[CANCER RESEARCH 35, 2729-2737, October 1975]

Effects of Human Sera on Reactivity of Lymphocytes in Microcytotoxicity Assays[1]

George E. Pierce and Bonita L. DeVald

University of Kansas Medical Center, Kansas City, Kansas 66103

SUMMARY

The effects of cancer patient and normal donor serum samples on the reactivity of patient and normal lymphocytes against both normal and malignant target cells were studied in microcytotoxicity assays. There were 17 of 140 cancer patient serum samples and 7 of 116 normal donor lymphocyte samples that selectively increased the growth of target cells in the presence of lymphocytes. This effect was most often noted with cancer patient serum against cultured tumor cells, but the effect was also noted against fibroblasts and with normal serum against both fibroblasts and tumor cells. Of 140 cancer-patient serum samples, 11 selectively decreased target cell survival in the presence of lymphocytes compared to medium and compared to other serum samples. In the absence of lymphocytes these serum samples were nontoxic. The effect was not observed with any of the normal serum samples studied. The lymphocyte-dependent serum toxicity appeared to be selectively directed against tumor target cells.

INTRODUCTION

Many studies have suggested that lymphocytes from cancer patients may be specifically toxic on autochthonous tumors or allogeneic tumors of the same histological type when tested in microcytotoxicity assays (1, 2, 6–8). Serum factors appear to exert important modulating effects on lymphocyte reactivity in these assays: serum from patients with extensive or with progressively growing tumors often blocks lymphocyte-mediated cytotoxicity (3, 5, 11, 12, 14, 15), but serum from certain patients, who are apparently cured or without evidence of disease when tested, may possess "unblocking" activity (9) or lymphocyte-"arming" activity (10).

Other investigators, however, have reported that lymphocytes from normal persons may be frequently toxic when tested in microcytotoxicity assays and that lymphocytes from cancer patients, although toxic, may not be specifically toxic on tumors (13, 16, 21, 22).

The results of microcytotoxicity assays of human tumor immunity obtained in this laboratory have been recently published (17). With the effector cell-to-target cell ratios used in these studies, most lymphocyte samples were slightly toxic when compared to the effects of medium only on target cell survival. There was, however, significant variation in the level of toxicity observed with different lymphocyte samples tested against any 1 target cell. The increased level of toxicity observed with certain lymphocyte samples was frequently selective, *i.e.*, noted against 1 or more but not against all target cells tested. Selectivity was noted with normal lymphocytes as well as with cancer patient lymphocytes and was noted against normal fibroblast as well as against tumor target cells. However, selective toxicity against tumor cells *versus* fibroblasts was noted more often with cancer patients than with normal lymphocytes. Furthermore, cancer patient lymphocytes were more frequently toxic on tumor cells but no more frequently toxic on fibroblasts than were normal lymphocytes, although there was little evidence for tumor type specificity in the toxicity noted with patient lymphocytes.

In the present communication the effects of serum samples from cancer patients and normal donors on lymphocyte reactivity in microcytotoxicity assay are reported.

MATERIALS AND METHODS

Lymphocytes. A total of 102 lymphocyte samples from 85 cancer patients and 104 lymphocyte samples from 48 normal volunteers were tested against cultured target cells. The cancer patients ranged from 32 to 72 years in age with a median age of 61 years. A variety of histological tumor types were represented, but 80% of the patients had either bronchogenic carcinoma or malignant melanoma. The normal volunteers were medical students or laboratory technicians with no history of significant illness. They ranged in age from 22 to 61 years with a median age of 29 years. Three-fourths of both groups were males.

Lymphocytes were separated from fresh heparinized blood by sedimentation with plasmagel and centrifugation over Ficoll-Hypaque (17). The final preparations contained 80 to 95% lymphocytes, 5 to 20% non-lymphocytes (primarily monocytes), very few polymorphonuclear leukocytes, and no erythrocytes. Lymphocyte viability counts ranged from 98 to 100% as determined by trypan blue exclusion; final concentrations were adjusted to 7.5×10^5 lymphoid cells/ml Waymouth's Medium MB-752/1 (Grand Island Biological Co., Grand Island, N. Y.).

Serum Samples. Serum samples were obtained from fresh clotted venous blood and were heat inactivated at 56° for 30 min prior to use. All serum samples were obtained and

[1] Supported by American Cancer Society Grant IC-54 and USPHS Grant CA-14775-02.

Received April 10, 1975; accepted June 13, 1975.

tested concomitantly with the lymphocyte samples.

Target Cells. Fresh sterile pieces of human tumors and skin were obtained at operation and grown in tissue culture. The culture techniques have been described (17). Forty-four different fresh tumor cultures were used as targets in the assays. The cultures were not continuously maintained for periods longer than 10 weeks; for subsequent use they were frozen in 15% dimethyl sulfoxide in medium and stored in liquid nitrogen. All target cells were cultured for *Mycoplasma;* only negative cultures were used in the assays.

Microcytotoxicity Assays. The Hellström microcytotoxicity assay (8, 20) was used. Target cell cultures, which had not yet reached confluence, were trypsinized and added to the wells of Falcon No. 3040 microtest plates in amounts of 150 or 300 cells/well. After 18 hr a majority of the target cells became attached to the bottoms of the wells, and the medium containing debris and non-attached cells was decanted. Heat-inactivated serum samples diluted 1:6 in Waymouth's medium were then added in amounts of 0.1 ml/well. Groups of wells incubated with medium only were included. After a 45-min incubation the sera were decanted, and lymphocytes in amounts of 1.5×10^5 were added in 0.2 ml Waymouth's medium per well. Groups treated with medium only, *i.e.*, no lymphocytes, were included.

Eight replicate wells were included for each group. After a 2-hr incubation, heat-inactivated normal AB+ human serum was added to a final concentration of 10%. Following a 40-hr incubation the plates were washed and stained with crystal violet, and the surviving (attached) target cells were counted with stereoscopic magnification. Each well was identified by a code which was broken only after completion of the counts. For each replicate group of wells the mean target cell count/well and its standard error were calculated, and the statistical significance of differences between the mean cell counts of any 2 different groups was determined by Student's *t* test.

For the purposes of this analysis an "experiment" is defined as the study of the effects of a group of lymphocyte samples, including 1 or 2 normal and 1 to 3 patient samples, tested simultaneously against 1 to 4 different target cultures. A "test" is defined as that fraction of an experiment in which the lymphocytes were studied against 1 particular target. Each test, therefore, is comprised of 1 or 2 normal lymphocyte-target cell "combinations" and 1 to 3 patient lymphocyte-target cell combinations. Each combination was studied after preincubation with medium, with 1 or 2 normal serum samples, and with 1 to 3 patient serum samples. In each experiment the lymphocyte and serum donors were the same (Table 1).

A total of 72 experiments were performed, each on a

Table 1

Survival of target cells in microcytotoxicity assays after preincubation with serum and subsequent exposure to lymphocytes

Results of experiment on September 7, 1973, demonstrating decreased survival of bronchogenic carcinoma target cells (HTO72) after exposure to lymphocyte samples from 2 cancer Patients, P_1 and P_2, in the presence of medium or normal serum but no decreased survival after exposure to these lymphocyte samples in the presence of serum from Patient P_2 (value in brackets). This effect was not observed against normal skin fibroblasts (HSO72) derived from the donor of HTO72. P_2 serum alone, in the absence of lymphocytes, did not promote survival of HTO72 cells compared to medium or other serum samples.

Target cells	Donor of lymphocytes	Target cell counts[a] after preincubation[b] with serum from			
		M[c]	N_1[d]	P_1	P_2
HT072	0[e]	108.6 ± 6.5[f]	117.2 ± 4.8	114.4 ± 5.4	112.6 ± 9.1
	N_1	81.7 ± 4.9	105.6 ± 3.6	101.9 ± 3.4	99.4 ± 5.0
	P_1	53.8 ± 3.2	79.6 ± 7.0	58.1 ± 2.8	72.7 ± 3.3
	P_2	67.1 ± 5.5	80.0 ± 4.4	75.9 ± 2.8	[100.7 ± 5.6]
HS072	0	181 ± 11	213 ± 12	226 ± 11	232 ± 27.3
	N_1	172 ± 11	182 ± 11	184 ± 4.9	178 ± 7.3
	P_1	122 ± 11	121 ± 7.4	116 ± 7.1	99 ± 4.8
	P_2	161 ± 13	164 ± 6.3	175 ± 9.0	164 ± 9.4

[a] Mean target cell count of 8 replicate wells.

[b] Target cells were preincubated for 45 min with 1:6 dilutions of heat-inactivated human serum samples. The dilute sera were then decanted and lymphocytes were added in amounts of 150,000/well. The plates were incubated for an additional 40 hr and then rinsed and stained, and the surviving target cells were counted.

[c] Wells preincubated in Waymouth's medium with 20% fetal calf serum; no human serum added.

[d] N_1, normal donor of serum and lymphocytes; P_1, Patient 154, a 56-year-old male who, at 2 months postoperative resection of bronchogenic carcinoma, showed no evidence of recurrence when tested; P_2, Patient 106, a 42-year-old male who, at 1 month postoperative resection of bronchogenic carcinoma, showed no evidence of recurrence when tested; target cells (HT072) are cultured bronchogenic carcinoma cells: target cells (HS072) are fibroblasts cultured from the normal lung of the donor of tumor HT072.

[e] In this group of wells plain medium was added in lieu of lymphocytes; *i.e.*, no lymphocytes were added.

[f] Mean ± S.E.

different day. A total of 140 cancer-patient and 116 normal volunteer serum samples were studied in 123 tests against tumor and 61 tests against fibroblast target cells.

RESULTS

Effects of Serum Only (*i.e.*, No Lymphocytes Added) on Target Cell Survival. In only 3 of 123 tests against tumor cells, and in only 1 of 61 tests against fibroblasts, was there a significant difference ($p < 0.05$) greater than 20% between mean target cell counts after exposure to one *versus* any other serum sample studied in the same test. In other words, different cancer patient and normal serum samples had remarkably constant or similar effects on target cell survival when the samples were heat inactivated and added to the target cells as 1:6 dilutions in medium for 45 min; target cells were then incubated for 40 hr in medium only, *i.e.*, without lymphocytes.

Effects of Serum on Lymphocyte Reactivity against Target Cells. Preincubation with certain serum samples, however, appeared to alter selectively the effect of lymphocyte samples on target cell survival. In 22 of 72 experiments, preincubation with a serum sample significantly increased (greater than 20% with $p < 0.05$) the mean cell count of 1 of the targets after exposure to a certain lymphocyte sample, when compared to the mean target cell counts obtained with that lymphocyte sample following preincubation with medium or with other serum samples.

The results of such an experiment are presented in Table 1. In this experiment lymphocytes from 2 lung cancer patients inhibited the growth of cultured bronchogenic carcinoma cells when tested following preincubation with medium and certain serum samples. Lymphocytes from the 1st cancer patient, P_1, were also toxic on skin fibroblasts derived from the target tumor donor, but the lymphocytes from the 2nd cancer patient were selectively toxic only on the tumor cells. This selective lymphocyte-mediated toxicity was blocked by preincubation with serum from the 2nd patient, P_2. In other words, preincubation with this patient's serum sample selectively increased the mean target cell count of wells exposed to his lymphocytes, when compared to preincubation with medium or with other serum samples.

Such an effect, however, was not observed just with cancer patient sera; the effect was also noted with certain normal sera. Furthermore, the reactivity of normal as well as of cancer patient lymphocytes was subject to modulation by serum samples, and the survival of fibroblasts as well as tumor target cells was increased.

Table 2 shows the results of a test on February 29, 1973, against normal skin fibroblasts HS032. In this test lymphocytes from normal donor N_2 were toxic against allogeneic skin fibroblasts, compared to lymphocytes from Normal Donor N_1 or Patient P_1, when added after preincubation with medium or with N_2 or P_1 serum. After preincubation with N_1 serum, however, N_2 lymphocytes were no longer toxic; the mean target cell count was increased, compared to the counts obtained after preincubation with medium or N_2 or P_1 serum, prior to addition of N_2 lymphocytes. Thus a blocking effect was observed with normal serum which abrogated the toxic effect of normal lymphocytes against a normal (nonmalignant) target cell.

Table 2

Survival of target cells in microcytotoxicity assays after preincubation with serum and subsequent exposure to lymphocytes

Results of test on February 29, 1973, demonstrating decreased survival of cultured skin fibroblasts (HS032) with normal lymphocytes N_2 compared to normal lymphocytes (N_1) or patient lymphocytes (P_1) in the presence of medium or N_2 or P_1 serum. In the presence of N_1 serum, however (value in brackets), there was no significant decrease in survival of HS032 fibroblasts after exposure to N_2 lymphocytes compared to N_1 or P_1 lymphocytes. A growth-promoting effect with normal serum N_1 was not observed on bronchogenic carcinoma cells (HT046).

Target cells	Donor of lymphocytes	Target cell counts[a] after preincubation[b] with serum from			
		M[c]	N_1[d]	N_2	P_1
HS032	0[e]	64.0 ± 2.4[f]	60.3 ± 4.5	59.1 ± 5.5	63.3 ± 2.8
	N_1	49.0 ± 3.0	47.4 ± 2.6	54.2 ± 3.3	54.6 ± 2.0
	N_2	36.0 ± 2.8	[48.1 ± 4.3]	29.3 ± 2.2	32.0 ± 2.6
	P_1	53.3 ± 3.1	54.1 ± 3.6	64.0 ± 2.4	53.2 ± 2.6
HT046	0	36.2 ± 2.6	35.5 ± 1.9	37.4 ± 2.9	38.6 ± 3.0
	N_1	26.0 ± 1.3	24.1 ± 1.7	28.3 ± 1.3	29.1 ± 1.9
	N_2	22.9 ± 1.8	22.9 ± 1.7	20.2 ± 1.8	20.4 ± 2.4
	P_1	26.1 ± 1.4	22.7 ± 1.0	27.3 ± 1.6	28.8 ± 1.6

[a] Mean target cell count of 8 replicate wells.

[b] Target cells were preincubated for 45 min with 1:6 dilutions of heat-inactivated human serum samples. The dilute sera were then decanted and lymphocytes were added in amounts of 150,000/well. The plates were incubated for an additional 40 hr and then rinsed and stained, and the surviving target cells were counted.

[c] Wells preincubated in Waymouth's medium with 20% fetal calf serum; no human serum added.

[d] N_1 and N_2, 2 normal donors of serum and lymphocytes; P_1, Patient 77, a 61-year-old male with nonresectable bronchogenic carcinoma; HS032 target cells are cultured skin fibroblasts; HT046 target cells are cultured bronchogenic carcinoma cells.

[e] In this group of wells plain medium was added in lieu of lymphocytes; *i.e.*, no lymphocytes were added.

[f] Mean ± S.E.

Sometimes a serum sample stimulated the survival of target cells after exposure to certain lymphocytes in the absence of lymphocyte-mediated toxicity (Table 3). Following preincubation of cultured bronchogenic carcinoma target cells, HT072, with serum from Patient P_2 with metastatic bronchogenic carcinoma, lymphocytes from Normal Donor N_1 produced an increased survival of the target cells compared to preincubation with medium or with other serum samples. The P_2 serum alone, in the absence of normal lymphocytes N_1, did not augment survival of the target cells compared to medium or other serum samples. In other words, the stimulating effect appeared to be a synergistic one between P_2 serum and N_1 lymphocytes. This combination of serum and lymphocytes did not augment survival of a different target cell, melanoma HT085.

Table 4 shows a summary of the selective serum blocking and stimulating effects. The growth-promoting effects were more frequently observed with cancer patient sera (17 of 140 samples) than with normal sera (7 of 118). Use of the terms "blocking" and "stimulating" does not imply 2 different types of reactions; with both, the net result was an

Table 3

Survival of target cells in microcytotoxicity assays after preincubation with serum and subsequent exposure to lymphocytes

Results from experiment on October 17, 1973, demonstrating a selective stimulation of bronchogenic carcinoma target cells (HT072) in the presence of normal lymphocytes (N_1) after preincubation with serum from Patient P_2, with metastatic bronchogenic carcinoma (value in brackets). A similar effect was not observed against tumor HT085, a melanoma.

Target cells	Donor of lymphocytes	Target cell counts[a] after preincubation[b] with serum from				
		M[c]	N_1[d]	N_2	P_1	P_2
HT072	0[e]	63.1 ± 2.6[f]	61.5 ± 5.1	65.6 ± 4.5	57.5 ± 5.0	59.0 ± 6.9
	N_1	53.4 ± 5.0	43.4 ± 2.6	51.3 ± 3.8	53.6 ± 3.5	[75.7 ± 6.2]
	N_2	50.6 ± 1.7	47.2 ± 2.2	62.9 ± 4.1	49.1 ± 3.2	59.9 ± 5.4
	P_1	58.3 ± 3.7	55.6 ± 1.7	61.0 ± 3.4	59.0 ± 5.5	51.8 ± 4.3
	P_2	54.6 ± 3.2	52.5 ± 1.8	54.1 ± 2.5	51.4 ± 2.4	54.4 ± 6.6
HT085	0	68.0 ± 2.3	62.3 ± 4.0	63.1 ± 2.3	59.9 ± 2.3	66.9 ± 2.9
	N_1	56.0 ± 2.7	55.8 ± 1.4	60.2 ± 2.8	60.0 ± 2.4	58.7 ± 3.0
	N_2	46.4 ± 1.9	54.0 ± 2.6	51.9 ± 3.7	58.0 ± 1.1	48.4 ± 1.8
	P_1	49.1 ± 2.5	52.1 ± 2.4	52.9 ± 2.2	59.7 ± 2.1	47.4 ± 1.8
	P_2	45.1 ± 1.6	51.0 ± 1.1	42.6 ± 1.7	57.4 ± 1.7	48.7 ± 2.4

[a] Mean target cell count of 8 replicate wells.

[b] Target cells were preincubated for 45 min with 1:6 dilution of heat-inactivated human serum samples. The dilute sera were then decanted and lymphocytes were added in amounts of 150,000/well. The wells were incubated for an additional 40 hr and then rinsed and stained, and the surviving target cells were counted.

[c] Wells were preincubated in Waymouth's medium with 20% fetal calf serum; no human serum was added.

[d] N_1 and N_2, 2 normal donors of serum and lymphocytes; P_1, Patient 118, a 60-year-old male who was postoperative resection of bronchogenic carcinoma with no evidence of recurrence; P_2, Patient 90, a 62-year-old female with nonresectable, metastatic bronchogenic carcinoma; target cells (HT072) are cultured bronchogenic carcinoma cells; target cells (HT085) are cultured melanoma cells.

[e] In this group of wells plain medium was added in lieu of lymphocytes; *i.e.*, no lymphocytes were added.

[f] Mean ± S.E.

increase in target cell survival after exposure to a lymphocyte sample following preincubation with a certain serum sample, compared to preincubation with medium or other sera. Only when the lymphocyte sample was toxic compared to other lymphocyte samples could the serum growth-promoting effect be called a blocking effect. With most of the growth-promoting serum samples, the effect was observed only with 1 lymphocyte sample (occasionally 2) against only 1 of 2 to 4 target cells. The serum growth-promoting effect was noted just as often with normal lymphocytes as with cancer-patient lymphocytes.

Of greater interest were reductions in mean target cell counts observed after preincubation with certain serum samples and subsequent exposure to lymphocytes. Table 5 shows an experiment illustrating synergistic toxicity of a patient serum sample with lymphocytes. When cultured bronchogenic carcinoma target cells HT046 and HT047 were preincubated with serum from Patient P_1, the mean target cell counts were reduced after exposure to all lymphocyte samples when compared to preincubation with medium or with the normal serum samples N_1 and N_2. Patient P_1 serum in the absence of lymphocytes, however, was not toxic on the tumor target cells. Patient P_1 (Patient 78) was a 68-year-old female with metastatic oat cell bronchogenic carcinoma. Her lymphocyte-dependent serum toxicity appeared to be specific for tumor targets since the effect was not observed against target HS047, skin fibroblasts autochthonous with tumor HT047.

Such an effect was observed with 11 of 140 cancer patient serum samples but with none of 116 normal samples. The 11 reactive patient samples and the target cells that they were active against in the presence of lymphocytes are shown in Table 6. Three of the samples were obtained from Patient 78. Each serum sample tested from this patient "armed" or made toxic normal and patient lymphocytes against bronchogenic carcinoma target cells. Serum toxicity was observed in 4 different tests against 2 different bronchogenic carcinomas with all lymphocytes tested. Her serum was nonreactive when tested against 3 different skin fibroblasts and, as shown in Tables 5 and 6, the effect was noted against only the tumor cells when tested against tumor cells and fibroblasts from the same donor. Serum from a 65-year-old female with a thymoma, Patient 75, markedly reduced the survival of 1 bronchogenic carcinoma target culture in the presence of all lymphocytes tested and significantly reduced the survival of another bronchogenic carcinoma in the presence of all lymphocytes tested, without affecting the survival of fibroblasts autochthonous with this tumor. Another patient with bronchogenic carcinoma, Patient 147, strongly reduced the survival of cultured bronchogenic

Table 4

Selective increase in target cell survival following preincubation with certain human sera prior to addition of lymphocytes in microcytotoxicity assays

Target cells were preincubated for 45 min with 1:6 dilutions of heat-inactivated human serum samples. The dilute sera were then decanted and lymphocytes were added in amounts of 150,000/well. The plates were incubated for an additional 40 hr and then rinsed and stained, and the surviving target cells were counted.

	No. of serum samples tested that produced an increase[a] in survival of target cells[b] in the presence of lymphocytes[c]	
Target cells	Cancer patient sera (140 samples tested)	Normal sera[d] (118 samples tested)
Tumor cells	11 (5 blocking, 6 stimulating)[e]	5 (3 blocking, 2 stimulating)
Fibroblasts	6 (3 blocking, 3 stimulating)	2 (1 blocking, 1 stimulating)

[a] Defined as a greater than 20% increase in mean target cell count after preincubation with the human serum sample, compared to preincubation with medium and compared to preincubation with 2 or 3 other human serum samples.

[b] In any given experiment preincubation with a serum sample increased the survival of only 1 of 2 to 4 target cells tested.

[c] The effect (increased target cell survival) was usually seen after exposure to only 1 or 2 of the 3 or 4 lymphocyte samples included in any 1 test, and the effect was observed just as often with normal as with patient lymphocytes.

[d] Obtained from normal volunteers, primarily medical students and laboratory technicians.

[e] If the serum sample abrogated toxicity of a certain lymphocyte sample, the effect was called "blocking." If the serum sample increased target cell survival after exposure to nontoxic lymphocytes, the effect was called "stimulating."

carcinoma in the presence of all lymphocytes tested while not reacting against melanoma cells or against allogeneic fibroblasts.

Serum samples from 3 other patients with bronchogenic carcinoma, Patients 84, 93, and 133, armed only 1 of 3 or 4 lymphocyte samples tested against bronchogenic carcinomas. Sera from a patient with carcinoma of the breast and from a patient with carcinoma of the bladder were toxic in the presence of lymphocytes tested against melanoma targets.

Only 1 serum sample, from Patient 124, was reactive against skin fibroblasts and this reactivity was observed with only 1 of 4 lymphocyte samples tested.

None of the 11 reactive serum samples were toxic compared to medium or to other serum samples when tested alone, in the absence of lymphocytes.

DISCUSSION

Microcytotoxicity assays of human cancer patients have yielded important information that suggests that the majority of patients exert cell-mediated reactions against their autochthonous tumors or against tumors of the same histological type (1, 2, 6–8) and that patient serum often modifies the reactivity of lymphocytes (3, 5, 9–12, 14, 15). Several investigators, however, have recently reported problems with these assays, including frequent toxic reactions with normal lymphocytes and nonspecific toxic reactions with cancer patient lymphocytes (13, 16, 21, 22). Our results with the microcytotoxicity assay in a study of a large number of cancer patients have been recently published (17). As experience was gained with the technique, it was possible to decrease but not eliminate the toxic reactions observed with normal lymphocytes. It was also possible to demonstrate that lymphocytes from cancer patients are more frequently toxic against tumor cells but no more frequently toxic against fibroblasts than are lymphocytes from normal donors, but it was not possible to demonstrate clearly tumor type specificity of the toxic reactions with patient lymphocytes.

The combined effects of patient and normal serum on the reactivity of lymphocytes were studied only in the most recent phase of our studies. Initially, it was anticipated that serum from patients with progressively growing tumors would block, or at least partially reduce, the toxicity of their lymphocytes when tested against tumor targets of the same histological type (11). Occasionally, serum from a cancer patient did block or reduce the toxicity of that patient's lymphocytes against a tumor of his own histological type (Table 1). However, such an effect was not a frequent finding. Furthermore, serum from controls as well as from cancer patients sometimes increased the target cell survival after exposure to a given lymphocyte sample (Table 2). The effect was not specific against tumor target cells; occasionally, survival of fibroblast target cells was affected in a similar manner.

It is possible that the increased growth, *i.e.*, blocking or stimulation, of target cells after preincubation with certain serum samples prior to exposure to lymphocytes, may have an immunological basis. The demonstration of a survival-promoting effect with normal serum, however, makes it difficult to attribute special or immunological significance to the blocking effects observed with patient serum in this study. Certain serum samples might selectively block the toxicity of certain lymphocyte samples, and certain combinations of serum and lymphocytes might act synergistically to promote target cell survival simply on a biochemical basis. In the present study, however, failure to demonstrate tumor-specific blocking with patient sera is not surprising, since many of the lymphocyte samples studied in this laboratory have not been specifically toxic on tumors of the same histological type (17). Elimination of the background or cross-reactive toxicity might permit a demonstration of type-tumor specific lymphocyte-mediated toxicity, a requisite for the detection of type-tumor specific serum blocking activity (1, 3, 11, 14, 15).

The observation that a few patient serum samples appeared to render toxic or increase the toxicity of lymphocytes is of interest. This effect was observed only with serum from patients, not with serum from normal donors. The effect usually was observed with all lymphocytes tested in any given experiment, and the effect was generally observed against tumor cells rather than fibroblasts. Serum from Patient 78, who had an oat cell bronchogenic carcinoma, consistently armed or made toxic lymphocytes from both

Table 5

Survival of target cells in microcytotoxicity assays after preincubation with serum and subsequent exposure to lymphocytes

Results of experiment on March 7, 1973, demonstrating lymphocyte-dependent toxicity of a patient serum sample against cultured bronchogenic carcinoma target cells.

Target cells	Donor of lymphocytes	Target cell counts[a] after preincubation[b] with serum from			
		M[c]	N_1[d]	N_2	P_1
HT046	0[e]	61.9 ± 4.5[f]	58.3 ± 3.8	56.1 ± 4.2	59.6 ± 3.2
	N_1	34.0 ± 1.9	39.1 ± 1.9	31.5 ± 2.1	20.8 ± 2.1
	N_2	29.1 ± 2.2	34.3 ± 2.1	31.1 ± 1.5	8.0 ± 1.1
	P_1	30.0 ± 1.9	34.4 ± 2.2	29.2 ± 1.8	3.1 ± 0.7
HT047	0	92.2 ± 4.7	88.6 ± 5.5	85.3 ± 4.9	83.4 ± 5.0
	N_1	51.1 ± 4.0	57.9 ± 4.1	43.9 ± 2.1	17.6 ± 1.8
	N_2	49.2 ± 3.1	46.6 ± 3.0	48.6 ± 2.9	14.5 ± 2.3
	P_1	50.3 ± 5.2	52.5 ± 6.6	48.5 ± 2.9	8.9 ± 1.3
HS047	0	90.6 ± 9.0	95.6 ± 6.6	90.3 ± 6.5	89.5 ± 5.3
	N_1	77.3 ± 4.6	73.4 ± 4.7	81.4 ± 3.5	79.1 ± 3.3
	N_2	85.4 ± 3.1	91.4 ± 4.8	83.0 ± 3.1	90.3 ± 4.3
	P_1	85.9 ± 5.7	89.3 ± 4.6	84.7 ± 3.6	83.3 ± 4.5

[a] Mean target cell count of 8 replicate wells.

[b] Target cells were preincubated for 45 min with 1:6 dilutions of heat-inactivated human serum samples. The dilute sera were then decanted and lymphocytes were added in amounts of 150,000/well. The wells were incubated for an additional 40 hr and then rinsed and stained, and the surviving target cells were counted.

[c] Target cells were preincubated with 20% fetal calf serum in Waymouth's medium; no human serum added.

[d] N_1 and N_2, 2 normal human donors of serum and lymphocytes; P_1, Patient 78, a 64-year-old female with oat cell (small cell undifferentiated) carcinoma of the lung who was tested 10 days postoperatively; HT046 is a bronchogenic carcinoma that was obtained from the cytology positive pericardial fluid of a 46-year-old male; HT047 is an undifferentiated carcinoma of the lung obtained from a mediastinal nodal metastasis in a 76-year-old male; HS047 is a culture of normal skin fibroblasts obtained from a donor of tumor HT047.

[e] In this group of wells plain Waymouth's medium was added in lieu of lymphocytes; *i.e.*, no lymphocytes were added to these wells.

[f] Mean ± S.E.

normal donors and cancer patients against cultured bronchogenic carcinoma cells. In those experiments in which it was possible to test against both tumor and fibroblast from the same donor, it appeared that the effect was specific or selective just for the tumor target. Serum from a patient with a thymoma appeared selectively to render lymphocytes toxic against cultured bronchogenic carcinoma cells but not against skin fibroblasts from the same donor. Serum samples from another patient with bronchogenic carcinoma made lymphocytes strongly toxic against cultured bronchogenic carcinoma but not against melanoma cells or allogeneic skin fibroblasts. The serum effect was not always tumor type specific, however, since serum from a patient with breast cancer and serum from a patient with bladder cancer increased the toxicity of lymphocytes against cultured melanoma cells. The selectivity of the patient serum toxic effect for tumor cells *versus* fibroblasts raises the possibility of an immunological mechanism, but a nonimmunological basis certainly cannot be excluded.

The serum toxic effect was clearly dependent upon the presence of lymphocytes; when tested alone in the absence of lymphocytes, the serum samples demonstrated no toxicity on target cells. Lymphocyte-dependent antibodies directed against normal tissue alloantigens have been demonstrated in serum from multiparous and from multiply transfused patients (23) and, recently, from renal allotransplant recipients (4). The possibility that the lymphocyte-dependent serum reactivity observed in the present experiments was directed against histocompatibility antigens cannot be eliminated, since each of the patients with this serum reactivity had a history of previous pregnancies and/or blood transfusions; possibly skin fibroblasts are more resistant to such alloimmune reactions than are tumor cells. The apparent specificity of the serum reactivity for tumor targets *versus* fibroblasts, however, is compatible with the suggestion that this effect represents a serum "arming" activity against tumor-associated antigens as previously described in both human (10) and animal (18, 19) tumor systems.

ACKNOWLEDGMENTS

The authors express their appreciation to B. Burnett, J. M. Anderson, and K. Graham for technical assistance and to L. Colvin for preparation of the manuscript.

All data obtained in these experiments have been deposited in the

Table 6

Serum samples that selectively decreased mean target cell survival after exposure to lymphocytes in microcytotoxicity assays

Target cells were preincubated with 1:6 dilutions of medium or heat-inactivated serum samples for 45 min prior to addition of lymphocytes. After a 40 hr incubation the plates were washed and stained and the number of attached target cells were counted. The percentage of reduction in mean target cell count after preincubation with the reactive[a] serum samples, compared to preincubation with medium or other serum samples in the presence of any given lymphocyte sample, is presented on the same line with the identification number of lymphocyte donor. None of the reactive serum samples alone, in the absence of lymphocytes, altered the target cell survival when compared to medium only or with other serum samples.

Date of experiment	Donor of reactive serum[a]		Donor of lymphocytes[b]		% reduction of mean target cell count after preincubation with reactive serum compared to medium prior to addition of lymphocytes on the following target cells						Nonreactive serum samples[c]	
	Patient[d]	Diagnosis	Donor	Diagnosis	Target cell	%	Target cell	%	Target cell	%	Patient	Diagnosis
3/7/73	78	Carcinoma of lung			HT046[e] carcinoma of lung		HT047 carcinoma of lung		HS047			
			Normal 48			39[f]		65[g]		0	Normal 48	
			Normal 28			72[g]		69[g]		0	Normal 28	
			Patient 78	Carcinoma of lung		90[g]		82[g]		3		
4/10/73	84	Carcinoma of lung			HT056 carcinoma of lung		HT046 carcinoma of lung		HS046			
			Normal 57			20		13		0		
			Normal 53			0		0		0	Normal 57	
			Patient 84	Carcinoma of lung		32[h]		0		0	Normal 53	
6/5/73	78	Carcinoma of lung			HT046 carcinoma of lung		HS060					
			Normal 64			28[h]		15			Normal 64	
			Normal 51			100[g]		5			Normal 51	
			Patient 98	Carcinoma of lung		68[g]		15				
6/12/73	93	Carcinoma of lung			HT060 carcinoma of lung		HS061					
			Normal 61			44[h]		7			Normal 61	
			Normal 65			0		4			Normal 65	
			Patient 93	Carcinoma of lung		10		7				
9/20/73	75	Thymona			HT046 carcinoma of lung		HT072 carcinoma of lung		HS072			
			Normal 56			61[h]		20[h]		13	Normal 56	
			Patient 75	Thymoma		100[h]		20[h]		4	Patient 70	Carcinoma of lung
			Patient 70	Carcinoma of lung		60[h]		23[h]		0		
9/26/73	78	Carcinoma of lung			HT046 carcinoma of lung		HT072 carcinoma of lung		HS072			
			Normal 55			62[h]		0		11	Normal 55	
			Patient 81	Carcinoma of lung		70[h]		19		0	Patient 81	Carcinoma of lung
			Patient 78	Carcinoma of lung		100[g]		6		0		
10/18/73	121	Carcinoma of breast			HT035 melanoma		HT085 melanoma		HT072 carcinoma of lung			
			Normal 56			0		6		4	Normal 56	
			Normal 73			21[h]		11		0	Normal 73	

Table 6—*Continued*

Date of experiment	Donor of reactive serum[a]		Donor of lymphocytes[b]		% reduction of mean target cell count after preincubation with reactive serum compared to medium prior to addition of lymphocytes on the following target cells						Nonreactive serum samples[c]	
	Patient[d]	Diagnosis	Donor	Diagnosis	Target cell	%	Target cell	%	Target cell	%	Patient	Diagnosis
			Patient 120	Carcinoma of stomach		41[h]		7		0	Patient 120	Carcinoma of stomach
			Patient 121	Carcinoma of breast		23[h]		19		0		
11/1/73	124	Carcinoma of bladder			HT085 melanoma		HT035 melanoma		HS072			
			Normal 67			22[h]		3		0	Normal 67	
			Normal 65			33[f]		22[h]		28[h]	Normal 65	
			Patient 123	Carcinoma of lung		66[g]		0		13	Patient 123	Carcinoma of lung
			Patient 124	Carcinoma of bladder		57[f]		8		0		
1/10/74	133	Carcinoma of lung			HT072 carcinoma of lung		HS072		HT035 melanoma			
			Normal 69			0		0		30	Normal 69	
			Normal 65			36[h]		0		23	Normal 65	
			Patient 133	Carcinoma of lung		0		0		35	Patient 134	Carcinoma of lung
			Patient 134	Carcinoma of lung		6		0		7		
3/5/74	146	Carcinoma of rectum			HT100 carcinoma of lung		HT104 carcinoma of lung		HS104			
			Normal 70			27[h]		0		0	Normal 70	
			Patient 146	Carcinoma of rectum		15		21		0	Patient 148	Carcinoma of lung
			Patient 147	Carcinoma of lung		18		0		7		
			Patient 148	Carcinoma of lung		0		40[h]		0		
	147	Carcinoma of lung	Normal 70			36[h]		0		0		
			Patient 146	Carcinoma of rectum		61[f]		0		3		
			Patient 147	Carcinoma of lung		69[f]		0		3		
			Patient 148	Carcinoma of lung		76[f]		0		4		

[a] Reactive serum samples refer to sera which decreased mean target cell survival in the presence of lymphocytes.

[b] Lymphocytes and serum samples were drawn and tested on the same day.

[c] Non-reactive serum samples are those that were studied in the same experiment but were found to have no effect on any target.

[d] Patients and normal donors are identified by code numbers throughout the study. Code numbers for serum donors correspond with those for lymphocyte donors. Patient code numbers, however, do not correspond with target cell code numbers.

[e] Target cells are identified by code numbers and prefixes. HT, Human Tumor; HS, Human Skin. Tumor and skin cultures with the same numbers were obtained from the same donor.

[f] The significance of the percentage of reductions (Student's t test), $p \leq .001$.

[g] The significance of the percentage of reductions (Student's t test), $p \leq 0.001$.

[h] The significance of the percentage of reductions (Student's t test), $p \leq 0.05$.

ASIS/NAPS, c/o Microfiche Publications, 440 Park Ave So., New York, N.Y. 10016.

REFERENCES

1. Baldwin, R. W., Embleton, M. J., Jones, J. S. D., and Langman, M. J. S. Cell-Mediated and Humoral Immune Reactions to Human Tumors. Intern. J. Cancer, *12:* 73–83, 1973.
2. Bloom, E. T., Ossorio, R. C., and Brosman, S. A. Cell-Mediated Cytotoxicity against Human Bladder Cancer. Intern. J. Cancer, *14:* 326–334, 1974.
3. Cohen, A. M., Ketcham, A. S., and Morton, D. L. Specific Inhibition of Sarcoma-Specific Cellular Immunity by Sera from Patients with Growing Sarcomas. Intern. J. Cancer, *11:* 273–279, 1973.
4. D'Apice, A. J. F., and Morris, P. J. The Role of Antibody-Dependent Cell-Mediated Cytotoxicity in Renal Allograft Rejection. Transplantation, *10:* 20–26, 1974.
5. Hellström, I., Hellström, K. E., Evans, C. A., Heppner, G. H., Pierce, G. E., and Yang, J. P. S. Serum Mediated Protection of Neoplastic Cells from Inhibition by Lymphocytes Immune to Their Tumor Specific Antigens. Proc. Natl. Acad. Sci. U. S., *62:* 362–368, 1969.
6. Hellström, I., Hellström, K. E., Pierce, G. E., and Bill, A. H. Demonstration of Cell Bound and Humoral Immunity against Neuroblastoma Cells. Proc. Natl. Acad. Sci. U.S., *60:* 1231–1238, 1968.
7. Hellström, I., Hellström, K. E., Pierce, G. E., and Yang, J. P. S. Cellular and Humoral Immunity to Different Types of Human Neoplasms. Nature, *220:* 1352–1354, 1968.
8. Hellström, I., Hellström, K. E., Sjögren, H. O., and Warner, G. A. Demonstration of Cell Mediated Immunity of Human Neoplasms of Various Histologic Types. Intern. J. Cancer, *7:* 1–16, 1971.
9. Hellström, I., Hellström, K. E., Sjögren, H. O., and Warner, G. A. Serum Factors in Tumor-Free Patients Cancelling the Blocking of Cell-Mediated Tumor Immunity. Intern. J. Cancer, *8:* 185–191, 1971.
10. Hellström, I., Hellström, K. E., and Warner, G. A. Incidence of Lymphocyte Mediated Tumor Cell Destruction by Certain Patient Sera. Intern. J. Cancer, *12:* 348–352, 1973.
11. Hellström, I., Sjögren, H. O., Warner, G., and Hellström, K. E. Blocking of Cell Mediated Tumor Immunity by Sera from Patients with Growing Neoplasms. Intern. J. Cancer, *7:* 226–237, 1971.
12. Heppner, G. H. Studies on Serum-Mediated Inhibition of Cellular Immunity to Spontaneous Mouse Mammary Tumors. Intern. J. Cancer, *4:* 608–615, 1969.
13. Heppner, G. H., Henry, E., Stolbach, L., Cummings, F., McDonough, E., and Calabresi, P. Problems in the Clinical Use of the Microcytotoxicity Assay for Measuring Cell-mediated Immunity to Tumor Cells. Cancer Res., *35:* 1931–1937, 1975.
14. Heppner, G. H., Stolbach, L., and Byrne, M. Cell-Mediated and Serum Blocking Reactivity to Tumor Antigens in Patients with Malignant Melanoma. Intern. J. Cancer, *11:* 245–260, 1973.
15. Levy, N. L. Use of an *in vitro* Microcytotoxicity Test to Assess Human Tumor Specific Cell Mediated Immunity and Its Serum-Mediated Abrogation. Natl. Cancer Inst. Monograph, *37:* 85–92, 1973.
16. Oldham, R. K., Siwarski, D., McCoy, J. L., Plata, E. J., and Herberman, R. B. Evaluation of a Cell-Mediated Cytotoxicity Assay Utilizing 125 Iododeoxyuridine-Labeled Tissue Culture Target Cells. Natl. Cancer Inst. Monograph, *37:* 49–58, 1973.
17. Pierce, G. E., and DeVald, B. L. Effects of Human Lymphocytes on Cultured Normal and Malignant Cells. Cancer Res., *35:* 1830–1839, 1975.
18. Pollack, S., Heppner, G., Brawn, R. J., and Nelson, K. Specific Killing of Tumor Cells *in Vitro* in the Presence of Normal Lymphoid Cells and Sera from Hosts Immune to the Tumor Antigens. Intern. J. Cancer, *9:* 316–323, 1972.
19. Pollack, S. Specific "Arming" of Normal Lymph Node Cells by Sera from Tumor Bearing Mice. Intern. J. Cancer, *11:* 138–142, 1973.
20. Takasugi, M., and Klein, E. A Microassay for Cell-Mediated Immunity. Transplantation, *9:* 219–227, 1970.
21. Takasugi, M., Mickey, M. R., and Terasaki, P. I. Reactivity of Lymphocytes from Normal Persons on Cultured Tumor Cells. Cancer Res., *33:* 2898–2902, 1973.
22. Takasugi, M., Mickey, M. R., and Terasaki, P. I. Studies on Specificity of Cell Mediated Immunity to Human Tumors. J. Natl. Cancer Instm, *53:* 1527–1538, 1974.
23. Yust, I., Wunderlich, J. R., Mann, D. L., and Terry, W. D. Identification of Lymphocyte-Dependent Antibody in Sera from Multiply Transfused Patients. Transplantation, *18:* 99–107, 1974.