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Correlation of Antitumor Chemoimmunotherapy with Serum Inhibition of Tumor Cell Destruction[1]

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ABSTRACT

The administration of cyclophosphamide and *Corynebacterium parvum* in combination results in tumor growth inhibition greater than that resulting from the use of either agent alone. The precise mechanism(s) by which this chemoimmunotherapy combination results in a synergistic inhibiting effect is not known. The possibility was entertained that the tumor effect might be related to a greater decrease in serum-mediated interference with cellular cytotoxicity, *i.e.*, "blocking" activity, by both agents in combination rather than by either alone. The present findings fail to support such an explanation. *C. parvum* by itself failed to decrease serum inhibition and in conjunction with cyclophosphamide resulted in an effect that was no greater than that produced by cyclophosphamide alone.

INTRODUCTION

Studies from this laboratory have demonstrated that administration of CY[3] and CP in combination results in tumor growth inhibition greater than that resulting from the use of either agent alone (11, 12). Greater retardation is achieved when CP is used in combination with alkylating agents rather than with antimetabolites, and the optimal result is achieved when CY is used with CP (7). The precise mechanism(s) by which the combination of CP and CY results in a synergistic tumor growth-inhibiting effect is not known. Several investigations have been carried out in an attempt to explain the findings. Some of these investigations have demonstrated an interference with the metabolism of CY by CP (10), whereas others have indicated a greater stimulation of bone marrow macrophage colony production during treatment (9). Another possibility that has been considered is that the effect is related to a decrease in serum-mediated interference with cellular cytotoxicity, *i.e.*, "blocking" activity. The present study was carried out to determine whether CY in combination with CP abrogates serum inhibition of cytotoxicity to a greater extent than does either agent alone. Should this abrogation occur, it could be responsible, at least in part, for the more effective inhibition of tumor growth observed with the therapeutic combination.

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[3] The abbreviations used are: CY, cyclophosphamide; CP, *Corynebacterium parvum*; RLNC, regional lymph node cells; LNC, lymph node cells.

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MATERIALS AND METHODS

Mice. Female C3HeB/FeJ mice, 8 to 12 weeks old, were housed in individual cages and were fed laboratory chow and water *ad libitum*.

Tumor. The tumor used was a mammary carcinoma arising in a C3H female and carried in transfer in C3HeB mice. Mice were inoculated with a suspension of 2×10^5 viable tumor cells in 0.1 ml Medium 199 s.c. in the left hind leg, distal to the popliteal node. When tumor diameters reached a size of approximately 5 mm, the mice were randomized into treatment groups so that each group contained mice with tumors of equivalent size.

CY. CY was prepared in sterile distilled water so that the desired amount was contained in 0.01 ml/g body weight. All injections of this agent were given i.p. every 7 days.

CP. Burroughs Wellcome Coparvax (supplied by Dr. J. K. Whisnant, Burroughs Wellcome Co., Research Triangle Park, N. C.) was administered in a dose of 1.4 mg (dry weight) of organisms in 0.2 ml of suspension i.p. every 7 days. When CP was used in conjunction with CY, it was given 4 days after each CY administration.

Preparation of RLNC's for Cytotoxicity Assay. Mice were killed by cervical dislocation. The popliteal and inguinal nodes of the tumor-bearing leg (regional lymph nodes) were removed, minced with scissors, and washed through an 80 mesh nylon screen with Earle's minimal essential medium containing 30% fetal calf serum.

***In Vitro* Cytotoxicity Testing.** Cytotoxicity was assayed by the method previously described (8). The test was carried out in microtest plate wells covered by a monolayer of tumor cells, which had been exposed to 1 of the 3 sera.

In all experiments 5×10^5 cells were added to each well. Each plate included wells with no added cells, LNC's from nontumorous mice, and RLNC's to be assayed. Since LNC's from non-tumor-bearing mice displayed a cytotoxicity $\leq 1\%$, these values have been omitted from the tables. The tumor specificity of the cytotoxicity has been previously established (6). Plates, coded to eliminate bias in observation, were incubated for 48 hr, stained with crystal violet, and examined. The wells were scored on a scale of 0 to 5 according to the area free of tumor cells: 0 indicated complete coverage, and 5 indicated complete absence of tumor cells. The mean ± S.E. was obtained for the findings from 30 wells. The results are presented as the percentage of tumor cell destruction, which was obtained by dividing the amount of tumor remaining in the treated wells by that in the nontreated control wells and subtracting the value obtained from 100. Data were analyzed by the Mann-Whitney *U* test.

Sera for Inhibition Studies. Blood was obtained from the mice providing the LNC. These mice were bled from the retroorbital plexus. The blood was allowed to clot at room temperature, and the serum was removed. All sera were inactivated for 30 min at 56° and diluted 1:5 with Dulbecco's phosphate-buffered saline. Blood from non-tumor-bearing mice provided normal serum. The method described by Hellström *et al.* (16) was used to determine serum inhibition. Target tumor cells in the microcytotoxicity plate wells were incubated with each serum to be tested, and an equal number were incubated with medium alone. After 45 to 60 min of incubation of the plates at 37°, the sera were removed, and the wells were washed once with phosphate-buffered saline. The cells to be assayed were then added to the wells. A sufficient number of plates were used to provide 30 wells treated with each serum-cell combination in each experiment.

RESULTS

Nodes and Serum from Similarly Treated Animals (Table 1). Maximum cytotoxicity was observed when target cells had been treated with normal mouse serum and RLNC's came from untreated tumor-bearing mice (Ia, IIa, and IIIa). Maximum inhibition of cytotoxicity occurred when both RLNC's and serum had been obtained from untreated tumor-bearing animals (Ib, IIb, and IIIb). When both serum and RLNC's were derived from CY-treated mice (Ic), cytotoxicity was greatly restored (Ib *versus* Ic, $p < 0.001$ for Days 1 through 4). At 3 or 4 days after the last treatment, it was 70 to 75% of that maximally obtained (Ia). CP treatment of serum and lymph node donor mice (IIc) restored cytotoxicity (IIb *versus* IIc, $p > 0.001$) but to a lesser extent than did CY. When both serum and RLNC's came from CY-CP-treated mice (IIIc), there was practically complete restoration of cytotoxicity, *i.e.*, abrogation of inhibition, by 4 days after treatment (IIIb *versus* IIIc, $p < 0.001$; IIIa *versus* IIIc, $p > 0.9$).

Effect of Treatment on RLNC's Cytotoxicity (Table 2). That we might determine whether the restoration of cytotoxicity resulting from the treatment described above was due to the effect of therapy on the cells used, we obtained the serum in the test system from normal (no tumor, no treatment) mice. Whereas cells from CY-treated donors (Ib) were only slightly ($p > 0.1$) more cytotoxic than were those from untreated tumor-bearing mice (Ia), CP treatment of donor mice resulted in a significant enhancement of cytotoxicity for the first 4 days after treatment (IIa *versus* IIb, p, 0.009 Day 4). When the CY-CP combination was used, the same augmented effect was observed at 4 days (IIIa *versus* IIIb, $p < 0.001$). This was greater than that resulting from CY treatment (p, 0.02), but it was not greater than that resulting from CP treatment (p, 0.2).

Effect of Treatment on Serum Inhibition of Cytotoxicity (Table 3). To determine whether the restoration of cytotoxicity was due to the effect of therapy on the serum used, we obtained all cells from untreated tumor-bearing mice. A significant ($p < 0.01$) abrogation of serum inhibition was observed for 4 days after CY therapy of serum donors (Ic); little effect was achieved when serum from CP-treated mice was used (IIc). When serum was obtained from mice treated with the CY-CP combination, decrease in cytotoxicity inhibition was no greater than that observed with serum from mice treated with CY alone (IIIc *versus* Ic, $p > 0.5$).

DISCUSSION

Serum inhibition of the cytotoxicity of lymphoid cells appears early in the growth of a tumor (3), is specific (14, 15), and increases progressively with tumor growth (3). It

Table 1

Effect of CY and/or CP on serum inhibition of cytotoxicity

Nodes and serum are from similarly treated animals.

Serum donor	RLNC donor (tumor-bearing)	% tumor cell destruction on following days after last treatment[a]						
		1	2	3	4	5	6	7
I. a. Nontumorous untreated	Untreated	73.0	68.9	68.6	69.0	69.9	69.2	
b. Tumor-bearing untreated	Untreated	34.8 (47.7)[b]	31.4 (45.6)	36.8 (53.6)	32.8 (47.5)	31.7 (45.4)	33.6 (48.6)	
c. Tumor-bearing CY-treated	CY-treated	50.8 (69.6)	49.1 (71.3)	52.2 (76.0)	51.9 (75.2)	34.2 (48.9)	36.8 (53.2)	
II. a. Nontumorous untreated	Untreated	74.9	71.3	70.0	74.9	71.3	70.0	
b. Tumor-bearing untreated	Untreated	24.5 (32.7)	19.3 (27.1)	21.8 (31.1)	24.5 (32.7)	19.3 (27.1)	21.9 (31.3)	
c. Tumor-bearing CP-treated	CP-treated	32.0 (42.7)	31.6 (44.3)	32.2 (46.0)	30.6 (40.9)	28.7 (40.3)	31.0 (44.3)	
III. a. Nontumorous untreated	Untreated			67.1	69.2			68.7
b. Tumor-bearing untreated	Untreated			28.1 (41.9)	37.5 (54.2)			27.3 (39.7)
c. Tumor-bearing CY-CP-treated	CY-CP-treated			45.2 (67.4)	68.9 (99.6)			36.0 (52.4)

[a] Average of 2 experiments.

[b] Numbers in parentheses, percentage of maximum tumor cell destruction (I, II, or IIIa).

Table 2

Effect of CY and/or CP on cytotoxicity of the RLNC's

RLNC donor (tumor-bearing)	% tumor cell destruction on following days after last treatment						
	1	2	3	4	5	6	7
I. a. Untreated	73.0	68.9	68.6	69.0	69.9	69.2	
b. CY-treated	71.6	72.0	73.0	73.7	72.0	69.6	
	(98.1)[a]	(104.5)	(106.4)	(106.8)	(103.0)	(100.6)	
II. a. Untreated	74.9	71.3	70.0	74.9	71.3	70.0	
b. CP-treated	81.6	83.5	78.1	84.3	70.6	60.9	
	(108.9)	(117.1)	(111.6)	(112.6)	(99.0)	(87.0)	
III. a. Untreated			67.1	69.2			68.6
b. CY-CP-treated			69.8	80.1			71.8
			(104.0)	(115.8)			(104.7)

[a] Numbers in parentheses, percentage of maximum tumor cell destruction (I, II, IIIa) for serum donor and normal mice.

Table 3

Effect of CY and/or CP on serum inhibition of cytotoxicity with RLNC's from untreated tumor-bearing animals

Serum donor	% tumor cell destruction on following days after last treatment[a]						
	1	2	3	4	5	6	7
I. a. Nontumorous	73.0	68.9	68.5	69.0	69.9	69.2	
b. Tumor-bearing	34.8	31.4	36.8	32.8	31.7	33.6	
	(47.7)[b]	(45.6)	(53.7)	(47.5)	(45.4)	(48.6)	
c. Tumor-bearing CY-treated	46.1	39.9	49.0	53.5	33.9	38.4	
	(63.2)	(57.9)	(71.5)	(77.5)	(48.5)	(55.5)	
II. a. Nontumorous	74.9	71.3	70.0	74.9	71.3	70.0	
b. Tumor-bearing	24.5	19.3	21.8	24.5	19.3	21.9	
	(32.7)	(27.1)	(31.1)	(32.7)	(27.1)	(31.3)	
c. Tumor-bearing CP-treated	22.4	27.5	24.6	22.4	13.8	22.1	
	(29.9)	(38.6)	(35.1)	(29.9)	(19.4)	(31.6)	
III. a. Nontumorous			67.1	69.2			68.6
b. Tumor-bearing			28.1	37.5			27.3
			(41.9)	(54.2)			(39.8)
c. Tumor-bearing CY-CP-treated			46.0	53.2			31.4
			(68.6)	(76.9)			(45.8)

[a] Average of 2 experiments.

[b] Numbers in parentheses, percentages of maximum tumor cell destruction (I, II, or IIIa) for RLNC donor and serum from nontumorous mice.

has been attributed to the presence of tumor antigen, serum antibody, and antigen-antibody complexes (1), which attach to tumor cells (2, 20) or are present on the surface of cytotoxic cells (5). Serum inhibition rapidly disappears after tumor excision (3) or after spontaneous regression (15), suggesting a role for antigen. Evidence from a variety of investigations provided the expectation that the use of CY in our model system would diminish serum inhibitory factor; presently reported findings confirm the validity of this consideration. There may be several explanations for the observation. Since CY is a potent B-lymphocyte depressant (21, 22) and antibody production is dependent on the functional integrity of those cells, the use of CY, by depressing the B-cell population, could have delayed antibody production, thus postponing serum inhibition of T-cell cytotoxicity (17, 18). The disclosure that blocking was at a minimum 4 days after serum donors were treated with CY correlates with other studies (4), relative to the time of depression of antibody synthesis after CY administration. It may also be postulated that CY, as a result of its direct cytotoxic effect on dividing tumor cells, could have reduced the input of tumor antigen, thereby decreasing the blocking activity of the serum. Another explanation may be related to the effect of CY on suppressor T-lymphocytes. Those cells have recently been shown to inhibit the *in vivo* differentiation of antigen-specific cytotoxic T-lymphocytes (19). Such an event may give rise to increased tumor growth with greater production of antigen, antigen-antibody complexes, and serum blocking. The reduction of suppressor cells by CY could conversely affect those events, thereby reducing serum inhibition. Since CP stimulates the reticuloendothelial system (13), the possibility was entertained that the use of that immunopotentiator might increase the clearance of tumor antigen or circulating antigen-antibody complexes, with the resultant reduction of serum inhibition of cytotoxicity. Moreover, it was considered that the synergistic inhibition of tumor growth observed when the CY-CP combination is used might be the result of each agent interfering with serum inhibition via different mechanisms; results of the present studies fail to support this possibility, for they have indicated that serum inhibition by the combination is no greater than that produced by CY alone. Thus,

since CP either alone or in combination with CY failed to decrease blocking, it is unlikely that clearance of serum antigen or antigen-antibody complexes was increased as a result of reticuloendothelial system stimulation by CP.

The increased cytotoxicity observed when the RLNC used for testing were derived from tumor-bearing donors treated with CY and/or CP rather than from untreated animals is of interest. Such findings may have been related to a diminution of tumor antigen and/or antigen-antibody complexes attached to the surface of the RLNC's or to a reduction in suppressor T-lymphocytes in that population as a result of CY. Whatever the mechanism involved, it is likely that these agents have antitumor effects through mechanisms other than interference with serum blocking.

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