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A kinetic study of the murine mixed lymphocyte reaction by 5,6-carboxyfluorescein diacetate succinimidyl ester labeling

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Abstract

Alternatives to the use of radioisotopes to measure cell proliferation in mixed lymphocyte reactions (MLR) are desirable to avoid the hazards and costs associated with radioisotope use. The versatile fluorochrome 5,6-carboxyfluorescein diacetate succinimidyl ester (CFSE) has been used to measure MLR and provides the opportunity to measure several different growth parameters. This study was aimed at determining which growth parameter is most practical and suitable for measuring murine MLR. The parameters measured were: the relative number of daughter T-cells, the relative number and frequency of reactive T-cell precursors and the relative number of mitotic events. Responder cells were CFSE-labeled unfractionated splenocytes from C57BL/6 mice. Stimulator cells included irradiated splenocytes from C57BL/6 (control), B6D2F$_1$ (haplo-allogeneic) or FVB/N (allogeneic) mice. Cultures were harvested daily for 1 week. Stimulator T-cells rapidly declined to less than 0.2–0.3% of the mixed population by day 2 of culture. Experimental groups had a significantly higher number of daughter T-cells and mitotic events after 2 days of culture with the number of daughter T-cells climbing exponentially after 5 days of culture. The number and frequency of reactive T-cell precursors were significantly higher in experimental groups on days 2–3, but this difference became insignificant by day 4. Among all the parameters, the relative number of daughter T-cells was the most practical for measuring MLR, after 5 days of culture, based upon the growth kinetics of responder T-cells and the survival of the stimulator cells.

Keywords: 5,6-carboxyfluorescein diacetate succinimidyl ester; Mixed lymphocyte reaction; Mouse

1. Introduction

The mixed lymphocyte reaction (MLR) measures the proliferative response of responder T cells against antigens present on allogeneic stimulator cells. It is presumed that the MLR is an in vitro analog of in vivo alloreactivity, and is widely used in transplantation

Abbreviations: MLR, mixed lymphocyte reactions; CFSE, 5,6-Carboxyfluorescein diacetate succinimidyl ester; ^{3}H-TdR, ^{3}H-thymidine; PBS, phosphate buffer saline; BSA, bovine serum albumin; FBS, fetal bovine serum; PI, propidium iodide.

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immunology to measure recipient T cell responses against donor tissues due to the mismatch of MHC antigens. Cell proliferation is an essential part of an immune response which can be measured in an allogeneic mixed lymphocyte culture as an indicator of immune reaction. There are numerous methods to measure changes in cell number. The most widely used method to measure proliferation is ^{3}H-thymidine (^{3}H-TdR) incorporation. However, due to the hazards and cost associated with the use and disposal of tritium, nonradioactive alternatives have been pursued.

Nonradioactive methods include the use of a range of fluorochromes, which have been used for measuring the proliferation of lymphocytes as well as for tracking migration and positioning in vivo (Parish, 1999). Among them, 5,6-carboxyfluorescein diacetate succinimidyl ester (CFSE) stands out as being the most versatile in terms of long-term cell tracking and quantifying proliferation either in vivo or in vitro (Lyons, 1999; Parish, 1999). CFSE can spontaneously and irreversibly couple to intracellular proteins and is equally distributed between two daughter cells when cells divide. Thus, proliferating cells can be tracked by flow cytometry based upon the sequential loss of fluorescence intensity (Lyons, 2000; Lyons and Parish, 1994). Furthermore, multiparameter flow cytometry allows for the examination of cell subsets within the dividing cell population as well as monitoring phenotypic changes associated with activation and cell division.

CFSE staining has been used as a replacement for ^{3}H-TdR incorporation to measure the MLR of cells of human and nonhuman-primate origins (Matthews et al., 2000; Popma et al., 2000; Nitta et al., 2001). In most cases, the outcome of the MLR has been determined based on the total numbers of daughter T-cells generated. However, the ability of CFSE to track cell division allows for other parameters of cell proliferation to be measured. These include estimates of the number and frequency of T-cell precursors that have responded in the MLR as well as the number of total mitotic events that have occurred. The utility of measuring these parameters in analyzing murine MLR has not been closely examined. Therefore, we studied the kinetics of murine T-cell responses in allogeneic MLR using CFSE labeling and multiparameter flow cytometry.

2. Materials and methods

2.1. Responder and stimulator

Two inbred strains of mice, C57BL/6 (H-2^{b}) and FVB/N (H-2^{q}), and one hybrid strain of mice B6D2F_1 (C57BL/6 × DBA/2, H-$2^{b/d}$) were purchased from Charles River (Wilmington, MA) or Simonsen (Gilroy, CA). They were 8–30 weeks of age when sacrificed and their spleens were harvested. Responder T-cells were from the C57BL/6 strain, whereas stimulators included C57BL/6 (syngeneic), B6D2F_1 (haplo-allogeneic) and FVB/N (allogeneic) strains. Splenocytes were harvested under sterile conditions and passaged through 70 μm cell strainers (Becton Dickinson, Franklin Lakes, NJ). Red cells were depleted by chemical lysis using ACK buffer, pH 7.2–7.4, consisting of 0.15 M NH_4CL, 1.0 mM $KHCO_3$ and 0.1 mM Na_2EDTA (Sigma, St. Louis, MO). The cell suspension was then washed twice with phosphate buffer saline (PBS) containing 0.3% bovine serum albumin (BSA) (PBS/BSA) (Roche Molecular Biochemicals, Indianapolis, IN). Responder cells were suspended at 2×10^7 cells/ml in PBS/BSA for CFSE labeling, whereas stimulator cells received 3000 cGy irradiation and were then resuspended at 6×10^6 cells/ml in culture medium.

2.2. CFSE labeling of responder cells

CFSE labeling of responder cells was undertaken as previously described (Lyons, 2000; Lyons and Parish, 1994). Immediately before labeling, 5 mM CFSE stock (Molecular Probes, Eugene, OR) in DMSO (Fisher Scientific, Fair Lawn, NJ) was thawed and diluted to 10 μM in a volume of PBS/BSA equal to that in which the responder cells were suspended. The two equal volumes were mixed to initiate labeling and periodically agitated at room temperature for 10 min. The labeling process was quenched by adding an equal volume of heat-inactivated fetal bovine serum (FBS, Hyclone, Logan, UT) to the sample. After 1 min, the CFSE-labeled cells were washed twice, recounted and adjusted to a concentration of 2×10^6 cells/ml in culture media.

2.3. MLR

The MLR was performed in 96-well U-bottom microtiter plates (Costar, Cambridge, MA). Culture medium consisted of RPMI 1640 supplemented with 10% heat-inactivated FBS, sodium pyruvate (110 mg/l), nonessential amino acids, L-glutamate (1 mM), 2-mercaptoethanol (5×10^{-5} M), penicillin (50 U/ml) and streptomycin (50 μg/ml) and *N*-acetyl cysteine (Sigma; 10 mmol/l, pH adjusted to 7.2). CFSE-labeled C57BL/6 responder cells were plated at 1×10^6 cells/ml in a volume of 250 μl per well and cocultured at a ratio of 1:3 with 3000 cGy irradiated C57BL/6, B6D2F$_1$ or FVB/N stimulator cells. The plates were then placed in a humidified 37 °C, 5% CO_2 incubator. The control experiment for accessing background fluorescence was set up by coculture of unlabeled C57BL/6 responder cells and irradiated stimulator cells under the same conditions. In order to assess the kinetic division of the responder cells and the viability of stimulator cells, cells were harvested from quadruplet wells on a daily basis.

2.4. Flow cytometric analysis

Cells harvested from each well were suspended in culture supernatant from the clone 2.4G2 hybridoma cell line (ATCC, Manassas, VA) that produces mAb against FcγII/FcγIII receptors to block nonantigen-specific binding of immunoglobulins. CFSE-labeled cells were incubated on ice for 30 min with PE conjugated anti-CD3 (Caltag Laboratories, Burlingame, CA). Cells were then washed three times in PBS/BSA with 0.01% NaN_3 (Sigma). Cells from each well were suspended in 150 μl PBS/BSA with 0.01% NaN_3 and 2 μg/ml propidium iodide (PI, Molecular Probes). Three-color flow cytometry was performed on a FACScan (BD Biosciences, San Jose, CA). Events for each well were acquired at a fixed speed (high setting) for 1 min to measure an equal volume from each sample, thereby providing a basis for the relative comparison of data collected for each sample. Dead cells were excluded from analysis based on staining with PI.

2.5. Data and statistical analyses

Data were analyzed using CellQuest acquisition and analysis software (BD Biosciences). The total numbers of events (cells) were determined by analyzing the data using dot plots and rectangular regions to define the cell populations. Histograms were used to track the divisions of CFSE-labeled cells where each peak of CFSE-fluorescence defined a single round of cell division. All data presented are from analyses of live (PI$^-$) cells only.

The relative numbers of T-cell precursors required for generating these daughter cells under each division peak was calculated by dividing the number of daughter-cell events by 2 raised to the power of the given round of division (2^n). The sum of all the calculated numbers of precursors from each division peak was used to represent the number of reactive T-cell precursors. Furthermore, the frequency of reactive T-cell precursors was estimated by dividing the number of reactive T-cell precursors by the number of total T-cells, which equals the number of undivided T-cells plus the number of reactive T-cell precursors. Alternatively, the number of total live T-cells measured on the first day of culture was also used to estimate the frequency of reactive T-cell precursors. The frequency values were multiplied by 100 to be represented as a percentage of all T cells. In addition, the mitotic events under each peak can also be determined by subtracting the number of reactive T-cell precursors from the number of daughter T-cells under each peak (Wells et al., 1997). Thus, the relative number of total mitotic events can be calculated as the sum of individual mitotic events under each peak, which equals the number of daughter T-cells minus the number of reactive T-cell precursors.

Independent sample *t*-test was used for comparing means of events and ratios between control and experimental groups. Paired-samples *t*-test was used to make a comparison between the two precursor frequencies generated on the basis of different denominators, one derived from the total T-cell precursors on the indicated day and the other from the mean undivided T-cells on day 1. A P-value < 0.05 was considered statistically significant.

3. Results

3.1. Proliferation of CD3$^+$ responder cells

Mixed lymphocyte cultures were harvested after 1–7 days and stained with CD3-PE to identify the T-cell component of the cultures. CD3 staining and CFSE fluorescence were detected by flow cytometry (Fig. 1).

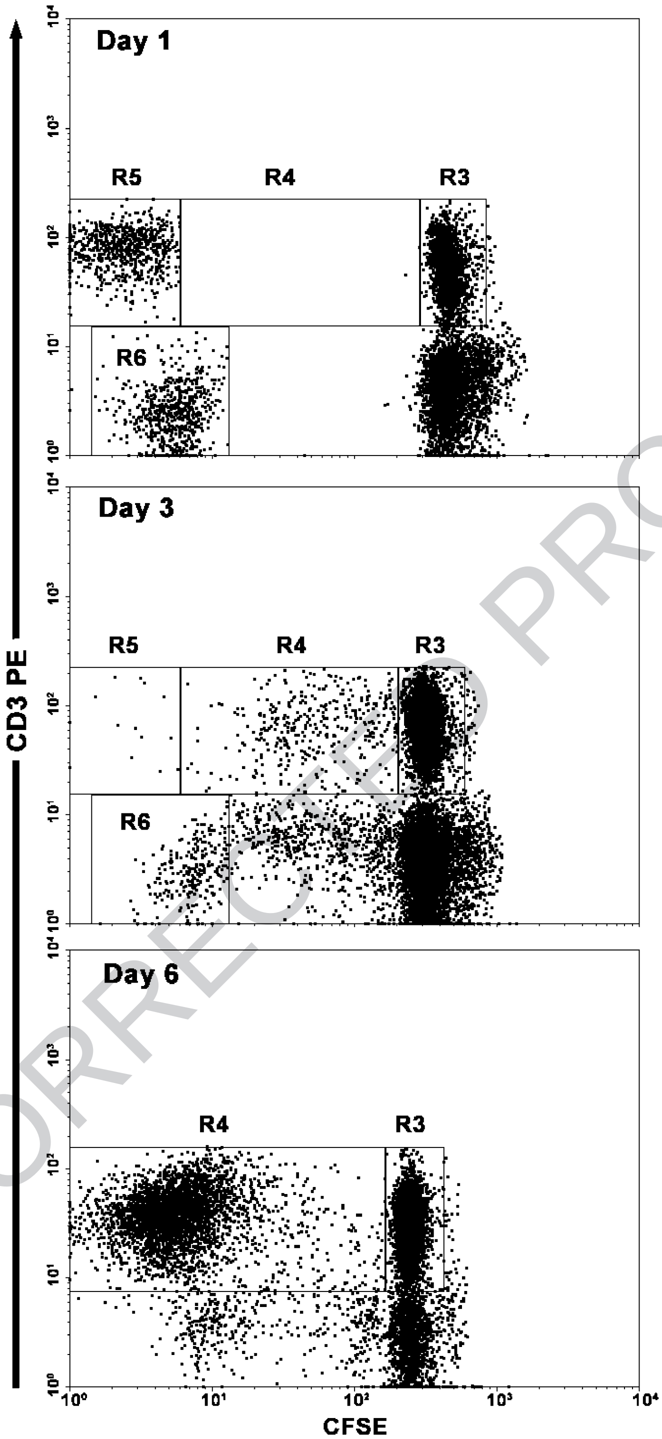

Fig. 1. Flow cytometric analysis of mixed lymphocyte cultures. Progressive loss of CFSE fluorescence by C57BL/6 responder $CD3^+$ T-cells, indicated by the regions R3 and R4, is shown for days 1, 3 and 6 of culture. Viable FVB/N stimulator cells are indicated by regions R5 and R6. The stimulator cells were mostly located at the area of low fluorescent intensity ($< 10^1$).

Daughter T-cells, derived from the responder splenocytes, could be differentiated from undivided T-cells by the intensity of CFSE staining. The relative numbers of daughter T-cells could be easily determined by electronic gating, as shown in Fig. 1. In the case of alloreactive MLR, the numbers of daughter T-cells showed a slow increase until day 4 and then an exponential expansion after day 5 (Fig. 2A). The syngeneic control group (C57BL/6) generated significantly lower numbers of daughter T-cells than the haploidentical $B6D2F_1$ and completely MHC-mismatched FVB/N groups beginning on the second day of culture onwards. There were no significant differences in the numbers of daughter T-cells between the $B6D2F_1$ and FVB/N groups at any time point. Thus, a T-cell proliferative response to MHC antigens could be readily detected by this method.

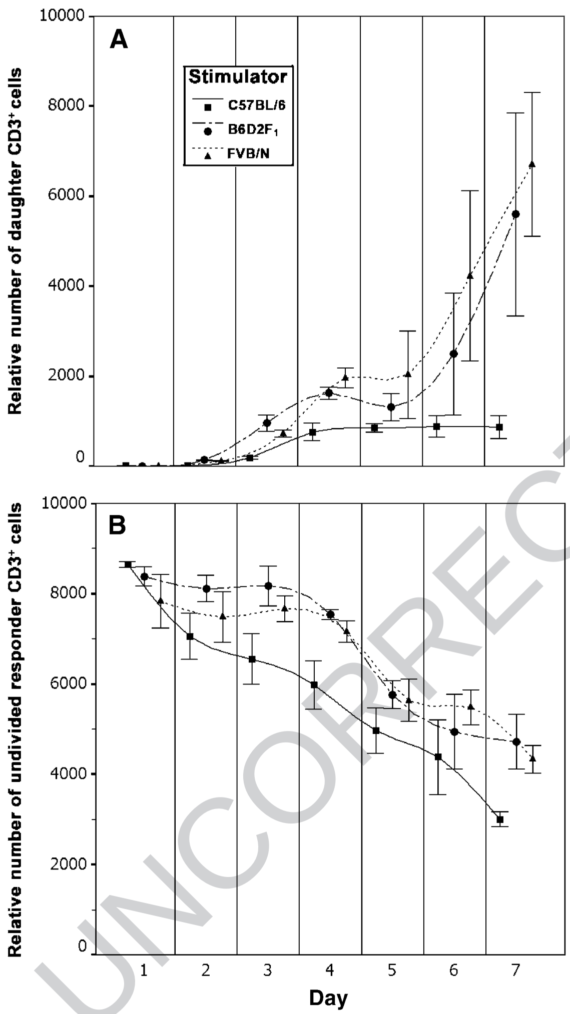

Fig. 2. Kinetics of expansion and survival of responder T-cells in mixed lymphocyte cultures. Accumulations of daughter T-cells from the responder population are shown in (A) and survival of undivided T-cells are shown in (B). Data are shown as the mean ± 2.0 standard errors of the mean (SE) of measurements made on replicate cultures.

Survival of the undivided $CD3^+$ cells in the mixed cultures was also analyzed (Fig. 2B). A linear decrease in the number of unresponsive T-cells was observed in the C57BL/6 group over the 7 days of culture analyzed. However, the $B6D2F_1$ and FVB/N groups had comparatively steady numbers of undivided $CD3^+$ cells until day 3. A slight drop followed in these groups on day 4, and then a linear decrease in the number of unresponsive T-cells was seen as in the C57BL/6 control group.

The kinetics of $CD3^+$ cell proliferation was analyzed in greater detail using histograms to view the levels of CFSE fluorescence (Fig. 3). On day 1 (24 h of culture), $CD3^+$ responder cells were uniformly stained with CFSE, indicating that these cells had not yet begun to divide. By the second day of culture, $CD3^+$ responders, stimulated by $B6D2F_1$ or FVB/N splenocytes, had begun to divide for up to three rounds of mitosis (Fig. 3 and data not shown). In contrast, responder T-cells stimulated with syngeneic C57BL/6 cells did not show any evidence of cell division for the first 2 days (Fig. 3). On days 3 and 4, four to six rounds of divisions could be detected in all the three groups. On day 5, $CD3^+$ cells of all three groups had divided at least for seven to eight times, resulting in a loss of fluorescent intensity on some responder cells to background levels. Thus, at this time point further resolution of subsequent cell division could not be accurately measured. On days 6–7, almost all the daughter T-cells, stimulated by $B6D2F_1$ and FVB/N splenocytes, had reached the area of background fluorescence due to many rounds of divisions.

Measured estimates of the relative number and frequency of reactive T-cell precursors are listed for a representative experiment in Table 1. These calculated measures are based on measurements of viable (PI^-) $CD3^+$ cells. A significantly higher number and frequency of reactive T-cell precursors were observed in the $B6D2F_1$ and FVB/N groups compared to the C57BL/6 group on days 2–3. However, the frequency of reactive T-cell precursors did not differ between

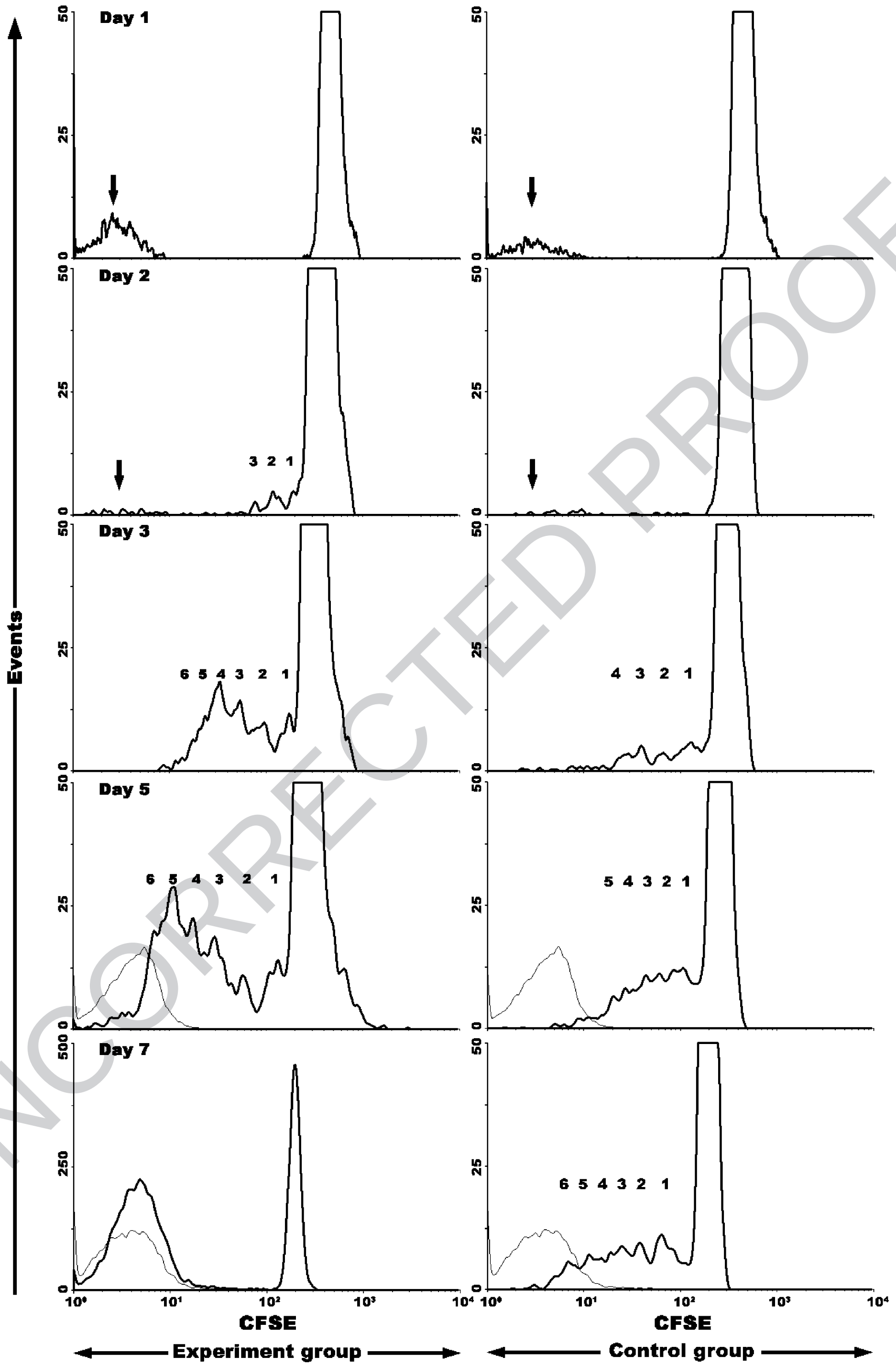
Day 1
Day 2
Day 3
Day 5
Day 7
3 2 1
6 5 4 3 2 1
4 3 2 1
6 5 4 3 2 1
5 4 3 2 1
6 5 4 3 2 1
Events
CFSE
CFSE
Experiment group
Control group

Table 1
Relative numbers and frequencies of reactive T-cell precursors as well as the number of mitotic events from MLR analyzed at 1–4 days of culture

Day	Stimulator	Relative Number of T-cell Precursors	Estimated T-cell Precursor Frequency (%)[a]	Relative Number of Mitotic Events
1	C57BL/6	4.13 ± 0.80	0.05 ± 0.01/0.05 ± 0.01	4.13 ± 0.80
	B6D2F1	2.38 ± 0.32	0.03 ± 0.01/0.03 ± 0.00	2.38 ± 0.31
	FVB/N	3.38 ± 0.55	0.05 ± 0.01/0.04 ± 0.01	3.38 ± 0.55
2	C57BL/6	5.00 ± 0.79	0.07 ± 0.01/0.06 ± 0.01[b]	5.00 ± 0.79
	B6D2F1	54.50 ± 1.85[c]	0.68 ± 0.02[c]/0.65 ± 0.02[c]	80.75 ± 2.39[c]
	FVB/N	47.25 ± 2.46[c]	0.64 ± 0.02[c]/0.60 ± 0.03[c]	67.25 ± 2.25[c]
3	C57BL/6	40.50 ± 2.06	0.63 ± 0.04/0.47 ± 0.02[b]	140.00 ± 10.79
	B6D2F1	152.75 ± 11.18[c]	1.87 ± 0.10[c]/1.82 ± 0.13[c]	759.75 ± 75.93[c]
	FVB/N	129.00 ± 3.49[c]	1.69 ± 0.04[c]/1.65 ± 0.04[c]	573.50 ± 32.17[c]
4	C57BL/6	141.00 ± 15.15	2.37 ± 0.18/1.63 ± 0.18[b]	591.00 ± 76.73
	B6D2F1	157.75 ± 3.59	2.08 ± 0.04/1.88 ± 0.04[b]	1411.75 ± 59.31[c]
	FVB/N	160.25 ± 6.20	2.24 ± 0.10/2.05 ± 0.08[b]	1720.75 ± 101.25[c]

Data are shown as the mean ± standard error of the mean for quadruplicate analyses performed on live cells.

[a] T-cell precursor frequencies were calculated by two methods. The first number represents the frequency calculated using the sum of reactive T-cell precursors and non-responsive T-cells measured on the day when the culture was harvested as the denominator. The second value was calculated using the mean number of undivided T-cells on day 1, when responder T-cells had not yet divided, as the denominator.

[b] $P<0.05$ comparison between first and second numbers, paired-samples t-test.

[c] $P<0.05$ compared with the C57BL/6 control group, independent-samples t-test.

control and experimental groups by the fourth day of culture. The frequencies of T-cell precursors were estimated by two methods, as described in Materials and methods. The linear decrease in undivided CD3$^+$ cells in the control group resulted in an overestimation of T-cell precursor frequency on days 2–4; whereas the relatively slow decrease of undivided CD3$^+$ cells in the experimental groups did not notably influence the frequency calculations until day 4.

The relative numbers of mitotic events that occurred in the cultures could also be calculated (Table 1). The B6D2F$_1$ and FVB/N groups exhibited higher division of T-cells on days 2–4 compared with the control group. This pattern was consistent with the measures of the relative number of daughter T-cells (Fig. 2A), which measures the outcome of these early mitotic events as a latter accumulation of daughter T-cells. By the 5th day of culture, it was difficult to accurately estimate the number and frequency of reactive T-cell precursors as well as the number of mitotic events. This is because T cells under peak 6 might contain daughter cells that had undergone more than six rounds of division and can no longer be discriminated from background fluorescence as indicated using an unstained control (Fig. 3).

3.2. Proliferation of responder CD3$^-$ cells

Responder CD3$^-$ cells also started to proliferate in the mixed culture beginning on day 2, reached a peak around days 3–4, and subsequently declined to a plateau (Fig. 4A). This proliferation pattern did not parallel that of responder CD3$^+$ cells. In B6D2F$_1$ and FVB/N groups, the ratio of CD3$^+$ to CD3$^-$ responder cells was usually less than 1 over the first 3 days, gradually increased to five on day 5, and abruptly amplified to over 10 on day 7. In contrast, the ratios from C57BL/6 control group sustained below 1 until

Fig. 3. Histogram plots gated on CD3$^+$ cells show the kinetics of cell division associated with an MLR. Histograms of CFSE fluorescence for T-cells stimulated by haploallogeneic B6D2F$_1$ splenocytes are shown in the left column and histograms for the syngeneic control group are shown for comparison in the right column. A peak of low fluorescence (arrow) represents viable stimulator T-cells observed during the first 2 days of culture. On days 5 and 7, cells with ≥6 rounds of division had reached a fluorescent intensity similar to the background autofluorescence of unstained responder T-cells (thin line). This overlap limited the resolution of further cycles of cell division and marked the maximal cycle that could be distinguished.

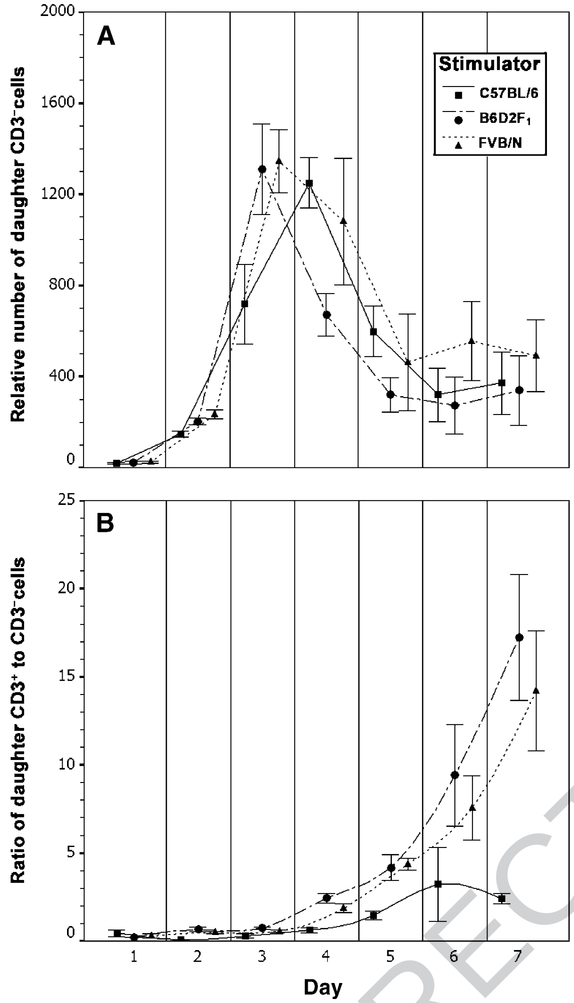

Fig. 4. Proliferation and contribution of CD3$^-$ responder cells to mixed lymphocyte cultures. Accumulations of daughter CD3$^-$ cells (non-T-cells) from the responder population are shown in (A). The ratio of daughter CD3$^+$ to CD3$^-$ cells is shown in (B). Data are shown as the mean ± 2.0 SE.

day 4, slightly increased to the peak of 3 on day 6, and then went down on day 7 (Fig. 4B).

3.3. Viability of stimulator cells

Viable stimulator cells in the mixed cultures were observed in the early days of culture and could be identified by their lack of CFSE staining. They remained after 1 day of culture, comprising both CD3$^-$ cells and CD3$^+$ cells (Fig. 1). CD3$^-$ stimulator cells exhibited a much slower decline and represented 2–3% of the total cell population on day 3 of culture (Fig. 5A). However, a rapid decline of viable CD3$^+$ cells to 0.2–0.3% of all viable cells was observed by the second day of culture, and less than 0.1% stimulator CD3$^+$ cells were present on days 3–4 (Fig. 5B). An MLR, in which the responders were not stained with CFSE, was also undertaken to trace the viability of stimulator cells by flow cytometry using differences in MHC antigens (H-2K$^{b,\ d,\ and\ q}$). Total viable stimulator cells in the mixed culture accounted for 11.5–23.6% on day 1, 2.9–7.6% on day 2, 0.5–0.7% on day 3, 0.5–1% on day 4, 0.2–0.5% on day 5 and 0.1–0.3% on days 6–7 (data not shown).

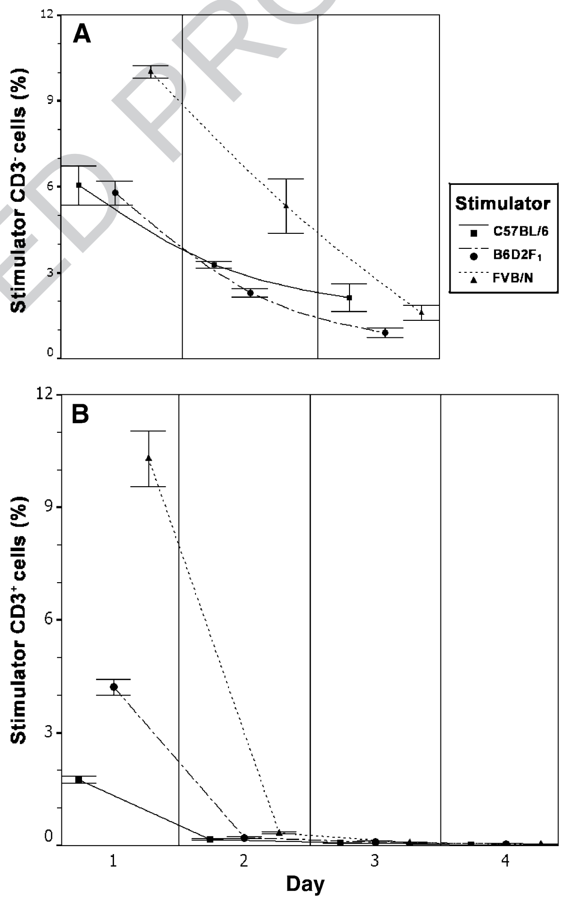

Fig. 5. Survival of stimulator cells in mixed lymphocyte cultures. The percentages of viable CD3$^-$ stimulator cells are shown in (A) and the percentages of viable stimulator CD3$^+$ T – cells are shown in (B). Data are shown as the mean ± 2.0 SE.

4. Discussion

Cell proliferation encompasses DNA synthesis, mitosis and an increase in cell number. ^{3}H-TdR incorporation measures cell proliferation by the incorporation of ^{3}H-TdR into DNA while cells are dividing, and has been a standard method for measuring cell proliferation in mixed lymphocyte cultures for decades. In view of the increasing costs of waste disposal and protective precautions associated with radioisotope use, developing an alternative for quantify cell proliferation, which can be as effective as, but less hazardous than ^{3}H-TdR, is welcomed. CFSE labeling stands out in many aspects as a versatile alternative to conventional ^{3}H-TdR incorporation. Most importantly, this method was shown to correlate well with ^{3}H-TdR incorporation (Fulcher and Wong, 1999; Popma et al., 2000). To extend the adoption of CFSE for use in measuring murine MLR, we closely examined a number of parameters associated with cell division to determine the optimal timing and methods to measure murine MLR.

^{3}H-TdR incorporation only measures cell division during a brief period of culture and provides no information on the types of cells growing or their degree of response. In contrast, CFSE labeling can provide more information such as the relative number of reactive T-cell precursors, the estimated frequency of reactive T-cell precursors, the relative number of mitotic events and relative number of daughter T-cells associated with an MLR. The proliferation of CFSE-labeled responder cells could be traced for at least 1 week, providing a detailed picture of the different stages of an MLR. Moreover, immunophenotypic analyses of the dividing cells insured that the measurement was specific to $CD3^+$ T-cells. This might be crucial in some cases because unfractionated tissues such as spleen, lymph node or fetal liver (Harris et al., 1994) have been used as the source of responder T cells and substantial non-T-cell proliferation might occur from such tissues. Under theses circumstances, data obtained by ^{3}H-TdR incorporation might variously reflect proliferation of both T and non-T cells. According to our results, non-T-cells displayed a different pattern of proliferation from T-cells. Clearly, non-T-cells are most apt to contribute to overall proliferation in the early days of a splenic MLR. Furthermore, cell proliferation in the control group tended to be more the result of non-T-cells than in the experimental groups, which could be readily distinguished and disregarded by the flow cytometric method.

The simplest method of quantifying an MLR using CFSE was found to be measurement of the accumulation of daughter $CD3^+$ cells. The magnitude of the T-cell proliferative response elicited by various stimulator conditions mainly depended upon the rounds of division the T-cell precursors had undergone rather than upon the frequency of reactive T-cell precursors. This argument was supported by the presence of two distinct peaks on days 6 and 7 in the histogram plots shown in Fig. 3. One peak represented quiescent T-cells and the other was daughter T-cells that had undergone at least seven to eight rounds of division. There was a relative paucity of daughter cells in between. T-cell proliferation was asynchronous, to a degree, likely due to variation in the time of entry into the first division cycle (Hasbold et al., 1999). Nonetheless, new T-cell precursors did not appear to be recruited after day 5 of culture. Accordingly, the alloresponse to MHC-mismatched stimulators led to an exponential expansion of daughter T-cells by eliciting more rounds of division than the nonspecific response in syngeneic group. Modest T-cell proliferation in the control group appeared later than in the experimental groups, becoming evident on day 3.

The number and frequency of reactive T cell precursors in an MLR could also be estimated using flow cytometry. Our estimated frequencies of reactive T-cell precursors was about 2%, which was within some published estimates of the percentage of alloresponsive precursors (Ford and Atkins, 1973; Ford et al., 1975; Matzinger and Bevan, 1977; Sherman and Chattopadhyay, 1993). These measurements were best performed on the third day of culture rather than at later time points that are better suited for measuring the accumulation of daughter T cells. This is because differences between the control and experimental groups were evident on days 2 and 3, but by day 4, these differences disappeared. The weak background proliferation observed in the controls by day 4 of culture might be due to nonspecific activation of the T cells by cytokines produced in the cultures or FBS used to supplement cultures. Another reason for early analysis of the cultures is that the death of some of the daughter cells and nonresponsive T-cells affects the estimates of the size and frequency of the precursor pool. Dead (PI^+) daughter and nonresponder cells were observed, but it was necessary to disregard these cells for analyses as the PI staining and high nonspecific binding of antibody by dead cells interferes with the analysis of CD3 expression. Since the estimation of the frequency of T-cell precursors relies on an accurate measurement of the total number of live T cells used to initiate the cultures, we tested two methods by which to calculate precursor frequencies. One method relied on a single flow cytometric analysis, which was used to estimate the total pool of T cells based on the calculated number of reactive T-cell precursors plus the number of undivided T-cells. A second method used an additional analysis after 1 day of culture, when the T-cell precursor pool had not yet begun to divide, to measure the total number of live T-cells. We chose to make this analysis after 1 day rather than on day 0 of culture as the CFSE staining procedure may result in the death of some T cells that is not immediately evident. Both methods of calculating the size of the total T-cell pool resulted in similar estimates of reactive T-cell precursor frequencies for the experimental groups, but the control group had a faster loss of nonresponsive T cells leading to higher estimates of precursor frequencies beginning on the second day of culture. Thus, choosing which method to use to calculate the size of the total T-cell pool depends on the types of MLR being compared.

Being able to estimate the number or frequency of reactive T-cell precursors may have specific application in studies aimed at measuring these values. However, this study clearly indicates that measuring the relative number of daughter T-cells is the best parameter for analyzing the magnitude of an MLR (Matthews et al., 2000; Popma et al., 2000). It is simple as well as straightforward without the requirement of extensive calculations. The number of mitotic events can also be used for comparing in vitro MLR because its pattern generally paralleled that of the accumulation of daughter T-cells at least during the first 4 days of cultures. However, it offered no clear advantages over the measuring the number of daughter T-cells and required labor-intensive calculations. Using the number of daughter T-cells, the differences between the control and experimental groups could be distinguished beginning on the second day of culture onwards, and were more pronounced after 5 days of culture. Furthermore, irradiated stimulators were nearly absent from culture after 3 days. Thus, quantifying MLR by the number of daughter T-cells after 5 days of culture could be easily performed with minimal interference from stimulator cells. These findings support the use of a flow cytometry as a reliable method to analyze murine MLR based on CFSE staining.

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