, and 23 (32%) exhibited *No suppression.*

The addition of TDC to MLR resulting in the pattern of *Selective suppression* (Fig. 1*B*) was an unanticipated result that requires further investigation. The TDC suspensions producing this pattern contained an average of 29% contaminating tumor-infiltrating mononuclear leukocytes (TIL). These TIL might have contributed to the suppression of the MLR of Ly from the patients and from some of the normal donors because TIL have been reported to suppress the response of autologous PBL to PHA (16). In that report, suppressor TIL were still active after treatment with mitomycin C, but data are not available as to whether the suppressor function of TIL can withstand the 4500 to 6000 R used in the present study for inactivation of DNA synthesis of the TDC suspensions containing the TIL. The 29% ± 21 ($\bar{X} \pm 1$ SD) of TIL contaminating the suspensions producing *Selective suppression*, however was not significantly different from the percent TIL in the suspensions producing *Specific* (34% ± 24), *Nonspecific* (31% ± 23), or *No suppression* (25% ± 20), and when these comparisons were performed within the tumor types, there was no significant difference in TIL contamination of the suspensions producing the different patterns (data not shown). Thus, the addition of TDC to Ly cultures of patients and normals produced patterns of interest that were consistent with immunologic reactions in 63% of the 74 cases (26 *Specific* and 21 *Selective suppression*) and produced *No suppression* in 23 cases.

With four patients we had the opportunity to perform criss-cross experiments with TDC preparations and Ly from more than one patient in the same experiment. Such an experiment is presented in Table III. When TDC from lung Ca patient 130 were added to the patient's own Ly culture, the MLR was significantly depressed (38%). When the same TDC were added to Ly cultures from renal Ca patient 131, however, or to cultures of any of the four normal donors, there was an enhancement of the MLR. Conversely, when TDC from patient 131 were added to the patient's own Ly, there was a significant decrease in MLR, whereas the same TDC produced no suppression when added to cultures from patient 130 or the normal donors. In another experiment (data not shown), we were able to test TDC from two renal Ca patients simultaneously, each suppressed the autologous MLR but not the MLR of the control patient or the normal donors. These data suggest that there is specificity

TABLE III

Effect of addition of TDC on MLR

Responder Lymphocytes/ Diagnosis[a]	Pooled Stimulator Lymphocytes	Tumor-derived Cells		^{3}H-TdR cpm at Day 6 of MLR[b]	% Change in Response
		#130	#131		
#130, lung Ca	+	−	−	28,953 ± 2,651	
	+	+	−	18,985 ± 1,801	−34[c]
	+	−	+	31,714 ± 2,940	+10
	−	+	−	6,947 ± 959	
	−	−	+	24,237 ± 1,209	
#131, renal Ca	+	−	−	21,939 ± 1,466	
	+	+	−	28,047 ± 1,125	+28[c]
	+	−	+	15,781 ± 2,171	−28[c]
	−	+	−	27,422 ± 2,662	
	−	−	+	1,639 ± 1,133	
J.G., normal	+	−	−	25,430 ± 1,116	
	+	+	−	37,601 ± 1,956	+48[c]
	+	−	+	37,728 ± 1,680	+48[c]
	−	+	−	25,216 ± 1,476	
	−	−	+	27,296 ± 1,474	
P.K., normal	+	−	−	20,799 ± 1,164	
	+	+	−	26,597 ± 1,357	+28[c]
	+	−	+	33,806 ± 1,206	+63[c]
	−	+	−	22,169 ± 367	
	−	−	+	24,297 ± 1,686	
L.H., normal	+	−	−	18,808 ± 1,408	
	+	+	−	32,693 ± 2,235	+74[c]
	+	−	+	36,459 ± 2,468	+94[c]
	−	+	−	29,083 ± 2,235	
	−	−	+	24,669 ± 1,072	
M.C., normal	+	−	−	15,370 ± 159	
	+	+	−	23,299 ± 2,972	+52[c]
	+	−	+	25,127 ± 516	+63[c]
	−	+	−	20,048 ± 2,071	
	−	−	+	17,273 ± 1,392	

[a] 62.5×10^3 responder Ly and 62.5×10^3 pooled irradiated (2250 R) stimulator Ly were incubated with or without irradiated (6000 R) TDC containing 62.5×10^3 TC in triplicate for 6 days.

[b] Mean cpm ± 1 SD. ^{3}H-TdR.

[c] $p < 0.05$.

involved in triggering the suppression of MLR.

Although the criss-cross experiments and the pattern of *Specific* and *Selective suppression* would seem to rule out the possibility of the TDC producing a nonspecifically toxic factor(s) (25, 26) that suppresses the MLR, the question remains as to the role of the major histocompatibility complex in this reaction. That is, autologous TDC were added to the cultures of the patient Ly, whereas the addition of those same TDC to Ly of control donors is an allogeneic combination. Thus, one might argue that the allogeneic stimulus provided to the normal Ly cultures is enough to offset a slight toxic or nonspecific suppressive effect of the TDC suspension, a stimulus that would be absent in the autologous patient-tumor combination. In murine models, allogeneic effects can be avoided by using cells from syngeneic animals immune to unrelated antigens as control responders. Devising such controls in humans is more difficult. We attempted to control for this difference by our selection of normal donors of different HLA-DR types so as to provide by chance some DR compatibility between the patient's TDC and the control Ly, and by attempting to find healthy identical twins of patients with solid tumors for these assays. Although analysis of the data has not been completed, we have not been able to demonstrate any increased HLA compatibility between the Ly of the patients and normal donors that are suppressed selectively.

The results of the one experiment in which a patient's identical twin was available for study are illustrated in Figure 2. The HLA identity of bladder Ca patient 128 and her identical twin was confirmed by HLA typing and mutual lack of responsiveness in one-way MLC. The addition of her TDC to her autologous Ly produced significant suppression of the MLR. The addition of those same TDC to Ly from her identical twin did not produce significant suppression. The addition of the patient's TDC to that of the normal donors also did not produce significant suppression. This experiment was repeated and the finidng was confirmed by using crypopreserved cells. These data would argue that the suppression observed, although slight, is disease related.

The MLR was chosen as a measure of *in vitro* immune responsiveness for this study because in addition to blastogenesis, the development of allocytotoxic T cells can be monitored, a phenomenon that is generally accepted as a specific *in vitro* immune function. Figure 3 illustrates an experiment in which there were sufficient TDC available from a patient with a pattern of *Specific suppression* to repeat the MLR and assay for the effects of addition of TDC on the generation of cytotoxic T cells. TDC were added to Ly cultures from the patient and normal donors in the MLR assay and simultaneously replicate cultures were performed for assessment of the effects of the addition of TDC on the generation of allocytotoxicity. In this example, the

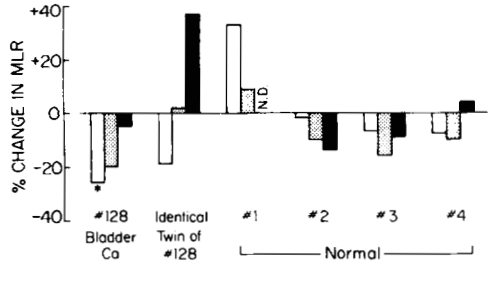

Figure 2. TDC from patient 128 with bladder Ca were added to the patient's Ly and Ly from her identical twin in addition to Ly from normal donors 1 through 4. 62×10^3 responder Ly were incubated with 62×10^3 stimulator cells with or without (▭) 62×10^3 TC; ▭ 32×10^3 TC; and ▬ 15×10^3 TC. * = $p < 0.05$.

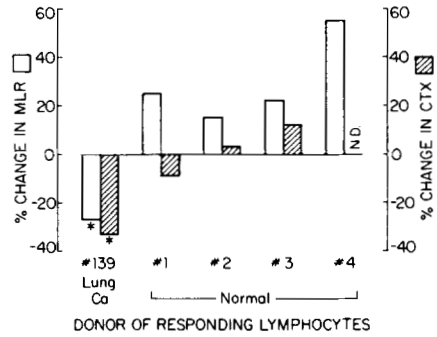

Figure 3. TDC from lung Ca patient 139 were added to replicate Ly cultures to determine their effect on the MLR and the effect of addition of these TDC on the ability to generate allocytotoxicity. * = $p < 0.05$.

addition of autologous TDC suppressed both the MLR and the generation of allocytotoxicity of Ly from lung Ca patient 139. The addition of the same TDC to the cultures from the normal donors enhanced the MLR and did not significantly affect the generation of cytotoxicity.

Extensive analysis of the relationship of the four patterns of MLR resulting from the addition of TDC to stage and course of disease must await accession of meaningful numbers and long term follow-up of patients with each type of Ca and stage of disease. Of the 29 transitional cell Ca patients in this study, however, there were 11 patients with papillary noninvasive Ca (stage 0) and 12 patients with papillary Ca invasive into the lamina propria (stage A), or muscle (stage B) of the bladder available for comparison. Only four of 11 stage 0 noninvasive Ca patients showed patterns of *Specific* or *Selective suppression* of the MLR when autologous TDC were added to their Ly cultures compared with 11 of the 12 stage A and B invasive Ca patients ($p < 0.05$). The TC viability was equal as was the non-tumor cell content (7% ± 5 *vs* 10% ± 9) of the TDC suspensions made from the two groups of patients.

DISCUSSION

In 26 of 74 cases, the addition of patients' TDC to autologous Ly resulted in patterns of suppression of the proliferative response similar to that seen in murine models when the relevant antigen is added to cultures (2, 3, 7, 8). In those cases, addition of the same TDC to Ly cultures from normal donors did not suppress, and sometimes enhanced, their MLR responsiveness. In 21 other cases, the addition of TDC suppressed the patient's MLR and also suppressed some, but not all, of the control Ly responses, an unexpected finding. In another 23 cases there was no suppression of the patients' or normal donors MLR when TDC were added to the cultures. In four cases, the addition of TDC resulted in *Nonspecific suppression*, i.e., the patient Ly and all the normal donor Ly were suppressed.

An especially intriguing finding was that only four of 11 patients with noninvasive bladder Ca had patterns of *Specific* or *Selective suppression* compared to 11 of 12 patients with superficially invasive Ca ($p < 0.05$). Whether this finding means there must be systemic exposure to the tumor to induce such cells or whether there is an inherent difference in the tumors themselves is not known.

There have been several recent developments that prompted us to perform these studies. Studies with murine models have demonstrated immunoregulatory cells arising as a consequence of exposure to nonoptimal doses of antigen, altered antigens, or prolonged exposure to antigens (1). Most important, such suppressor cells have been found in both viral and chemically induced murine tumor models and can be demonstrated *in vitro* by co-culture of Ly from tumor-bearing hosts with the relevant

tumor cells (7, 8). In addition, *in vivo* these Ts abrogate the cellular immune response to immunogenic tumors and facilitate tumor growth (5, 6, 8–11). Recently, there have been reports of similar antigen-specific suppressor cells reactive to bacteria and alloantigens in man (17–21, 27). These demonstrations of antigen-specific and/or genetically restricted Ts in humans to non-Ca antigens along with the unequivocal demonstration of such cells in the murine models prompted us to look for such cells in humans with Ca.

The preliminary studies presented here would argue that tumor-induced suppressor cells exist in patients with Ca in addition to the suppressor cells of unknown specificity that have been demonstrated in blood and tumors (13–16). In those studies, blood Ly or TIL were tested either with or without mitogen activation rather than after antigen exposure for their ability to suppress the proliferative response of blood Ly from the patients or from healthy individuals. There are no published studies of which we are aware in which investigators have determined if addition of autologous TDC to the patient's blood Ly would diminish the immune responsiveness of those Ly as reported in this study. There have been a number of reports on partial success in attempts to generate or reactivate cytotoxic T cells by co-culture of patients' Ly with autologous TC at the Ly to TC ratios used here (28, 29). Those reports, however, do not discuss whether or not a diminished blastogenic response in Ly cultures containing TC and proliferative stimuli was ever observed. Perhaps the inability to generate tumor-directed cytotoxic cells in some of the patient cultures in those studies was due to reactivation of suppressor cells.

The possibility that the suppression reported here is due to "nonspecific" toxic effects by the TC suspension was considered and found to be unlikely. If the suppression was due to a nonspecific toxic factor (25) or prostaglandin (26), thymidine, etc., all the Ly donors' MLR would be decreased and the selective and specific patterns would not be seen. Also, the experiments with the identical twins should not show specificity. If the suppression of MLR was due to the responding Ly being stimulated to release interferon, suppression of MLR could occur, but *in vitro* generation of allocytotoxicity should be unaffected or enhanced rather than suppressed (30, 31) (see Fig. 3). The TIL content of the suspensions producing the different patterns of suppression was not significantly different, which eliminates crowding as an explanation for the decrease in response seen in some and not in other patients.

Another possibility to be considered, not eliminated by the one set of identical twins, is that the diminished immune responsiveness observed in some patient and normal Ly populations when cultured with TC might be indicative of an immune response/suppressor gene triggered by the TC and not a consequence of prolonged antigenic stimulation generating suppressor cells. Although such genes have been described in murine models (1), it is only recently that such a phenomenon has been described in humans (23, 32). Of particular interest is the report by Fainboim *et al.* (23), who demonstrated the presence of an HLA-linked suppressor-activating determinant(s) that triggers some Ly to fail to respond in MLC. Removal of $OKT8^+$ cells (a subset known to contain suppressor cells) from the nonresponder Ly converted the remaining Ly to responders.

To prove formally that tumor-induced Ts or that TC bearing suppressor-activating determinants exist in humans, further studies are needed. Additional studies on identical twins, families, and HLA-identical siblings will be informative. Controls for specificity with the use of "normal" tissue cells are difficult. With some renal Ca patients we have attempted to compare the effects of addition of normal cells to the effects of addition of TC on the MLR of the patient and controls. Enzyme-dissociated irradiated normal kidney cells were uniformly toxic to lymphocyte cultures, however, resulting in decreased Ly cell numbers and viability (unpublished data). Selective deletion of a subset of responding Ly resulting in restoration of responsiveness in MLR or in the ability to generate cytotoxic T cells in the presence of TC would be convincing evidence of such suppressor cells.

Given the assumption that some human tumors are antigenic (33), along with the knowledge of the signals necessary for induction of Ts in murine models, the presence of a tumor growing in a human over time should induce the generation of such suppressor cells *in vivo*. Generation of suppressor cells would eventually result in an "eclipse" or diminution of the effector phase of the immune response against the tumor allowing for more rapid growth and the development of metastasis.

Acknowledgments. We especially thank Heather Huppe, Vicki McDonald, and Lyle Sorensen for their excellent technical assistance; Harriet Langsford for preparation of the manuscript; and Drs. R. P. Anderson, G. Brannen, R. Correa, R. Gibbons, L. Hill, and J. A. Hansen for their cooperation in these studies.

REFERENCES

1. Germain, R. N., and B. Benacerraf. 1980. Helper and suppressor T cell factors. Springer Semin. Immunopathol. 3:93.
2. Brondz, B. D., A. V. Karaulov, I. F. Abronia, and Z. K. Blandova. 1981. Requirements for induction of specific suppressor T cells and detection of their H-2 antigen-binding receptors by fractionation on target cell monolayers. Scand. J. Immunol. 13:517.
3. Rich, S. S., and R. R. Rich. 1976. Regulatory mechanisms in cell-mediated immune responses. III. 1976. I region control of suppressor cell interaction with responder cells in mixed lymphocyte reactions. J. Exp. Med. 143:1672.
4. Tada, T., M. Taniguchi, and C. S. David. 1976. Properties of the antigen-specific suppressor T-cell factors in the regulation of the antibody response of the mouse. IV. Specific subregion assignment of the gene(s) that codes for the suppressive T-cell factor in the H-2 histocompatibility complex. J. Exp. Med. 144:713.
5. North, R. J. 1982. Cyclophosphamide-facilitated adoptive immunotherapy of an established tumor depends on elimination of tumor-induced suppressor T cells. J. Exp. Med. 155:1063.
6. Enker, W. E., and J. L. Jacobitz. 1980. *In vivo* splenic irradiation eradicates suppressor T-cells causing the regression and inhibition of established tumor. Int. J. Cancer 25:819.
7. Glaser, M., H. Kirchner, and R. B. Herberman. 1975. Inhibition of *in vitro* lymphoproliferative response to tumor-associated antigens by suppressor cells from rats bearing progressively growing Gross leukemia virus-induced tumors. Int. J. Cancer 16:384.
8. Greene, M. 1980. The genetic and cellular basis of regulation of the immune response to tumor antigens. *In* N. Warner, ed. Contemporary Topics in Immunology, Vol. 11. Plenum Press, New York. Pp. 81–116.
9. Greene, M. I., M. E. Dorf, M. Pierres, and B. Benacerraf. 1977. Reduction of syngeneic tumor growth by an anti-I-J alloantiserum. Proc. Natl. Acad. Sci. USA 74:5118.
10. Kinsky, R. G., H. T. Duc, G. Chaouat, and G. A. Voisin. 1981. Involvement of suppressor cells in active enhancement of allografted tumors. *In vivo* (adoptive transfer) and *in vitro* (MLR) evaluation. Synergy with enhancing antibodies. Cell. Immunol. 58:107.
11. Reinisch, C. L., S. L. Andrew, and S. F. Schlossman. 1977. Suppressor cell regulation of immune response to tumors: abrogation by adult thymectomy. Proc. Natl. Acad. Sci. USA 74:2989.
12. Broder, S., and T. A. Waldmann. 1978. The suppressor network in cancer. N. Engl. J. Med. 299:1335.
13. Engleman, E. G., C. Benike, R. T. Hoppe, and H. S. Kaplan. 1979. Suppressor cells of the mixed lymphocyte reaction in patients with Hodgkin's disease. Transplant. Proc. 11:1827.
14. Hersh, E. M., Y. Z. Patt, S. G. Murphy, *et al.* 1980. Radiosensitive, thymic hormone-sensitive peripheral blood suppressor cell activity in cancer patients. Cancer Res. 40:3134.
15. Jerrells, T. R., J. H. Dean, G. L. Richardson, J. L. McCoy, and R. B. Herberman. 1978. Role of suppressor cells in depression of *in vitro* lymphoproliferative responses of lung cancer and breast cancer patients. JNCI 61:1001.
16. Vose, B. M., and M. Moore. 1979. Suppressor cell activity of lymphocytes infiltrating human lung and breast tumurs. Int. J. Cancer 24:579.
17. Bean, M. A., Y. Kodera, K. B. Cummings, and B. R. Bloom. 1977. Occurrence of restricted suppressor T-cell activity in man. J. Exp. Med. 146:1455.
18. Bean, M. A., M. Akiyama, Y. Kodera, B. Dupont, and J. A. Hansen. 1979. Human blood T lymphocytes that suppress the mixed leukocyte culture

reactivity of lymphocyte from HLA-B14-bearing individuals. J. Immunol. 123:1610.
19. Brankovan, V., M. A. Bean, P. J. Martin, *et al.* 1983. The cell surface phenotype of a naturally occurring human suppressor T-cell of restricted specificity: definition by monoclonal antibodies. J. Immunol. 131:175.
20. Mehra, V., L. H. Mason, J. P. Fields, and B. R. Bloom. 1979. Lepromin-induced suppressor cells in patients with leprosy. J. Immunol. 123:1813.
21. Engleman, E. G., J. M. Andrew, and H. O. McDevitt. 1978. Suppression of the mixed lymphocyte reaction in man by a soluble T-cell factor. J. Exp. Med. 147:1037.
22. Thomsen, A., E. Dickmeiss, B. K. Jakobsen, P. Platz, L. P. Ryder, and A. Svejgaard. 1978. Low responsiveness in MLC induced by certain HLA-A antigens on the stimulator cells. Tissue Antigens 11:449.
23. Fainboim, L., D. Jaraquemada, H. Festenstein, and J. A. Sachs. 1981. MHC-specified lymphocyte activating and suppressor activating determinants in human mixed lymphocyte reactions. Scand. J. Immunol. 14:655.
24. Boyum, A. 1968. Isolation of mononuclear cells and granulocytes from human blood. Scand. J. Clin. Lab. Invest. 21:77.
25. Werkmeister, J., R. Zbroja, W. McCarthy, and P. Hersey. 1980. Detection of an inhibitor of cell division in cultures of tumour cells with immunosuppressive activity *in vitro.* Clin Exp. Immunol. 40:168.
26. Goodwin, J. S., A. D. Bankhurst, and R. P. Messner. 1977. Suppression of human T cell mitogenesis by prostaglandin. Existence of a prostaglandin-producing suppressor cell. J. Exp. Med. 146:1719.
27. Rocklin, R. E., A. L. Sheffer, D. K. Greineder, and K. L. Melmon. 1980. Generation of antigen-specific suppressor cells during allergy desensitization. N. Engl. J. Med. 302:1213.
28. Vanky, F., S. Argov, and E. Klein. 1981. Tumor biopsy cells participating in systems in which cytotoxicity of lymphocytes is generated. Autologous and allogeneic studies. Int. J. Cancer 27:273.
29. Zarling, J. M., P. C. Raich, M. McKeough, and F. H. Bach. 1976. Generation of cytotoxic lymphocytes *in vitro* against autologous human leukemia cells. Nature 262:691.
30. Kadish, A. S., F. A. Tansey, G. S. M. Yu, A. T. Doyle, and B. R. Bloom. 1980. Interferon as a mediator of human lymphocyte suppression. J. Exp. Med. 151:637.
31. Fradelizi, D., and I. Gresser. 1982. Interferon inhibits the generation of allo-specific suppressor T lymphocytes. J. Exp. Med. 155:1610.
32. Nishimura, Y., and T. Sasazuki. 1983. Suppressor T cells control the HLA-linked low responsiveness to streptococcal antigen in man. Nature 302:67.
33. Old, L. J. 1981. Cancer immunology: the search for specificity—G. H. A. Clowes memorial lecture. Cancer Res. 41:361.