Journal of Supramolecular Structure 13:525–532 (1980)
Control of Cellular Division and Development: Part B: 79–86

Long-Term Maintenance of "Cloned" Human PLT Cells in TCGF With LCL Cells as a Feeder Layer

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The long-term maintenance of T cells "cloned" by limiting dilution in TCGF was enhanced by the use of irradiated autologous lymphoblastoid cell line (LCL) cells as well as irradiated LCL cells of the individual to which the T cells were originally primed. It was possible to obtain more than 1×10^{12} cells from a "clone" seeded at one cell per well. Some of the clones tested express primed LD-typing activity.

Key words: T lymphocytes, HLA-D, cloning, TCGF, PLT lymphocyte typing

Long-term growth of human T lymphocytes can be accomplished through the use of T-cell growth factor (TCGF). This factor is present in conditioned medium of PHA-stimulated mixtures of allogeneic peripheral blood lymphocytes [1, 2]. We have used such conditioned media for the propagation of T cells that have been activated in vitro to allogeneic cells. Such cells following agar or limiting dilution cloning have been demonstrated to retain functional PLT-type proliferative specificity [3, 4] and cytotoxic activity [4–6]. Often, however, during the maintenance of T cells in TCGF, proliferation will decline, and eventually death of the cell population occurs. While the expansion of cells in TCGF can be demonstrated with relative ease, when attempting to clone cells from these populations, either through the use of soft agar or by limiting dilution, it has been difficult to obtain greater than 1×10^8 cells.

In an effort to increase the number of progeny obtained from a clone, we examined the ability of irradiated cells used as "feeder" cells in the presence of TCGF to improve or enhance the growth of cloned T cells. Five cell types were used: normal lymphocytes autologous with the original responder, normal lymphocytes of the specific stimulator used to prime the cells, EBV-transformed lymphoblastoid cell line (LCL) cells autologous with the responder or the specific stimulator, and the continuous human T-cell line CCRF-HSB-2. Maximum growth of cloned cells in TCGF was supported by irradiated LCL lines; and through the use of these EBV-transformed cells as feeders, it was possible to obtain up to 1×10^{12} cloned cells. Such cloned cells grown with irradiated LCL cells in TCGF retain primed LD typing (PLT) reactivity. It has been suggested that cloned PLT reactive cells may be capable of allowing definition of HLA-encoded antigens with a degree of

Received March 7, 1980; accepted July 23, 1980.

0091-7419/80/1304-0525$01.70

specificity previously unobtainable with the average PLT reagent [4]. Clones of T cells that have been primed to alloantigens in a primary MLC might thus facilitate fine structure dissection and analysis of the HLA-D region encoded antigens.

MATERIALS AND METHODS

T-Cell Growth Factor

TCGF was either obtained from Associated Biomedics Systems (Buffalo, NY) or produced in our own laboratory. It was concentrated fourfold and then dialyzed, first against 20 volumes of 0.15 M NaCl and then against 10 volumes of Hank's balanced salt solution. After filter sterilization, the TCGF was stored at −20°C. TCGF medium was prepared by dilution of the TCGF in RPMI-1640 with 25 mM Hepes and addition of human serum to a final concentration of 15%. The appropriate dilution of the TCGF was one that induced maximum ^{3}H-thymidine uptake in long-term cultures of T cells [7].

PLT Activation and Cell Cloning

Peripheral blood lymphocytes were isolated from heparinized blood of healthy volunteers by Ficoll-Hypaque gradient centrifugation [8]. One $\times 10^7$ cells to be primed from individual A, were added to 1×10^7 irradiated (4,000 R) stimulator cells from individual B in 15 ml RPMI-1640 with 25 mM Hepes supplemented with 15% human serum (HS-RPMI) in Falcon number 3013 culture flasks. On day 5, the large blast cells were isolated by unit gravity sedimentation [9] and plated at a dilution of 1 cell per well in Terasaki microwells (Cooke 236-72) in TCGF medium. The remaining small lymphocytes obtained from the gradient were pooled, irradiated with 4,000 R and added to each microwell at a concentration of 1×10^4 feeder cells per well. When growth of the clone was observed through the use of a phase-contrast microscope, the contents of a microwell were transferred to a 7-mm well (Linbro 76-001-05) and stimulated with 1×10^5-irradiated lymphocytes from individual B. Again, when growth was visualized, the contents of a 7-mm well were transferred to a 17-mm well (Linbro 76-003-05) and stimulated with 2×10^5-irradiated lymphocytes from individual B. When growth was observed, the contents of each well were split into 2 wells and fresh TCGF medium was added to each. After the initial addition of Bx cells to the 17-mm wells, no additional stimulator or irradiated feeder cells were added until growth in TCGF medium had declined.

Feeder Layer Cells

Five cell types were used as irradiated feeder layers: Ax, normal lymphocytes autologous with the cloned cells; Bx, normal lymphocytes from the same donor as that used to originally sensitize the cloned cells; A-LCLx and B-LCLx, lymphoblastoid cells obtained by conversion of A and B lymphocytes respectively with the Epstein-Barr virus [10]; and an unrelated T-cell line CCRF-HSB-2 obtained from Dr. Jun Minowada, Roswell Park Memorial Institute, Buffalo, NY. The T-cell line was derived from a patient with acute lymphocytic leukemia. The normal lymphocytes were irradiated with 4,000 R and the continuous lines with 8,000 R prior to their use as feeders. Throughout this paper "autologous" refers to cells from the same donor as the individual used for the responder in the initial sensitization.

Day 0	ABx PLT cell culture
Day 5	1 g gradient isolation of large blast cells 1 blast cell per well in Terasaki microwell plates with irradiated feeder cells
Day 15	transfer to 7-mm wells with Bx feeder cells
Day 20	transfer to 17-mm wells with Bx feeder cells
Day 32	transfer to flasks with TCGF medium alone
Day 49	transfer to 17-mm wells with various irradiated test feeder cells in TCGF medium (see Table I)
Day 80	transfer to flasks with A or B LCLx feeder cells

The clones have continued to multiply in the presence of the irradiated LCL feeders in TCGF medium for more than 120 days.

Fig. 1. Expansion and maintenance of cloned T cells. The cloned cells were fed every 3–4 days with fresh TCGF medium.

PLT Reactivity of Cloned Cells

The cloned cells were tested as given in Results for their PLT reactivity [11]. Briefly, 1×10^4 cloned test cells were incubated with 5×10^4-irradiated stimulator cells in V-bottom wells (Linbro 76-021-05) in 15% HS-RPMI. After 40 hours, the cultures were labeled with 2 μCi per well ^{3}H-thymidine for 8 hours. Values given are mean counts per minute of quadruplicate samples. The clonal nature of the cells we are using is not established; re-cloning has not been performed. For simplicity of expression we have referred to the populations as clones.

RESULTS

Visual examination of microwells 10 days after seeding with an average of 1 blast cell per well in TCGF-medium revealed that 10% of the wells seeded had actively growing cultures, a finding consistent with previous work [12]. Figure 1 summarizes the handling of the clones. The contents of wells with cells growing were transferred to 7-mm wells and stimulated with 1×10^5-irradiated lymphocytes from individual B. After an additional 5 days, 68% of the 7-mm wells contained actively growing cultures. The contents of these wells were transferred to 17-mm culture wells and stimulated with 2×10^5-irradiated lymphocytes from individual B. For the following 2 weeks, each of these wells was split into 2 wells every 3–4 days and fresh TCGF-medium added. No additional feeder lymphocytes were added during this period. Clones grown for 28 days in culture yielded 1×10^7 cells or more, as previously reported [4]. Results that are representative of the crisis in cell growth seen when clonal progeny are cultured in TCGF-medium without feeder cells are shown in Figure 2. Between day 28 and day 35 there was an increase in cell numbers in the clones. However, between days 35 and 42 one of the clones demonstrated cell growth and the other maintained the cell number at which it was seeded. Between days 42 and 49, clone 6 decreased in cell number, and clone 4 maintained at the level at which it was seeded, even though each week the clones were recultured under identical conditions.

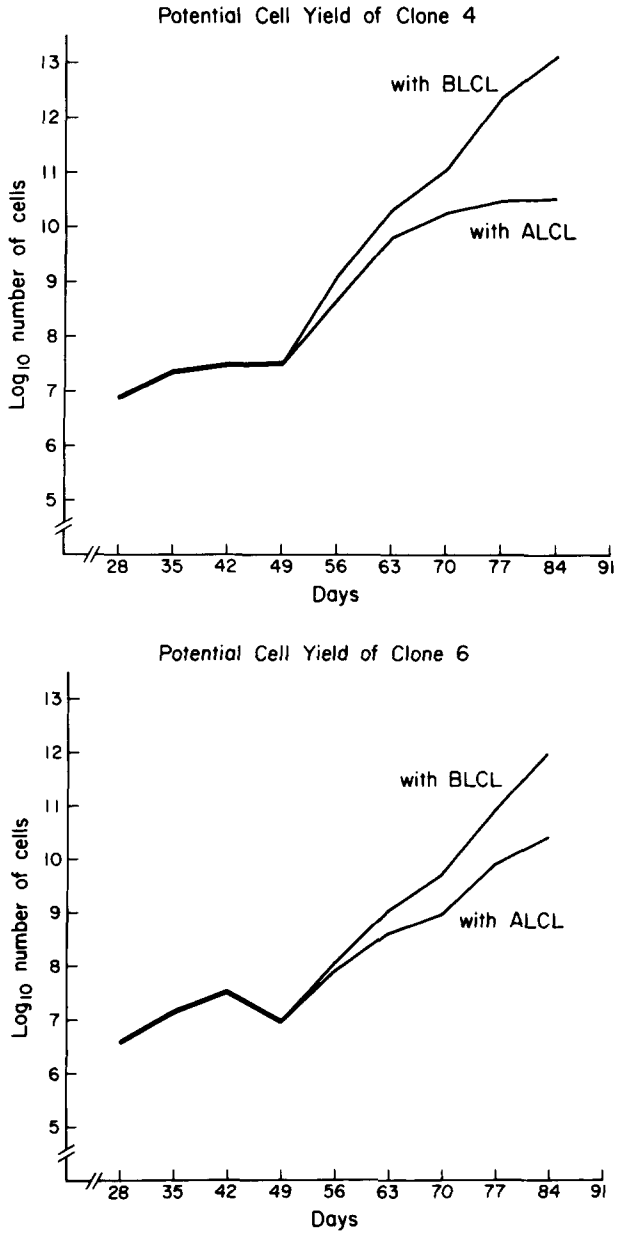

Fig. 2. Potential cell yield of clones 4 and 6 had the total number of cloned cells obtained each week been recultured.

In an attempt to keep the clones proliferating, 7 weeks after initiation of culture, 5 different cell types were used as irradiated feeder cells in the presence of TCGF-medium in the cultures. Table I presents the number of cells obtained, after one week, per 1×10^5 cloned cells seeded with the different irradiated feeder cells. The control, with only TCGF medium, did not support the continued growth of any of the clones nor did the T-cell line

TABLE I. Growth of Cloned Cells in TCGF With or Without Feeder Cells*

	Clone			
Feeder cell	4	6	7	8
None	0.2[a]	0.2	0.2	0.8
A	0.4	2.8	1.6	0.4
B	1.6	2.8	3.6	0.4
A LCL	8.4	15.0	36.0	9.6
B LCL	12.0	39.0	68.0	26.0
HSB-2	0	0.4	0.8	1.2

*Each clone was seeded into 6 wells (Linbro 76-033-05) (1×10^5 cells/well). Irradiated feeder cells were added as follows:

A	6×10^5 autologous normal lymphocytes 4,000 R.
B	6×10^5 normal specific stimulator lymphocytes 4,000 R.
A LCL	1×10^5 autologous LCL cells 8,000 R.
B LCL	1×10^5 specific stimulator LCL cells 8,000 R.
HSB-2	1×10^5 cells of T-cell line HSB-2 8,000 R.

[a]Number of cells obtained $\times 10^{-5}$ per 1×10^5 cells seeded.

CCRF-HSB-2. Irradiated autologous normal cells supported growth in 2 clones, and irradiated specific stimulator lymphocytes did so in 3 clones. The cell increase, however, was not as great as that observed with LCL cells as feeder cells. Irradiated autologous LCL cells induced an 8- to 36-fold increase in cell number, whereas the LCL line of the specific stimulator induced a 12- to 68-fold increase. These results are representative of the relative effects of the various feeder cells on growth of clones.

Clones have been maintained in 17-mm wells by seeding 1×10^5 cloned cells with either 1×10^5 autologous or specific stimulator-irradiated LCL cells in 2 ml of TCGF medium. Each well is split into 2 wells after 3 days and an additional 1 ml of TCGF medium is added to each well. One week after initial seeding, the contents of the 2 wells are combined and a cell count is made. The cloned cells are then reseeded at 1×10^5 cloned cells per well with 1×10^5 feeder cells – conditions identical to the previous week.

Larger numbers of cells can be grown in flasks (Corning 25100). Three $\times 10^5$ cloned cells and 3×10^5-irradiated LCL feeder cells are combined in 10 ml of TCGF medium; after 1 week a cell count is made, and the cells are reseeded under identical conditions as 7 days earlier.

Cell counts made on cultures reseeded each week during the 13 weeks were used to calculate the theoretical number of cells (Fig. 2) that could be obtained from 2 clones had the total number of cloned cells obtained each week been reseeded in fresh TCGF medium with irradiated LCL feeder cells. As shown in Table I and Figure 2, the LCL feeder cells autologous with the clone induced growth; however, the LCL line of the specific stimulator induced an even higher level of cell division. It would be possible with both clones to obtain greater than 1×10^{10} cells when A-LCLx is used as a feeder, and 1×10^{12} cells when B-LCLx is used as a feeder. We have added irradiated LCL feeder cells at various times before the logarithmic growth of cloned cells in TCGF alone has declined. The feeder cells, when added between the third and fourth weeks of culture, prolong the logarithmic growth rate for up to 13 weeks of culture.

The PLT reactivity of 2 clones that had been maintained in TCGF medium with either A-LCLx or B-LCLx from day 26 on is presented in Table II. Clone A-1 responded to Bx, the original stimulating cell, as well as to Cx and Dx, while Clone A-5 responded to

TABLE II. PLT Reactivity of Cloned T-Cells

Clone[a]	Feeder cell	Stimulator[b]	Day 26	Day 33	Day 41	Day 48
A-1	B LCL	Ax	97 ± 26[c]	445 ± 157	334 ± 304	207 ± 93
		Bx	1,893 ± 296	3,895 ± 256	2,808 ± 312	22,702 ± 2,478
		Cx	1,908 ± 242	2,236 ± 428	1,772 ± 324	NT[d]
		Dx	1,190 ± 209	954 ± 125	535 ± 213	NT
A-5	A LCL	Ax	NT	141 ± 43	391 ± 409	392 ± 113
		Bx	NT	13,958 ± 738	7,687 ± 358	19,564 ± 2,750
		Cx	NT	100 ± 52	156 ± 91	NT
		Dx	NT	138 ± 29	292 ± 129	NT

[a]Clones A-1 and A-5 originated from an ABx primary culture with subsequent limiting dilution in Terasaki wells at 1 cell per well for A-1 and 5 cells per well for A-5.

[b]Cx and Dx are lymphocytes from randomly chosen unrelated individuals.

[c]Values given are mean counts per minute of quadruplicate samples ± SD.

[d]NT, not tested.

only the original stimulating cell. This indicates that B has a common or cross-reactive determinant with C and D, and B also has a unique determinant not shared with either C or D.

DISCUSSION

Human T-lymphocytes sensitized to alloantigens grow in vitro in the presence of TCGF [2, 4]. When cloning of these cells is attempted by limiting dilution, the use of an irradiated feeder layer greatly enhances the efficiency of cloning [12]. Once the clones are established and proliferating, growth for the first several weeks in culture is rapid. However, after the clones have undergone approximately 25 divisions, growth diminishes substantially, often resulting in loss of the culture. This occurs even though the TCGF that is used is active in allowing other clones – those that have not been in TCGF for as long – to proliferate.

In an attempt to keep cloned cells proliferating, 5 different cell types were used as irradiated feeder layers. While the normal Ax and Bx cells did enhance growth over the level observed with clones given no feeders or feeder cells of a T-cell line, the use of x-irradiated EBV-transformed cells allowed growth at levels often 10 times higher than the normal cells. The reason for the enhanced growth is not known, although LCL cells are known to be potent stimulators in an MLC response [13] and enhance TCGF production when included in the mixture of allogeneic cells used to produce TCGF [12]. Cloned cells that are primed to a specific alloantigen could not be antigenically stimulated by LCL cells autologous with the cloned cells but only by the alloantigen on the LCL cells of the specific stimulator; this could explain the greater growth of progeny of any one clone given B-LCLx as feeders, as opposed to A-LCLx as feeders. We have not determined if the use of LCL cells along with TCGF medium selects for a specific type of T cell. It is our experience that TCGF medium alone will not maintain cloned human PLT reactive cells past day 50 in culture; whereas the use of the feeder cells prolongs their life span in vitro.

It is evident that the multiplying cells are from the original clone and not from the irradiated feeder layer. First, greater than 85% of the lymphocytes obtained rosetted with sheep red blood cells and thus are not LCLs. Second, the cloned cells, after growing in the presence of the LCL feeder layer, will die within 72 hours after being transferred to HS-RPMI, medium known to support the growth of LCL cells. Third, growth of cloned cells was dependent on TCGF as well as the feeder layers. Clones cultured in HS-RPMI with irradiated LCL feeders, but without TCGF did not proliferate. Similarly, when clones were cultured in TCGF but deprived of feeders, proliferation declined (data not shown).

When PLT cells are cloned by limiting dilution, approximately 25–30% of the clones express PLT specificity. Of 10 clones chosen for their specificity at day 26, all maintained their PLT reactivity through day 48. The activity of 2 representative clones is presented in Table II. The PLT reactivity of the 2 clones presented demonstrates how limiting dilution cloned PLT cells can simplify the PLT response and enhance the usefulness of the assay. The original ABx 10-day PLT population responded highly to Bx and Cx, and to a much lower level to Dx (data not presented). This pattern is similar to the response of clone A-1, indicating that B and C, as well as B and D, have a common determinant. There must also be a determinant unique to B – that is, not shared with C or D – and clone A-5 recognizes this unique determinant.

Even though the true clonal nature of these T-cell populations has not been established, the populations are capable of giving a highly specific PLT response, which may enable dissection of HLA-D region-encoded determinants that has not been possible with reagents previously available. The use of irradiated LCL cells as feeder cells in conjunction with TCGF medium enables prolongation of the life-span of an individual clone, as well as an increase in the number of cloned cells obtained.

ACKNOWLEDGMENTS

We thank Dr. C. Garrison Fathman for helpful discussions in the initiation of this work and Dr. Guy Bonnard, who first suggested to us the inclusion of LCL cells in the production of TCGF.

This work was supported by March of Dimes Birth Defects Foundation grant 6-256 and NIH grants CA-09106, AI-08439, CA-16836, and AI-15588. This is paper number 217 from the Immunobiology Research Center and paper number 2407 from the Laboratory of Genetics, University of Wisconsin, Madison, Wisconsin 53706. H. Inouye was supported by Tokai University School of Medicine, Japan.

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