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Lymphoblastoid Cell-Induced Suppression of Human Peripheral Blood Leukocyte Mitogenic Responses (41943)

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Abstract. Two lymphoblastoid tumor cell lines, the Burkitt lymphoma derived BJAB cell line which is free of Epstein–Barr virus (EBV) and B95-8 cells, which are marmoset lymphocytes transformed by EBV isolated from an infectious mononucleosis patient, were studied in regards to their effects on the blastogenic responsiveness of normal human peripheral blood leukocytes stimulated *in vitro* with mitogens. Mitomycin C treated tumor cell suspensions, when cocultured with normal human blood leukocytes, markedly depressed the expected blastogenic responses *in vitro* to concanavalin A, pokeweed mitogen, and phytohemagglutinin. In addition, cell-free sonicates from the cell lines also depressed blastogenic responsiveness of the leukocytes *in vitro*. Heating the sonicates for 10 min at 100°C markedly diminished the suppressive properties of the sonicates, as did ultraviolet light irradiation. The suppressive activity of the B95-8 sonicates was pelleted by high speed centrifugation as compared to the activity of sonicates derived from the BJAB cells. Further studies are warranted to determine the nature and mechanism of suppression of blastogenic responsiveness of normal human leukocytes by soluble components derived from such lymphoblastoid cell lines.

Burkitt's lymphoma occurs in Equatorial Africa as an acute disease, generally related to infection by Epstein–Barr (EB) virus (1, 2). Lymphoid cells from Burkitt's lymphoma patients may be readily cultured *in vitro*, and various continuous cell lines have been established. In addition, EBV is capable of infecting B lymphocytes *in vitro* and immortalizing such cells so they proliferate in culture essentially indefinitely (3). Various studies have been performed concerning the nature and mechanism of malignancy in Burkitt's lymphoma and EBV induced oncogenesis. Patients with Burkitt's lymphoma may show diminished immune responses, at least during some stages of the disease process (4). In this regard, numerous other studies, especially with laboratory animals, have shown that lymphoid cell malignancy is often associated with marked alteration of the immune response mechanism (5). The relationship between immunodysfunction and lymphoid cell malignancy is not understood. Nevertheless, it seems evident that many tumor cells, especially those derived from the lymphoid system, may influence the immune response system itself, in either a positive or a negative manner. In terms of EBV infection, Werkmeister *et al.* have reported that supernatant fluids from an EBV transformed cell line, Daudi, inhibited responsiveness of normal human lymphoid cells to phytohemagglutinin (6). In the present study, two continuous lymphoma cell lines, one induced *in vitro* by EBV and the other derived from a patient with Burkitt's lymphoma but showing no evidence of the presence of the EBV genome, were examined regarding their effects on the blastogenic responsiveness of normal human blood leukocytes *in vitro*. Both intact tumor cells and cell-free preparations were studied.

Experimental Methods. *Cell lines.* Two lymphoblastoid cell lines, the B95-8 cell line which was induced initially by transformation of marmoset lymphocytes with EBV from a patient with infectious mononucleosis and which sheds the virus in culture, and the BJAB cell line which was derived from a Burkitt's lymphoma patient but which has no evidence of virus, were utilized for the study (3, 4). The cells were maintained as suspensions at a concentration of 10^6 nucleated cells/ml at −70°C until used. Aliquots of the cell suspensions were thawed and the cells cultured in sterile RPMI-1640 medium containing 10% fetal calf serum and anti-

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biotics. The cells were recultured every 4 days with fresh medium.

Cell suspensions and sonicates. Suspensions of the cell lines were cultured for 48–96 hr at 37°C and the harvested cells were washed several times with medium. They were resuspended to a concentration of 10^6 viable cells/ml medium and then treated for 30 min at 37°C with 10 μg mitomycin C. The cell suspensions were then washed three times with medium by centrifugation and resuspended to a concentration of 10^6 viable cells/ml. Cell-free sonicates were prepared from untreated freshly harvested cell suspensions by 6 pulses of 30 sec each with a Raytheon sonicator. Cell debris was removed by centrifugation at 1500*g* for 10 min and the clarified supernatant was used as the cell-free sonicate.

Blastogenic assay. Peripheral blood leukocytes were obtained from normal volunteers, ages 20–50. The leukocytes were obtained by the standard Ficoll–Hypaque gradient centrifugation method. For the blastogenic test 10^6 leukocytes were placed in 0.2 ml medium in individual wells of 96-well flat bottom microtiter plates. The cells were stimulated by addition of 0.1 ml of a 5.0 μg concentration of either concanavalin A (Con A), pokeweed mitogen (PWM), or phytohemagglutinin (PHA). The plates were incubated for 72 hr at 37°C in a humidified atmosphere. Each culture was then pulsed for 18 hr with 0.5 μCi [^{3}H]thymidine (Schwartz Biochemical, Boston, Mass.). The amount of radioactivity taken up into the cellular DNA was determined for three or more duplicate cultures by standard liquid scintillation counting using a Packard spectrophotometer, Model 3380 (7). To determine the effects of the tumor cells, an equal volume of graded numbers of one or the other cell line was added to cultures of normal peripheral blood leukocytes at the time of culture initiation. Other cultures were incubated with 0.1 ml of graded dilutions of the cell-free sonicate preparations.

Experimental Results. The lymphoblastoid cells markedly suppressed the blastogenic responsiveness of normal peripheral blood leukocytes stimulated *in vitro* with any one of the three mitogens. As is evident in the representative experiments in Table I, culture of 10^6 normal human leukocytes with 10^5 mitomycin C treated B95-8 or BJAB cells resulted in marked diminution of the expected response to Con A, PWM, or PHA. The cell-free sonicates at a dilution of 1:10 also markedly depressed the responses, although to a somewhat lesser degree than occurred with the tumor cell suspensions. It seemed noteworthy that there was a six to eight times greater background uptake of thymidine by normal peripheral blood leukocytes cocultured with mitomycin C treated tumor cells as compared to leukocytes incubated alone. The mitomycin treatment of the tumor cells inhibited their capacity to take

TABLE I. EFFECT OF LYMPHOBLASTOID CELLS AND CELL-FREE SONICATES ON BLASTOGENIC RESPONSES OF NORMAL HUMAN PERIPHERAL BLOOD LEUKOCYTES TO PLANT MITOGENS

Addition to culture[a]	No mitogen (control)	Blastogenic response[b]					
		Con A		PHA		PWM	
		cpm	SI	cpm	SI	cpm	SI
None (control)	374 ± 46	34,560 ± 1500	92.4	39,450 ± 2650	105.5	9,261 ± 130	24.7
B95-8							
Cells (10^5)	2910 ± 460	12,400 ± 976	4.3	3,572 ± 180	1.2	6,260 ± 149	2.2
Sonicate (1:10)	1084 ± 96	6,182 ± 430	5.7	21,560 ± 1170	19.8	10,530 ± 1230	9.7
BJAB							
Cells (10^5)	3142 ± 275	8,163 ± 530	2.6	41,240 ± 2360	13.2	16,430 ± 978	5.1
Sonicate (1:10)	523 ± 32	4,872 ± 370	9.4	17,420 ± 2140	33.2	7,860 ± 163	15.0

[a] Cultures of 10^6 normal human peripheral blood leukocytes treated *in vitro* with indicated tumor cells (10^5) or 0.1 ml of a 1:10 sonicate from 10^6 tumor cells at time of *in vitro* immunization with indicated mitogen.

[b] Average cpm ± SD, and SI for three to four cultures per group 72 hr after culture simulation with 5.0 μg of indicated mitogen.

up thymidine, although slight uptake still occurred. Furthermore, in the presence of any one of the three mitogens the mitomycin treated lymphoma cells incorporated only low levels of thymidine. In contrast, when nontreated lymphoma cells, either with or without mitogen stimulation, were cultured *in vitro* with thymidine, significant uptake occurred over a period of 3–4 days, indicating that the cells were proliferating rapidly. Thus it was necessary to use the mitomycin C treated cells for the coculture experiments. In this regard, when the SIs were calculated for cultures containing mitomycin treated lymphoma cells plus normal leukocytes, marked depression of the thymidine uptake occurred, as compared to the cultures without the lymphoma cells. This was evident for all mitogen stimulated cultures containing lymphoma cells, even when the higher background cpm of such cultures was compared to controls.

Cell-free sonicates, when added to the peripheral blood leukocytes, resulted in some stimulation of background thymidine uptake, but to a lower degree than that occurring in cultures with the tumor cells. The sonicates also resulted in markedly depressed SIs as well as lower thymidine uptake upon stimulation with the mitogens (Table I).

The suppressive effect of the sonicates was labile to heating at 100°C for 10 min or treatment with uv light (Table II). In addition, when the B95-8 derived sonicate was centrifuged at 100,000*g* for 30 min, more suppressive activity was found in the pellet than in the supernatant. In contrast, the BJAB sonicate yielded pellets which were less suppressive than the pellets from the B95-8 sonicates.

The time of addition of the cell-free sonicates to normal peripheral blood cultures markedly affected blastogenic responses. As is evident in Table III, addition of 0.1 ml of a 1:10 dilution of sonicate on the day of culture initiation resulted in maximum suppression. Addition of sonicate to cultures on Day +1 or +2 resulted in only a slight to moderate inhibition.

Discussion. Many transformed cell lines have been established from Burkitt's lymphoma patients. In addition, lymphocytes from marmosets and normal individuals have been transformed by exposure to EBV *in vitro* (8). As is evident from the results of the present study, two of these cell lines, i.e., B95-8 and BJAB cells, were found to interfere

TABLE II. EFFECT OF HEATING, uv LIGHT, OR CENTRIFUGATION ON INHIBITORY EFFECT OF CELL-FREE SONICATES FROM LYMPHOBLASTOID CELLS ON BLASTOGENIC RESPONSE OF HUMAN PERIPHERAL BLOOD LEUKOCYTES STIMULATED *IN VITRO* WITH PHA

Addition to culture[a]	Treatment *in vitro*	Blastogenic response		
		No stimulator (cpm)	PHA[b]	
			cpm	SI
None (control)	—	396	32,568	82.2
B95-8 sonicate	None	675	926	1.4
	Heat (100°C)	325	19,568	60.2
	uv light	481	22,432	46.6
	Centrifugation			
	Supernatant	650	18,653	28.7
	Pellet	730	9,126	12.5
BJAB sonicate	None	1136	22,355	19.2
	Heat (100°C)	428	24,872	58.2
	uv light	530	35,316	66.6
	Centrifugation			
	Supernatant	612	21,430	25.4
	Pellet	507	20,642	40.4

[a] Cultures of 10^6 normal human peripheral blood leukocytes treated *in vitro* with 0.1 ml of indicated cell sonicate, with or without prior treatment as indicated.

[b] Average cpm and SI for three to four cultures per group 72 hr after *in vitro* stimulation with 5 μg PHA.

TABLE III. EFFECT OF TIME OF ADDITION OF CELL-FREE LYSATES FROM LYMPHOBLASTOID CELLS ON BLASTOGENIC RESPONSE OF NORMAL HUMAN BLOOD LEUKOCYTES TO PLANT MITOGENS

Addition to culture[a]	Day added	Blastogenic response[b]			
		Con A		PHA	
		cpm	Percentage of control	cpm	Percentage of control
None (control)	—	35,860	—	47,938	—
B95-8 sonicate	0	1,240	3	1,436	3
	+1	19,738	55	22,790	48
	+2	32,156	90	38,431	80
BJAB sonicate	0	3,974	11	2,934	6
	+1	18,312	51	38,760	81
	+2	37,310	104	44,793	93

[a] Culture of 10^6 normal human peripheral blood leukocytes treated with 0.1 ml of cell-free sonicate on day indicated after culture stimulation.

[b] Average cpm for three to four cultures per group 72 hr after culture stimulation with 5.0 μg of indicated mitogen.

with the blastogenic responsiveness of normal human peripheral blood lymphocytes upon coculture at relatively low ratios. The same cell lines, when sonicated to yield a cell-free extract, resulted in preparations which markedly depressed the blastogenic responses of normal human blood cells. Incubation of peripheral blood lymphocytes from normal individuals with the cell-free sonicates derived from these two cell lines resulted in diminution of the expected blastogenic response.

The B95-8 cells were initially derived from EBV transformed normal marmoset lymphocytes. These cells shed small amounts of virus and contain the virus genome (3, 4). The cell-free sonicate from these cells was markedly immunosuppressive. This did not appear due to nonspecific factors, such as cold thymidine. Heating the cell-free preparations for as little as 10 min at 100°C markedly diminished the suppressive effects. In addition, centrifugation of the cell-free sonicate at a 100,000*g* for 30 min resulted in pelleting of a significant portion of the suppressive activities. Exposure of the sonicate to uv light for 20 min also diminished the suppressive effects. Thus it seemed possible that a factor associated with a virus or possibly EBV itself may be involved in mediating suppression of the blastogenic response.

It is of interest that similar sonicates prepared from BJAB cells also suppressed the responsiveness of normal peripheral blood leukocytes *in vitro*. This cell line does not shed virus and does not contain any detectable viral genome (4). This suggests that it is unlikely that EBV per se could be involved in the suppressive effects by these cells. It also does not seem plausible that EBV is present in a noninfectious state in these cells or that a provirus component or genome may be involved. The properties of the cell-free sonicates from the BJAB cells are relatively similar to that of the B95-8 cells in that suppressive activity was inhibited by heating at 100°C. However, the pellet obtained by centrifugation at 100,000*g* was much less suppressive. Also it appears that the sonicate from BJAB cells was less suppressive for normal peripheral blood leukocytes as compared to sonicates from B95-8 cells.

It seems unlikely that the depressed responsiveness to mitogens by leukocyte cultures with either the tumor cells or their sonicates was due to alteration in the relative stimulation of background vs mitogen-induced blastogenesis. Since mytomycin C treated tumor cells were utilized for the coculture experiments, it is improbable that the tumor cells could have contributed significantly to the uptake of thymidine after stimulation with the mitogens. In addition, the BJAB cells were somewhat less inhibitory of

thymidine uptake by mitogen stimulated cells in comparison to the effects of the B95-8 cell line.

Additional studies characterizing the cell-free sonicates in terms of specific components responsible for suppression of the blastogenic responsiveness of normal human leukocytes *in vitro* to mitogens are warranted. In addition, study of the effects of these materials on other immune parameters also appears warranted. For example, the effects of these preparations on antibody forming capacity of leukocytes and/or macrophage functions should be investigated. Nevertheless, it is of interest from the results of the present study that Burkitt's lymphoma and EBV induced tumor cells, as well as cell-free sonicates from these cells, have depressive activity for normal human leukocytes *in vitro*.

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