Brief Definitive Report

AUTOLOGOUS MIXED LYMPHOCYTE CULTURE REACTIONS AND GENERATION OF CYTOTOXIC T CELLS*

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It has been known for a number of years that lymphoblastoid cells established in continuous culture will stimulate the proliferation of lymphocytes from the donor from whom the cell line was derived (1–2). This observation has been interpreted as reflecting the expression of viral antigens, blast-associated antigens, derepressed genetic material, or new HLA specificities on lymphoid cells in continuous culture. However, the recent demonstration that purified B lymphocytes stimulate autologous T-lymphocyte proliferation (3, 4) suggests that the stimulatory capacity of autologous lymphoid cells depends on their lineage and not on their establishment in continuous culture. For this reason, we have reevaluated the capacity of B-lymphoid cell lines and purified B cells to stimulate the proliferation of autologous T lymphocytes and to generate cytotoxic T lymphocytes.

Materials and Methods

Lymphoid Cell Lines (LCL). LCL were initiated and maintained as previously described (5). Briefly, mononuclear cells in RPMI 1640 with 20% fetal bovine serum (FBS) were mixed with supernate from the EBV producing cell line B95-8, and were incubated at 37°C. Half of the medium was replaced each week, and with establishment of the LCL stationary cultures at a density of 5–10 × 10^5 cells/ml were maintained. Three autologous lymphoid cell lines were established from individuals whose peripheral blood lymphocytes were available for continuous testing. These were Lig, Letch, and Hoff. All of these grew well in continuous culture and were utilized several years after initial establishment.

HLA Typing of LCL and Peripheral Blood Lymphocytes (PBL). HLA typing of PBL and LCL were kindly performed by Dr. M. Fotino at the New York Blood Center, by a two-stage microcytotoxicity technique. Cell lines present special problems in HLA typing because of their B-cell alloantigens. Specificities listed represent those also present on autologous PBL; any extra reactions encountered are not included. The apparent HLA specificities of the cells used in these experiments were as follows: Lig A1,A2,B8,B12; Letch A1,A2,B7,B8; Hoff A2,B12,B13; 4265 A2,AW32,B7,B12; 8392 A1,A10,B7,BW17; B35M A1,A3,B7,B8; CMS A9,AW24,B7,BW15; AG A1,A2,B12,BW17; RV A2,B12,BW18; and JH A1,A11,BW5. Typing for B-cell antigens was kindly performed by Dr. R. Winchester and Dr. A. Gibofsky (6). Results with sera previously found to be correlated with HLA-D typing by mixed lymphocyte culture (MLC) allowed tentative assignment of the following DW associated specificities: Lig 1,3; Letch 3,–; Hoff 3,3 or 3,4; 4265 1,4; and B35M 3,3.

Purification of B Cells and T Cells. After initial separation on Ficoll-Hypaque gradients,

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lymphocytes were fractionated by using E-rosette separation techniques. The nonrosetting layer was composed primarily of Ig-bearing cells and monocytes. The percent of T-cell contamination determined by re-rosetting ranged from 2 to 15%. The rosetting population was of uniform lymphocyte morphology and was 90–98% pure T cells by re-rosetting.

Preparation of MLC. The micromethod used was a modification of the technique described by Hartzman et al. (7). Unidirectional MLCs were established in triplicate, each culture well consisting of 0.2 ml of culture medium containing 1×10^5 responding lymphocytes and either 1×10^5 irradiated (3,000 rads) stimulating lymphocytes or 3×10^4 irradiated (6,000 rads) stimulating LCL cells. Primary cultures were incubated for 144 h at 37°C in a humidified CO_2 environment. Cultures were pulsed with [^{3}H]thymidine, harvested, and assayed for thymidine incorporation as previously described (4). Cells were used as cell-mediated lympholysis (CML) effectors or primed responder cells in secondary MLC after culture in small flasks. Cultures consisted of 20 ml of medium containing 1×10^7 responder cells and either 1×10^7 stimulating lymphocytes or 3×10^6 stimulating LCL cells. Cultures for generation of CML effector cells were stopped after 6 days, except for autologous purified B-cell stimulation, when cultures were incubated for 7 days. Secondary cultures were established after lymphocytes had been incubated for 10 days.

In third party cell experiments, heat treatment of PBL was done at 45°C for 90 min (8). Viability was greater than 90% after treatment. The number of viable heat-treated cells added to a culture was equal to the number of responder cells.

Peripheral blood lymphocytes to be used as CML target cells were incubated at 1×10^6 cells/ml in standard culture medium without mitogen, for 6 or 7 days.

CML Assay. A modification of the standard radioactive chromium (^{51}Cr) release assay (9) was used. Cultured PBL and LCL to be used as target cells were washed and incubated with an equal volume of ^{51}Cr-labeled sodium chromate; followed by washing, resuspension in medium, and kept at 4°C. Effector cells were harvested from MLC flasks, washed three times in PBS, and resuspended in RPMI 1640 with 20% FBS. Assay was performed in triplicate in V-bottom microtiter plates, each well containing 0.1 ml of target cell suspension and 0.1 ml of effector cell suspension. Target cells without effector cells were mixed with 0.1 ml medium to obtain spontaneous ^{51}Cr-release, and with 0.1 ml detergent to obtain maximal ^{51}Cr-release.

After addition of cells, plates were spun at 40 *g* for 5 min, then incubated for 4 h at 37°C in a humidified 5% CO_2 environment. After incubation, plates were spun at 200 *g* for 10 min at 4°C. 0.1 ml of supernate was carefully removed from each well and counted in a Packard Auto-Gamma scintillation counter (model no. 5385, Packard Instrument Co., Inc., Downers Grove, Ill.).

The percentage of specific lysis was calculated for each effector to target cell ratio tested as described previously (9). The percent CML values obtained in triplicate determinations were plotted versus the $\log_{10}$ of the effector to target cell ratio. A dose-response curve was obtained by using points from the four effector to target cell ratios tested in each case. A lytic unit (LU) was arbitrarily defined as that number of effector cells required to yield 30% lysis.

Results

Lymphocyte Proliferation Stimulated by Autologous LCL or Purified B Cells. Lymphocyte proliferation is markedly stimulated by autologous or allogeneic LCL. This was documented not only by the increased incorporation of [^{3}H]thymidine but also by the three to fivefold increase in the number of lymphocytes present at the end of the culture period. Allogeneic and autologous LCL stimulated lymphocyte proliferation to a similar degree. Maximal thymidine incorporation occurred after 5 or 6 days of culture for both allogeneic and autologous LCL stimulation.

Thymidine incorporation at the peak of the autologous and allogeneic response was always greater with LCL stimulators than with purified B-cell stimulators from the cell line donors. Kinetic studies by Weksler and Kozak (10) have shown that the peak response to allogeneic purified B cells occurs on day 5 or 6 while the peak response to autologous purified B cells occurs on day 6 or 7. Similar results were obtained in the present study.

TABLE I

MLC with Autologous LCL and Purified B-Cell Stimulators: Cytotoxic Cell Generation

MLC Combination	^{3}H, cpm	% CML against PBL target cells									
		Lig		Letch		AG		RV		JH	
		100:1	25:1	100:1	25:1	100:1	25:1	100:1	25:1	100:1	25:1
Lig + Lig LCL_x	77,380 ± 6,270	−2 ± 1	0 ± 1	−2 ± 2	−1 ± 2	1 ± 3	−1 ± 0	1 ± 1	0 ± 2	3 ± 2	3 ± 1
CD + Lig LCL_x	69,040 ± 470	46 ± 3	35 ± 2	−2 ± 1	−1 ± 2	48 ± 2	33 ± 4	39 ± 1	27 ± 2	−1 ± 2	2 ± 1
Letch T + B_x	19,950 ± 1,980	2 ± 2	1 ± 2	−2 ± 1	1 ± 2	3 ± 2	2 ± 2	1 ± 2	−2 ± 1	−5 ± 3	−2 ± 1
		% CML against LCL target cells									
MLC Combination	^{3}H, cpm	Letch		Lig		Hoff		4265		8392	
		80:1	20:1	80:1	20:1	80:1	20:1	80:1	20:1	80:1	20:1
Letch T + B_x	19,950 ± 1,980	−6 ± 2	−2 ± 1	−2 ± 2	−1 ± 3	−1 ± 2	1 ± 2	−2 ± 1	2 ± 0	−5 ± 2	−5 ± 2
Lig + Lig LCL_x	62,800 ± 3,700	38 ± 2	26 ± 3	23 ± 4	18 ± 3	37 ± 2	24 ± 4	34 ± 3	26 ± 9	45 ± 6	33 ± 2
Letch + Letch LCL_x	47,280 ± 8,040	21 ± 4	19 ± 4	32 ± 4	31 ± 6	35 ± 5	24 ± 3	48 ± 4	39 ± 1	26 ± 4	18 ± 2
Hoff + Hoff LCL_x	58,200 ± 3,300	42 ± 2	35 ± 3	29 ± 1	23 ± 3	23 ± 1	18 ± 2	34 ± 4	29 ± 2	30 ± 3	23 ± 1
Letch + Lig LCL_x	64,080 ± 19,100	14 ± 1	12 ± 3	82 ± 6	69 ± 7	46 ± 6	35 ± 5	61 ± 6	49 ± 3	27 ± 5	20 ± 3

X = Stimulator cells X irradiated.

Secondary responses (maximal on day 3) could be stimulated by autologous LCL after primary stimulation by B cells. The reverse experiment with primary stimulation by LCL and secondary B-cell challenge gave similar effects. Allogeneic cells also gave an accelerated secondary response after primary stimulation with either autologous LCL or B cells, although the degree of stimulation was less than with the secondary autologous additions.

Cytotoxic T-Cell Generation. No cytotoxic T cells were detectable after stimulation with autologous purified B cells or autologous B-lymphoid cell lines when peripheral blood target cells were used. Numerous experiments showed this was always the case. Representative experiments are shown in Table I. In 10 experiments, mean peak thymidine incorporation by T cells cultured with purified autologous B cells (19,409 ± 5,651 cpm) was significantly greater than the sum of the thymidine incorporated by T cells alone (1,143 ± 867 cpm) and irradiated B cells alone (1,039 ± 551 cpm). Despite this proliferative response, no cytotoxicity was developed against autologous or allogeneic LCL or PBL. In contrast the allogeneic MLC always developed specific CML towards PBL or LCL bearing the stimulating alloantigens.

Similar results were found when autologous LCL were used as stimulators. Thus, despite the extremely high thymidine incorporation by lymphocytes cultured with autologous LCL no cytotoxicity was observed against PBL from the donor of the stimulating LCL or against any other peripheral blood cells. When lymphoid cell lines were used as target cells, some nonspecific killing was observed when LCL were also used to stimulate autologous T cells. However the cytotoxicity against the LCL used as stimulator was always less than the cytotoxicity against heterologous LCLs (Table I). This was not the case when autologous B cells were used as stimulators; no killing above baseline values was observed against LCL targets.

The nonspecific cytotoxicity generated in the autologous LCL culture was considerably below the cytotoxicity generated in the allogeneic LCL culture. This was particularly evident when the results were calculated as LU per 10^3

cells. After culture with autologous LCL, the LU generated were less than 2 when stimulating LCL used as targets and 13–143 when heterologous LCL were used as targets. After culture with allogeneic LCL, the specific LU generated ranged from 830 to 10,000 when the stimulating LCL was used as target.

Cytotoxic Cell Generation in Third Party Systems. The autologous MLC with purified B-cell stimulators or LCL is a proliferative response without the generation of significant cytotoxic T cells. Experiments were performed to determine if the autologous MLC would provide a proliferative stimulus for CTL generation in the presence of nonstimulating third party cells with foreign HLA antigens. Heat-treated lymphocytes possess HLA-A and HLA-B determinants, but neither stimulate the MLC nor by themselves generate cytotoxic lymphocytes (CTL) (8). A variety of experiments with different cell combinations in autologous MLC with purified B-cell stimulators were performed in the presence and absence of allogeneic heat-treated cells. As had been previously reported, heat treatment completely abolished the capacity of B cells to stimulate proliferation of T cells and the generation of cytotoxic T cells. However, the addition of these cells to the autologous MLC reactions in each case resulted in the generation of specific CTL's directed against target cells bearing the HLA determinants of the heat treated cell used. No cytotoxicity to autologous cells was found. Similar results were obtained with the autologous LCL system. In Table II, two representative experiments are shown. The pattern of cytotoxicity in the autologous MLC plus heat-treated allogeneic cell combination was identical to the standard allogeneic MLC control.

Discussion

In the present study long-term lymphoid cell lines were obtained from three individuals who were available for continued studies of the autologous MLC reaction. This permitted the stimulatory capacity of B-lymphoblastoid line cells and purified B cells from peripheral blood from the same individual to be compared in autologous MLC reactions. Both systems have been studied independently, lymphoid cell lines a number of years ago (1, 2) and normal B cells more recently (3, 4). The latter studies suggest that the earlier LCL autologous stimulation studies may have been misinterpreted and that the two systems are analogous. The present work centered on the production of cytotoxic cells in these autologous systems. No cytotoxic cells were generated during the autologous MLC when purified B cells were used as autologous stimulators regardless of whether peripheral blood cells or cell lines were used as target cells.

No specific cytotoxic cells were detected when autologous LCL were used as stimulators in the autologous MLC although some general cytotoxicity against a variety of cell lines was observed. The level of cytotoxicity was low in comparison with the level of specific cytotoxic reactivity stimulated by allogeneic LCL. It also was evident that cytotoxicity generated was nonspecific as it was lower against the stimulating LCL than against heterologous lines. The exact cause of this cytotoxicity remains unclear. It is known and was evident in the present study that certain cell lines are very susceptible to lysis as target cells. This may have been one factor in the cytotoxicity observed but certainly not the only factor in view of the lower cytotoxicity against the

TABLE II
Generation of Specific CTL by Combination of an Autologous MLC and Heat-Treated Allogeneic Lymphocytes

MLC Combination	^{3}H Uptake, cpm	% CML					
		PBL Targets					
		Letch		Hoff		CMS	
		100:1	25:1	100:1	25:1	100:1	25:1
Letch T	290 ± 140	−3 ± 2	−3 ± 1	0 ± 1	−1 ± 1	−1 ± 0	−1 ± 0
Letch T + B_X	29,230 ± 2,600	−5 ± 2	−3 ± 0	−1 ± 0	−1 ± 2	−1 ± 1	−2 ± 0
Letch T + Δ Hoff PBL	1,620 ± 420	−9 ± 2	−7 ± 1	−6 ± 1	−1 ± 1	−6 ± 2	−6 ± 1
Letch T + B_X + Δ Hoff PBL	18,800 ± 660	−14 ± 1	−10 ± 2	29 ± 2	23 ± 3	−8 ± 1	−5 ± 3
Letch T + Hoff PBL_X	36,680 ± 2,390	−4 ± 3	−1 ± 2	37 ± 2	28 ± 1	2 ± 1	0 ± 1
CMS T	129 ± 65	−4 ± 1	−4 ± 2	2 ± 1	1 ± 1	−1 ± 1	1 ± 1
CMS T + B_X	19,170 ± 1,180	−6 ± 2	−2 ± 1	−3 ± 1	−3 ± 2	−2 ± 0	−1 ± 3
CMS T + Δ Letch PBL	173 ± 39	−8 ± 2	NT	−4 ± 1	NT	−5 ± 2	NT
CMS T + B_X + Δ Letch PBL	20,666 ± 1,650	41 ± 4	25 ± 2	28 ± 1	22 ± 2	−1 ± 1	−4 ± 0
CMS T + Letch PBL_X	28,574 ± 3,073	36 ± 3	20 ± 2	32 ± 3	21 ± 1	2 ± 2	0 ± 1

* NT = not tested.

stimulating line. The latter finding also argues against a role for cytotoxicity against EB viral antigens in the cell lines. However, this is not ruled out and studies are underway to answer this question.

The results of experiments involving heat-treated third-part cells indicate clearly the distinction between the proliferative and cytotoxic phases of the MLC response. More importantly they show that the interaction between unmodified autologous cell populations in collaborating with a foreign antigenic determinant can generate cytotoxic reactivity which cannot be generated by the foreign determinant itself. This raises the possibility that the autologous MLC may be of significance in vivo. Cells bearing foreign antigens may exist in vivo as a consequence of viral infection or neoplastic transformation. The autologous MLC in localized areas may provide the proliferative stimulus needed for the generation of CTL in vivo against such cells. Recently several groups (11, 12) have found that lymphocytes from some normal individuals sensitized to MHC identical leukemia cells could, in the presence of an allogeneic proliferative stimulus, differentiate into specific CTL against the leukemia cells. The autologous MLC proliferative stimulus might also be used to create in vitro specific cytotoxic cells against specific neoplasms without the introduction of allogeneic cells.

Summary

Autologous mixed lymphocyte culture (MLC) reactions were studied utilizing autologous purified B cells and autologous established B lymphoid cell lines as stimulating cells. Similar results were obtained although somewhat greater stimulation of lymphocyte proliferation was found with the autologous lymphoid cell lines. Cytotoxic T cells were not generated against the stimulating cells in either case when peripheral blood cells were used as targets. A low cytotoxicity was detected when lymphoid cell lines were used both as stimulators and target cells. However this was nonspecific and was always greater for heterologous lines than for the stimulator line.

Third-party cell experiments demonstrated that the autologous reaction could serve as a proliferative stimulus for specific cytotoxic lymphocyte generation. Heat-treated allogeneic lymphocytes that alone do not stimulate proliferation or cytotoxic T-cell generation in MLC reactions when added to the autologous system produced specific cytotoxic cells. The separation of the proliferative phase from the cytotoxic cell generation was especially striking in these experiments. Possible uses of this system for the generation of specific cytotoxic cells to other nonstimulatory cells are discussed.

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