GROWTH PROPERTIES AND ALLOANTIGENIC EXPRESSION OF MURINE LYMPHOBLASTOID CELL LINES*

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A number of genetically determined cell surface components have been described which are expressed on murine lymphocytes (reviewed in references 1–4). As examples of selective gene action between various cell types or the same cell type under different microenvironmental influences these cell surface components have been termed "differentiation alloantigens" (5, 6).

The alloantigens Thy-1 and thymus leukemia (TL)[1] are intriguing components for study in that they reflect differentiational events of thymocytes and thymus-derived lymphocytes. Thy-1, originally described by Reif and Allen (7) can be detected on thymocytes and peripheral T lymphocytes as well as brain and epidermal cells (7, 8). The expression of Thy-1 on peripheral lymphocytes in vivo requires processing of lymphocytes by the thymus (9). Two allelic specificities (Thy-1.1, formerly theta-AKR and Thy-1.2, formerly theta-C3H) have been detected in mice (7) while only the Thy-1.1 specificity has been detected in rats (10).

TL alloantigens can be detected in normal mice only on thymocytes (11). Three phenotypes have been described; TL.2 (BALB/c and DBA/2), TL.1,2,3 (A/J and C58), or TL^- (AKR and B6) (12). Leukemic mice may express TL on peripheral lymphocytes, even if the strain is TL^-, and the specificity TL.4 is often expressed by leukemic cells (13).

A number of murine lymphoblastoid cell lines have been adapted to culture (14–16). These cultured cell lines have been maintained in stationary or shaker flasks for long periods, and many of the lines continue to express their cell surface components (17–20). For many studies it would be advantageous to acquire large quantities of these lymphoblastoid cell lines. The requirement can be best fullfilled by adapting the cells to grow in suspension cultures (21–23). This was the impetus for the present study in which we evaluated the quantitative expression of Thy-1 and TL on lymphoblastoid cell lines maintained in

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[1] *Abbreviations used in this paper:* AD, absorption dose; FBS, fetal bovine serum; HS, horse serum; TL, thymus leukemia.

suspension cultures, and investigated the effect of various culture conditions on the expression of these alloantigens.

Materials and Methods

Cell Lines. Lymphoblastoid cell lines were obtained from several sources (Table I). These lines had been maintained previously in stationary or shaker cultures. Upon receipt the cultures were adapted to grow in suspension culture using RPMI-1640 enriched with either 10% horse serum (HS) or fetal bovine serum (FBS) as the growth medium (23). All lines evaluated for alloantigen expression were cultured in RPMI-1640 plus 10% FBS, except for RADA1, designated in Table I as RADA1-HS, which could not be propagated with FBS and was cultured in medium supplemented with 10% HS.

Antisera. Antiserum specific for TL-1,2,3 was raised by injecting ASL1 ascites tumor cells (TL.1,2,3) into hybrid mice derived from mating male A/TL$^-$ with female C57BL/6 mice which were also TL$^-$ (8).

Antiserum specific for Thy-1.2 was raised by injecting AKR/Cum thymocytes (Thy-1.2) into AKR/Jax (Thy-1.1) mice (7, 24). Specific antiserum for Thy-1.1 was raised by reversing the above injection scheme. After the appropriate injection schedule, mice were bled from the retroorbital plexus, and the serum was collected, aliquoted and stored at −70°C until used.

Absorption. Cells used for absorption were washed in RPMI-1640 medium and adjusted to a concentration of 4×10^8 cells/ml for TL.1,2,3 absorption and 2×10^8 cells/ml for Thy-1 absorptions. Serial dilutions of the cell suspension were made and incubated for 2 h on ice with an equal volume of the appropriate dilution of antiserum (1/800, TL.1,2,3; 1/80, Thy-1.1; and 1/30, Thy-1.2). The absorption mixture was frequently stirred by use of a Vortex mixer (Scientific Industries, Inc., Queens Village, N. Y.) during the incubation period. After centrifugation for 2 min at 8,000 *g* on a Brinkmann 3200 centrifuge (Brinkmann Instruments, Inc., Westbury, N. Y.) the supernate was removed and subsequently assayed for residual antibody by the cytotoxicity assay. The residual activity remaining in the absorbed antiserum was expressed as:

$$\% \text{ absorption} = 100 - \left(\frac{\% \text{ cells dead by absorbed serum}}{\% \text{ cells dead by unabsorbed serum}} \times 100\right).$$

The percent absorption was plotted vs. the number of cells used for absorption. The number of cells which reduced the cytotoxic activity of a milliliter of specific antiserum at a defined dilution by 50% was taken as the absorption dose (AD_{50}) (25). As a control, cells were used for absorption which lacked the specificity against which the antiserum was directed.

Cytotoxicity Assay. The cytotoxicity assays were conducted as described by Boyse et al. (26). 50 μl of the appropriate target cell suspension (A/J, TL.1,2,3; AKR/Jax, Thy-1.1; or AKR/Cum, Thy-1.2 thymocytes) at a concentration of 5×10^6 cells/ml were added to a microtest tube, followed by 50 μl of the absorbed or unabsorbed antiserum. Finally, 50 μl of a dilution of guinea pig serum, previously tested for high killing in the presence of antiserum and less than 5% killing alone, were added as a source of complement (C). The reagents were mixed on a Vortex mixer and incubated at 37°C for 45 min. The number of cells killed was determined by adding 100 μl of 0.16% trypan blue solution and counting the number of cells stained vs. the total (27). Controls always consisted of reacting antiserum against a given specificity with cells expressing another specificity.

Cell Culture. Before each set of experiments the cell lines were recovered from stocks which had been frozen previously in FBS plus 10% dimethylsulfoxide and stored in liquid nitrogen. The cells were rapidly thawed and washed two times in RPMI-1640 plus 10% FBS. The cell density was adjusted to approximately 1×10^6 cells/ml by addition of RPMI-1640 plus 10% FBS before inoculation into a 50 ml Bellco spinner flask (Bellco Glass, Inc., Vineland, N. J.). An overlay of 5% CO_2 in air was made, and the cultures were incubated at 37°C. When the cell concentration reached $2–4 \times 10^6$ cells/ml the cell suspension was transferred to a 100 ml spinner flask with the addition of 50 ml of fresh medium. When the cells in this culture reached $2–4 \times 10^6$/ml, 50 ml of the cell suspension were transferred to a 250 ml spinner flask, and 200 ml of fresh medium were added. The cells were harvested when the cell concentration was $1–2 \times 10^6$ cells/ml and the expression of Thy-1 and TL was then determined by absorption.

Cells Sizing. Cells were sized using a Bio/Physics Cytograft (model 6300 A; Bio/Physics Systems Inc., Mahopac, N. Y.). A cell suspension of 5×10^5 cells/ml was used, and the gain controls were both

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set on high. A Distribution Analyzer (model 2100; Bio/Physics Systems Inc.) was used to analyze the number of cells in each channel. The number of cells per channel was plotted vs. the channel number.

Results

Table I is a list of the cells lines, strain of mouse, method of tumor induction, and the source from which they were received. To date, 14 cell lines (RADA1 from two sources, see Table I) have been analyzed, representing eight strains of mice and five methods of tumor induction.

TABLE I
Origin of Murine Lymphoblastoid Cell Lines

Cell line	Donor strain	Method of tumor induction	Source
L-258	C3H	Gross virus	D. Imagawa
RADA	A/J	Radiation	P. Ralph
H-111	C3H	Gross virus	D. Imagawa
L-251A	C57BR	Gross virus	D. Imagawa
R1	C58	Spontaneous	R. Hyman
ASL1-W	A/J	Spontaneous	M. Wolcott
S49	BALB/c	Mineral oil	R. Hyman
S1A	BALB/c	Mineral oil	R. Hyman
SCRF 61_B	NZB	Spontaneous	R. Lerner
SCRF 65_C	NZB	Spontaneous	R. Lerner
P-388	DBA/2	Carcinogen	P. Ralph
BW5147	AKR	Spontaneous	P. Ralph
H-1210	DBA/2	Carcinogen	W. Bodmer
H-99A5	AKR	Gross virus	D. Imagawa
RADA1-HS	A/J	Radiation	M. Wolcott

The antisera used to study the expression of Thy-1 was raised in two sublines of the AKR strain of mouse. Fig. 1 demonstrates the specificity of these antisera. Anti-Thy-1.1 serum, raised in AKR/Cum mice against AKR/Jax thymocytes, could be absorbed by AKR/Jax thymocytes or BW5147 (Thy-1.1) cells while cells expressing Thy-1.2, AKR/Cum thymocytes, L-251A, or R1 cells did not exhibit any absorption at the highest concentration of cells used (1×10^8 cells/ml antiserum) (Fig. 1 A). Conversely, only cells positive for Thy-1.2 were able to absorb the anti-Thy-1.2 serum, raised in AKR/Jax mice against AKR/Cum thymocytes, and no absorption could be demonstrated for cells expressing Thy-1.1 (Fig. 1 B).

Absorption assays for TL.1,2,3 are shown in Fig. 2. Cells expressing TL.1,2,3 (A/Jax and C57BL/TL$^+$ thymocytes, ASL1, R1, and L-251A) all significantly absorbed the cytotoxic activity of the antiserum. Cells which are TL$^-$ (AKR/Cum thymocytes and BW5147 cells) did not demonstrate any absorption at the highest concentration of cells used (2×10^8 cells/ml antiserum). S49 cells expressing TL.1,2 were able to absorb only 22% of the cytotoxic activity of the antiserum at a concentration of 2×10^8 cells/ml, when tested against A/Jax thymocytes.

It has previously been suggested that culture conditions result in an alteration

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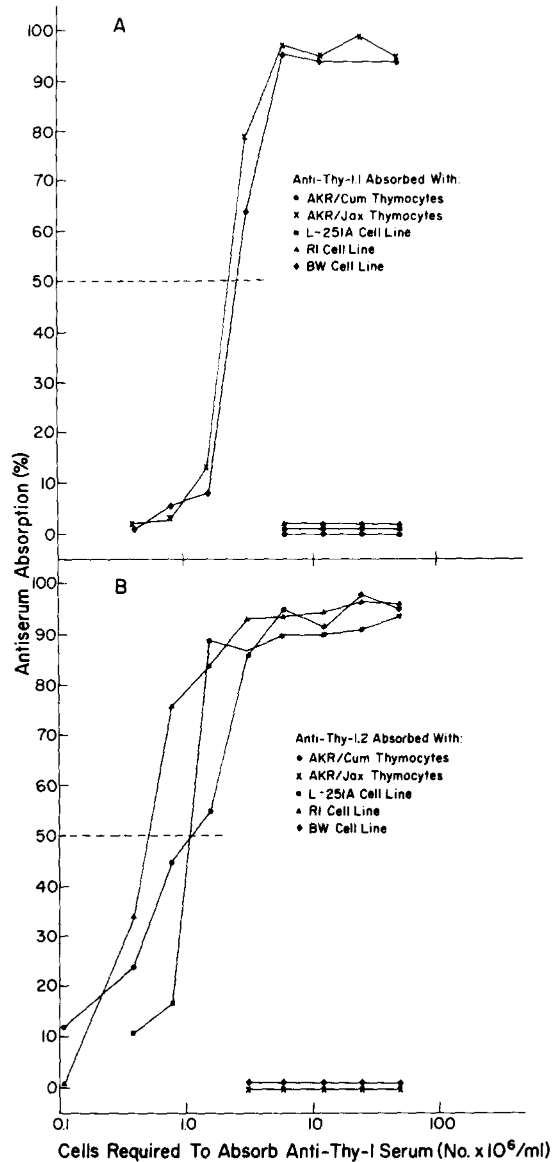

FIG. 1. Absorption assays of normal thymocytes and lymphoblastoid cell lines using (A) anti-Thy-1.1 serum (1:80) absorbed with the indicated cells and tested against AKR/Jax thymocytes and (B) anti-Thy-1.2 serum (1:30) absorbed with the indicated cells and tested against AKR/Cum thymocytes. The end point of the assay was taken as the number of cells required to reduce by 50% the cytotoxicity of the antiserum, i.e., the absorption dose (AD_{50}).

of the expression of Thy-1 or TL (23). This possibility was investigated further. The growth of the L-251A cell line in a 14 liter fermentor as shown in Fig. 3, resulted in a variation of the Thy-1.2 AD_{50} units from 750 $\times$ 10^3 cells/ml antiserum at initiation of the culture to 350 $\times$ 10^3 cells/ml antiserum during the logarithmic growth phase and, finally, an AD_{50} of 950 $\times$ 10^3 cells/ml antiserum

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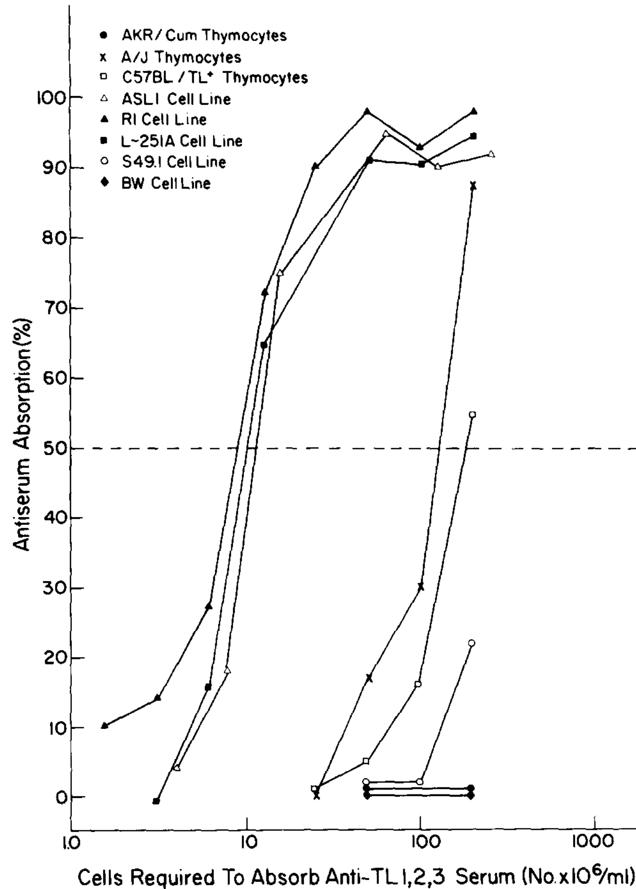

FIG. 2. Quantitative absorption assays using anti-TL.1,2,3 serum (1:800) and tested against A/Jax thymocytes.

during the stationary phase. Only a slight variation was detected for the AD_{50} of TL during the various phases of growth.

The type of serum supplement used in culturing the cells was also investigated to determine the effect on the expression of Thy-1 and TL. When the L-251A cell line was cultured in RPMI-1640 enriched with either 10% HS or 10% FBS, a distinct difference was noted for the expression of TL.1,2,3 (Fig. 4). The cells grown in medium enriched with FBS had a 15-fold greater capacity to absorb anti-TL.1,2,3 serum than cells grown in HS. However, less than a two-fold difference was detected in the AD_{50} values of Thy-1.2 for cells grown in HS- or FBS-enriched medium. Presently we cannot ascertain what factor(s) in the serum supplements is affecting the expression of TL although L-251A cells grown in FBS were larger than those grown in HS (unpublished observation), the variation in size could be related to the differences in the AD_{50} values for Thy-1.2 but not TL.1,2,3.

In quantifying the expression Thy-1 and TL on the cell lines, we considered the serum supplement and phases of the growth curve when the cells were collected. In all cases, except the RADA1-HS cell line, the cells were adapted to growth in

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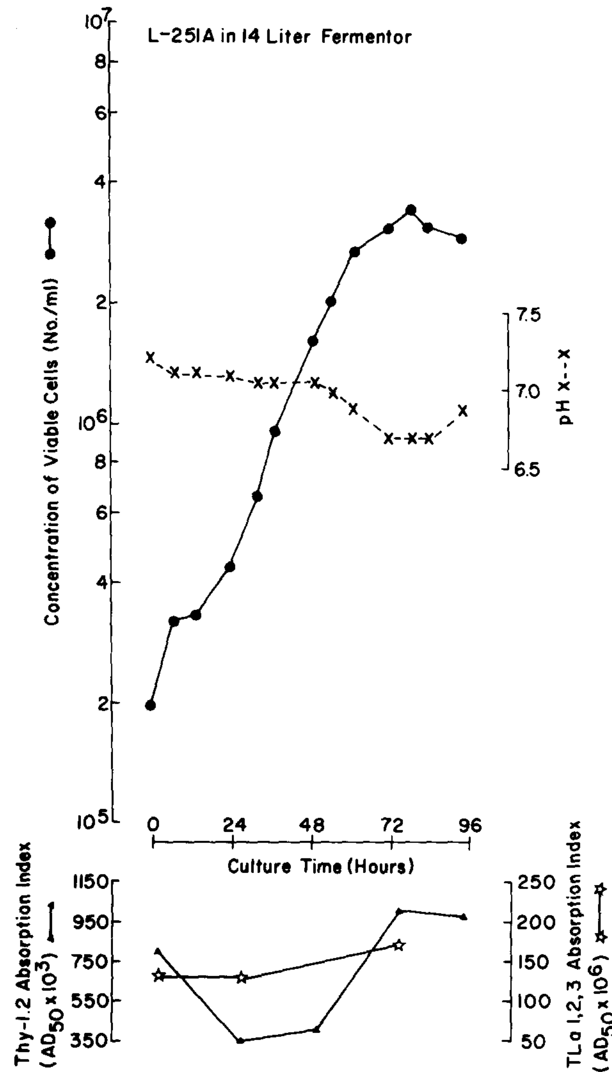

FIG. 3. The growth of L-251A in a 14 liter New Brunswick fermentor utilizing RPMI-1640 supplemented with 2% L-glutamine and 10% HS as the growth medium. CO_2 was continuously added to the vessel by overlay until the pH reached 7.0. No additional gas was added until the pH fell below 7.0 at which time a mixture of 5% CO_2 plus 95% air was introduced by overlay throughout the remainder of the culture time. Gas flow rate was 85.7 ml/min. The absorption index for Thy-1.2 and TL.1,2,3 was determined at various times during the growth cycle as indicated. Impeller speed was 300 rpm.

RPMI-1640 plus 10% FBS. The cell lines were brought from frozen stocks and grown to a cell density of 1–2 $\times$ 10^6 cells/ml. This concentration represents the logarithmic phase of growth, which experience has shown the cells to express the greatest concentration of Thy-1 and TL. These cells have the capacity of growing to densities of 4–6 $\times$ 10^6 cells/ml under these conditions.

Thy-1 and TL quantitative absorption values for the cell lines, as well as normal thymocytes, are depicted in Table II. Greater than 10-fold difference could

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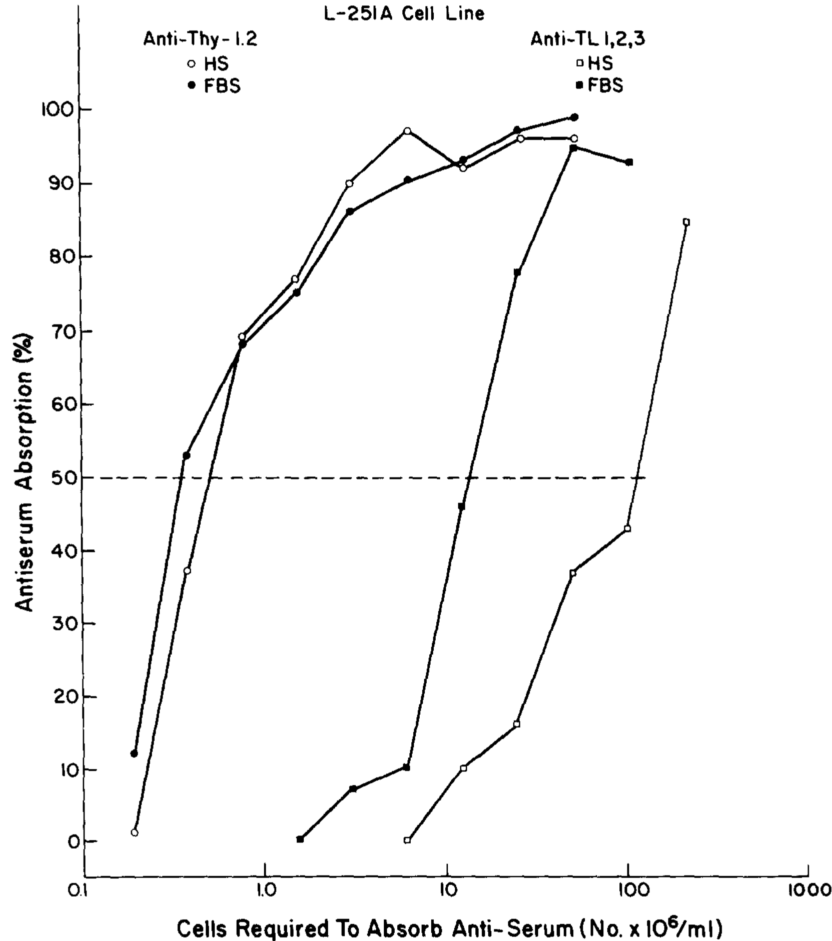

FIG. 4. Effect of serum type on the expression of TL and Thy-1 alloantigens. L-251A cells cultured in RPMI-1640 supplemented with either 10% FBS or HS in a 250 ml Bellco spinner flasks. Cells from each flask were used for absorption of Thy-1.2 and TL.1,2,3.

be detected in the expression of TL between the various cell lines examined. No cell line which was derived from a mouse with TL.2, TL.1,2,4, or T.1,2 phenotype was found to effectively absorb anti-TL.1,2,3 serum. This was partly due to the fact that the anti-TL serum and the target cells were both TL.1,2,3. S49 and S1A, two TL.1,2 cell lines, did exhibit some absorption, but only 22 and 39% respectively, and most likely reflected the inability of these cells to absorb antibody against TL.3 which would result in the cytotoxicity of the A/Jax thymocytes. In all cases the amount of TL.1,2,3 expressed by the cultured cells was greater than that expressed by normal A/Jax thymocytes.

The presence of Thy-1 was detected on all cell lines except L-1210. The variation in Thy-1 was greater than with TL, showing over a 450-fold difference. Of the cell lines expressing Thy-1.2, eight had equal or greater expression of Thy-1.2 than normal AKR/Cum or AKR/Jax thymocytes, while four cell lines expressed less. Thy-1.1 was present on both of the cell lines derived from AKR

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TABLE II
Absorptive Capacity of Murine Lymphoblastoid Cell Lines

Cell line	Allotypic expression of mouse strain		AD_{50} (cells/ml × 10^3)	
	TL	Thy-1	TL	Thy-1
A/Jax thymocytes	1,2,3	2	175,000	1,310
AKR/Cum thymocytes	—	2	Negative	1,070
AKR/Jax thymocytes	—	1	Negative	[2,440]*
L-258	—	2	Negative	89
H-111	—	2	Negative	188
RADA	1,2,3	2	Negative	511
R1	1,2,3	2	11,700	585
RADA-HS	1,2,3	2	20,310	633
ASL1	1,2,3	2	9,500	731
L-251A	1,2,3	2	13,600	1,070
S49	1,2	2	(22%)‡	1,070
S1A	1,2	2	(39%)	1,950
SCRF 61_B	1,2,3	2	100,000	3,020
SCRF 65_C	1,2,3	2	106,000	15,600
P-388	1,2	2	Negative	40,600
L-1210	1,2	2	Negative	Negative
BW5147	—	1	Negative	[2,730]
H-99A5	—	1	Negative	[20%]

* Brackets indicate the AD_{50} values for Thy-1.1 whereas those values for Thy-1.2 are without brackets.

‡ Parentheses indicate that the chosen end point for the assay (50% absorption) was not reached at the greatest cell concentration used (2 × 10^8 cells/ml for anti-TL.1,2,3 and 1 × 10^8 cells/ml for anti-Thy-1) so the percent absorption obtained at these concentrations is depicted.

mice. One of these cell lines, BW5147, expressed an amount of Thy-1.1 equal to that detected on normal AKR/Jax thymocytes, while the other, H-99A5, resulted in only 20% absorption of anti Thy-1.1 serum at 1 × 10^8 cells/ml.

The relative size of normal thymocytes as compared to the lymphoblastoid cell R1 and L-251A was derived by size distribution analysis on a Bio/Physics Cytograf (model 6300A) (Fig. 5). Although actual sizing was not determined, the size distribution plots demonstrate that the lymphoblastoid cell lines were approximately twice the size of a normal mouse thymocyte which has a mean vol of 145 μm^3 (27). With respect to size, the lymphoblastoid cell lines were more heterogeneous than mouse thymocytes.

To determine if L-251A cells could be propagated in spinner culture for a long period of time and would still express Thy-1 and TL, they were cultured in a 14 liter New Brunswick fermentor (New Brunswick Scientific Co., Inc., New Brunswick, N. J.) utilizing RPMI-1640 plus 10% HS (Fig. 6), as previously described (23). This experiment was terminated on the 26th day, only because the fermentor was needed for other experiments. The culture was cut in late logarithmic or early stationary phases of growth by removing 8 liters of the cell

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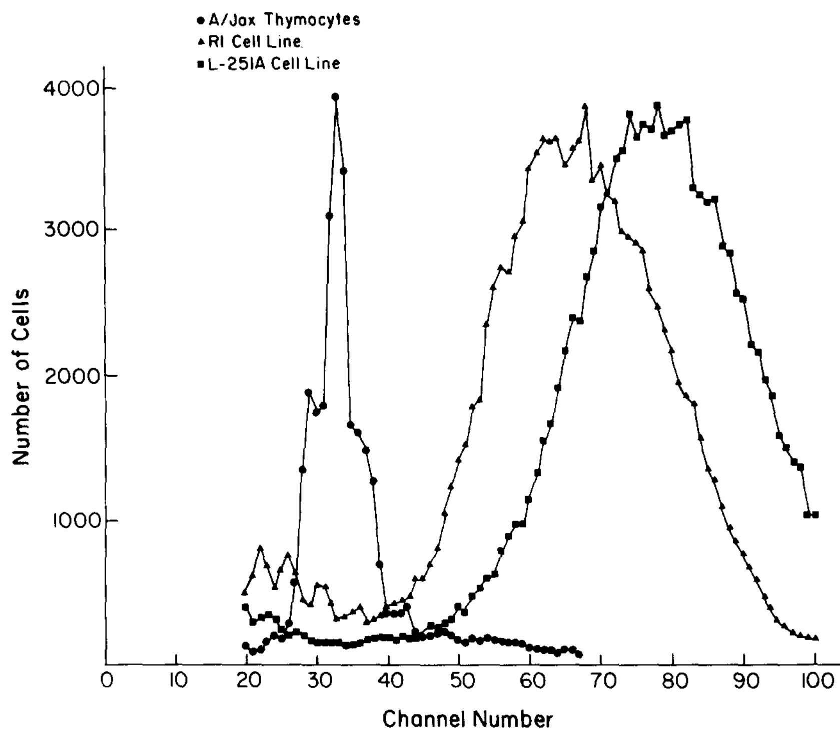

FIG. 5. Relative cell size distributions of normal thymocytes, R1, and L-251A cells.

suspension and replacing this with fresh medium. Since the fermentor had a 12 liter working capacity the cutting resulted in a threefold dilution of the cell concentration. The AD_{50} for both TL and Thy-1.2 did not vary significantly during the entire period. This experiment demonstrated that lymphoblastoid cells can be cultured in large volume for a duration of time and still retain TL and Thy-1.2 surface alloantigens.

Discussion

Several murine lymphoblastic cell lines were evaluated on a quantitative basis to determine their expression of TL and Thy-1 cell surface alloantigens. The procedures of culturing, harvesting, and assaying were standardized since it was demonstrated that culture conditions could alter the expression of the alloantigens. The cell line used to demonstrate culture variation was L-251A which expressed both TL.1,2,3 and Thy-1.2. Culturing these cells in FBS-enriched medium resulted in a 15-fold greater AD_{50} value for TL than when these cells were grown in HS.

The expression of Thy-1.2, but not TL, was shown to be dependent on the phase of the growth curve when the cells were collected for absorption. Cikes (28) also found the expression by YCAB murine lymphoma cells of *H-2* and Moloney leukemia virus-determined surface antigens to be dependent on different phases of growth. The expression of these antigens was shown to be three times greater during the late logarithmic phase than during the logarithmic phase by

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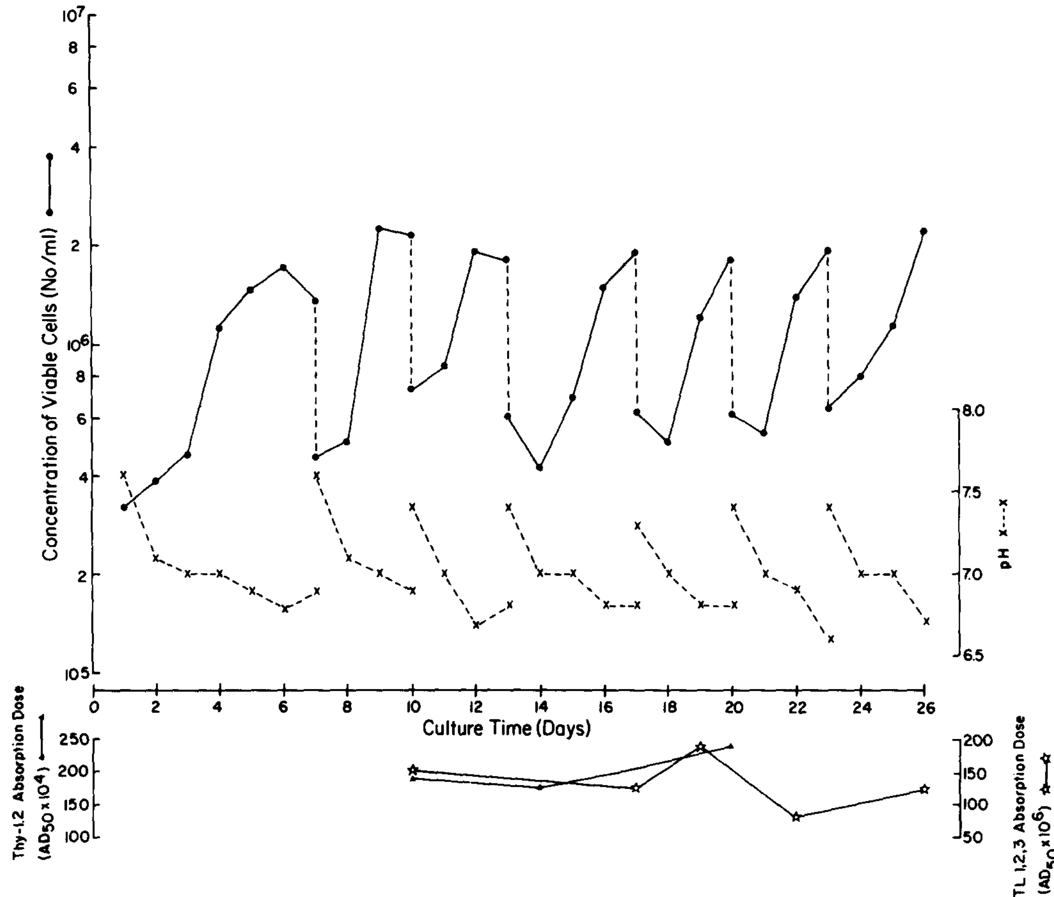

FIG. 6. The long-term culture of the L-251A cell line in a 14 liter New Brunswick fermentor utilizing RPMI-1640 supplemented with 10% fetal calf serum as the growth medium. Other culture conditions were as described in Fig. 3.

direct cytotoxicity (28) and quantitative antibody absorption (29). Lerner et al. (30) demonstrated this same relationship for viral antigens and showed that the difference in cytotoxicity was not due to differences in C activation but the quantity of cell surface antigen. In contrast to these observations, HL-A expression on human lymphoblastoid cells was greatest during logarithmic phase of growth and decreased in stationary phase (31, 32). In the present study a threefold greater absorptive capacity was demonstrated for Thy-1.2 during logarithmic growth phase than during lag or stationary phases, while TL did not vary significantly with change in growth phases. No attempt was made to synchronize the cells before absorption; however, the cells for growth curve studies were usually taken from late logarithmic or early stationary phase cultures. Since cells in suspension will enter G_1 phase of the cell cycle upon release from rest (18, 33), our cultures may have represented a more synchronous than asynchronous population of cells. Variation in antigenic expression during the cell cycle using synchronized cell cultures has been demonstrated for *H-2* (34)

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and temporal coexpression with cell cycle variation for *H-2* and Moloney leukemia virus-determined cell surface antigens (35). Pellegrino et al. (36) did not observe HL-A antigens of cultured human diploid cells to vary significantly during the growth cycle. Everson et al. (32) found HL-A expression to be maximal in S and G_2 phases as compared with G_0 or G_1 cultures. The former group of investigators speculated that the increase in volume in S phase cells accounted for the difference in total antigen expression. Although variation in size of the cells may result in some differences in the antigenic expression, this is questionable in our experiments since TL and Thy-1.2 did not vary together. Cytotoxicity studies with *H-2* antigens were unable to demonstrate any correlation between the cytotoxicity index and an increase in surface area (19, 37).

The quantitative absorptions of mouse lymphoblastoid cell lines demonstrate large differences in the expression of Thy-1 on the cells. In order to rank these cell lines, they were all cultured in the same medium and collected during the logarithmic phase of growth. A comparison between the expression of TL and Thy-1 cannot be accurately deduced from our data, since different lots of antiserum will result in differences in their absorptive capacity. However, in our system it always took 8–20 times more cells to reach an AD_{50} value to TL than Thy-1. This is consistent with the observations of Hämmerling and Eggers (38) who demonstrated 10-fold more Thy-1 than TL on the thymocyte membrane.

Lymphoblastoid cell lines can be grown in large volumes on a continuous basis and still retain their TL and Thy-1 surface antigens. Over a period of 26 days only slight variation was noted. Two important aspects of continual culture were control of pH and maintainence of the cells at a concentration less than stationary (23). Late stationary cultures when cut entered a lag phase, and the growth which followed was retarded. Our experience to date suggests that murine lymphoblastoid cell lines can be grown for long periods of time on a modest scale (14 liter fermentor). Studies are not in progress to determine if the parameters described in the present report will hold for growth in a 70 liter and a 250 liter fermentor.

Summary

Murine lymphoblastoid cell lines were evaluated for their expression of Thy-1 and thymus leukemia (TL) differentiation alloantigens. Two culture conditions were shown to affect this expression. Cells grown in fetal bovine serum (FBS)-enriched medium expressed up to 15 times the amount of TL as cells grown in horse serum (HS)-enriched medium. Thy-1 expression was less affected by the type of serum used for culture. The phase of growth when the cells were harvested, was demonstrated to affect the expression of Thy-1. The expression of Thy-1.2 for one cell line examined, L-251A, during logarithmic growth was threefold greater than cells collected during either lag or stationary growth. When culture conditions were standardized a ranking of the amount of Thy-1 and TL expressed by several cell lines was made. All cell lines, except one, L-1210, expressed Thy-1. There was a 450-fold difference in the expression of Thy-1 between the cell lines evaluated. Seven cell lines expressed TL-1,2,3 with a ninefold difference in the amount of expression. The L-251A cell line was cultured in a 14 liter fermentor for a 26 day period. During this

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time TL and Thy-1 expression did not vary significantly, demonstrating that lymphoblastoid cell lines can be cultured on a continuous basis and will continue to express their surface alloantigens.

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