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LIMITING DILUTION ANALYSIS OF ALLOANTIGEN-REACTIVE T LYMPHOCYTES. II. EFFECT OF CORTISONE AND CYCLOPHOSPHAMIDE ON CYTOLYTIC T LYMPHOCYTE PRECURSOR FREQUENCIES IN THE THYMUS

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A minimal estimate of the frequency of cytolytic T lymphocyte precursors (CTL-P) in the thymus was determined by application of Poisson statistics to limiting dilution analysis. A mean CTL-P frequency of 1/1467 was obtained for C57BL/6 (H-2^b) thymus cells activated by DBA/2 (H-2^d) irradiated spleen cells and assayed against P-815 mastocytoma (H-2^d) target cells. CTL-P frequencies were also obtained for spleen, nylon wool column purified spleen, peripheral blood, and lymph node cell populations. The effect of in vivo drug treatments on CTL-P frequencies was then examined. Cortisone at 100 mg/kg dramatically increased the CTL-P frequency in thymus by more than 20-fold despite a drastic reduction in the number of total thymus cells. The same cortisone treatment did not affect the CTL-P frequency in spleen. In contrast, cyclophosphamide at 300 mg/kg decreased the CTL-P frequency in spleen by more than 10-fold without affecting that in thymus. Cyclophosphamide at 100 mg/kg did not produce any significant change. A detailed explanation of the calculation of CTL-P frequencies is provided and their validity is discussed.

Keywords: thymus; cytolytic T lymphocyte precursor; frequency; limiting dilution analysis; cortisone; cyclophosphamide

INTRODUCTION

The thymus is now well known and accepted as the principal organ responsible for the maturation of lymphopoietic stem cells into immunocompetent T lymphocytes active in a wide variety of cell-mediated immune phenomena [1]. The identification of functional subpopulations of T lymphocytes and the delineation of their developmental pathways in the thymus, however, have not yet received comparable acceptance despite significant progress toward their elucidation [2–5]. Continued progress in this area depends upon the level of resolution of the experimental system used to distinguish T lymphocyte subpopulations and to follow their maturation and circulation throughout the immune system. Quantitation of actual numbers of functionally active T lymphocytes would provide much higher resolution

than does quantitation of units of functional activity. Fortunately, advances in leukocyte culture methods have recently permitted the application of limiting dilution techniques to the enumeration of precursors of several functional subpopulations of T lymphocytes. In the previous article of this series [6], we reported the development of a limiting dilution analysis system for the determination of alloreactive cytolytic T lymphocyte precursor (CTL-P *) frequencies. Modelled upon earlier limiting dilution assays reviewed by Miller et al. [7], our system differs from them by the use of secondary mixed leukocyte culture supernatant (2° MLC SN) [8] as a stimulatory reagent enabling limiting numbers of responding precursor cells to produce measurable CTL activity in mixed leukocyte microcultures (micro-MLC). In the present article, we report the frequencies of CTL-P in thymus and various other lymphoid tissues. Since both cortisone (Cor) and cyclophosphamide (Cy) are immunosuppresive drugs which have been used to select T lymphocyte subpopulations [3—5], we also investigated the effect of these drugs on the CTL-P frequencies in thymus and spleen.

MATERIALS AND METHODS

Mice

Mice of the inbred strains C57BL/6 and DBA/2 were obtained from the animal colony maintained at the Swiss Institute for Experimental Cancer Research, Lausanne, Switzerland.

Preparation of supernatant from secondary MLC

As detailed elsewhere [8], 2° MLC SN was prepared by mixing 10×10^6 viable cells recovered from pools of 10—14 day primary C57BL/6 anti-DBA/2 MLC with 40×10^6 irradiated DBA/2 spleen cells in 10 ml culture medium in tissue culture flasks (25 cm^2; 1461, Nunc, Roskilde, Denmark). Supernatants were collected after 24 h of incubation and sterilized by filtration.

Mixed leukocyte microcultures

Cultures were established in Dulbecco's modified Eagle's medium (DMEM) supplemented as described previously [9] with additional amino acids and 5×10^{-5} M 2-mercaptoethanol. This culture medium was further supplemented with 10% (v/v) fetal bovine serum (FBS) and 50% (v/v) 2°

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* Abbreviations used in this paper: CTL, cytolytic thymus-derived lymphocyte; CTL-P, precursor of CTL; micro-MLC, mixed leukocyte microculture; 2° MLC SN, supernatant from secondary MLC; FBS, fetal bovine serum; DMEM, Dulbecco's modified Eagle's medium; Cor, cortisone; Cy, cyclophosphamide; NWC, nylon wool column.

MLC SN. Micro-MLC (11—33 per group) were established by mixing limiting numbers of C57BL/6 responding leukocytes with 1×10^6 irradiated (2000 rads) allogeneic DBA/2 spleen leukocytes in a final volume of 0.2 ml in round bottomed microwells (Greiner, Nurtingen, West Germany). Cultures were wrapped in aluminum foil to minimize evaporation and maintained for 7 days at 37° C in a water saturated atmosphere of 5% CO_2 in air.

Target cells

P-815 mastocytoma cells of DBA/2 origin were maintained in culture and labelled with $Na_2{}^{51}CrO_4$ as described previously [9]. Labelled cells were washed 3 times and resuspended at a concentration of 20×10^3/ml in DMEM supplemented with 5% (v/v) FBS and 10 mM Hepes buffer.

Assay for cytolytic activity

A modification of the ^{51}Cr release assay [9] was used to determine CTL activity. After 7 days of culture, 0.1 ml of medium was removed from each microwell and replaced by 0.1 ml of ^{51}Cr labelled P-815 mastocytoma target cells. The plates were centrifuged and then incubated for $3\frac{1}{2}$—$4\frac{1}{2}$ h at 37° C. Cytolysis was assessed by counting the radioactivity of 0.1 ml of supernatant for 1—2 min in a well-type scintillation counter. For the determination of spontaneous release, control microcultures containing irradiated DBA/2 spleen leukocytes, 2° MLC SN, and FBS in the absence of responding leukocytes were assayed in the same manner. Micro-MLC were defined as positive for CTL activity when ^{51}Cr release exceeded the mean spontaneous ^{51}Cr release by at least 3 SD. Specific lysis was calculated as described previously [9].

Calculation of CTL-P frequencies

Minimal estimates of CTL-P frequencies were calculated by analysis of the Poisson distribution relationship between the percentage of cytolytically negative or non-responding microcultures per group and the number of C57BL/6 responding leukocytes per microculture. The zero order term Poisson equation [7] was linearized to the form, $\ln y = -fx + \ln a$, where y is the percentage of non-responding cultures, x is the number of responding leukocytes, f is the CTL-P frequency equal to the negative of the slope, and a is the y-axis intercept theoretically equal to 100. Experimental x- and y-values were fitted to this equation by the least-squares method. Results were reported as the 3 values determined by linear regression analysis: the frequency f, the y-axis intercept a, and the coefficient of determination r^2. A more detailed explanation of these calculations is found in the Appendix.

In vivo drug treatments

All in vivo drug treatments consisted of a single i.p. injection followed by sacrifice of the mouse and removal of the thymus and spleen 2 days later. (Contamination of the thymus by the parathymic lymph nodes was avoided by separating them from the thymus after their identification by concentration of India ink: $\frac{1}{2}$ h before sacrifice, each mouse received a 0.2 ml i.p. injection of a 50% (v/v) ink suspension in DMEM.) For the Cor treatment, each mouse received 100 mg/kg of aqueous hydrocortisone acetate (Hydrocortifor; Vifor SA, Geneva, Switzerland). For the Cy treatment, each mouse received 100 or 300 mg/kg of cyclophosphamide saline (Endoxan-Asta; Asta-Werke AG, Brackwede, West Germany). Limiting dilution analyses were performed on pools of 3 thymuses or spleens per determination.

Nylon wool column (NWC) purification

Spleen cells were purified on NWC according to the method of Julius et al. [10]. Limiting dilution analyses were performed on pools of 2 spleens per determination.

RESULTS

Frequency of CTL-P in thymus and other tissues

Limiting numbers of C57BL/6 responding leukocytes and 1×10^6 irradiated allogeneic DBA/2 spleen cells were cultured for 7 days in the presence of 50% 2° MLC SN and 10% FBS. The CTL activity of each microculture was then assayed individually against ^{51}Cr labelled P-815 mastocytoma (DBA/2) target cells. Fig. 1 presents the results from representative limiting dilution analyses for normal thymus and normal spleen. Micro-MLC were defined as positive for CTL activity when ^{51}Cr release exceeded the mean spontaneous ^{51}Cr release by at least 3 SD, where the spontaneous release was determined from control microcultures containing irradiated stimulating cells, 2° MLC SN, and FBS in the absence of responding cells. These control microcultures did not generate any CTL activity [6]. A dose–response effect can be clearly seen for both thymus and spleen experiments. The percentage of cytolytically positive microcultures ranged from 58% at 2000 thymus responding cells to 3% at 100 cells and from 75% at 750 spleen responding cells to 29% at 250 cells. The mean specific ^{51}Cr release of all positive cultures was 35% for the thymus experiment and 47% for the spleen experiment. The percentage of non-responding microcultures per group was then calculated from the proportion of cytolytically negative microcultures at each dose of C57BL/6 responding leukocytes. The logarithm of the percentage of non-responding microcultures was plotted against the dose of responding leukocytes according to Poisson statistics and curves were then

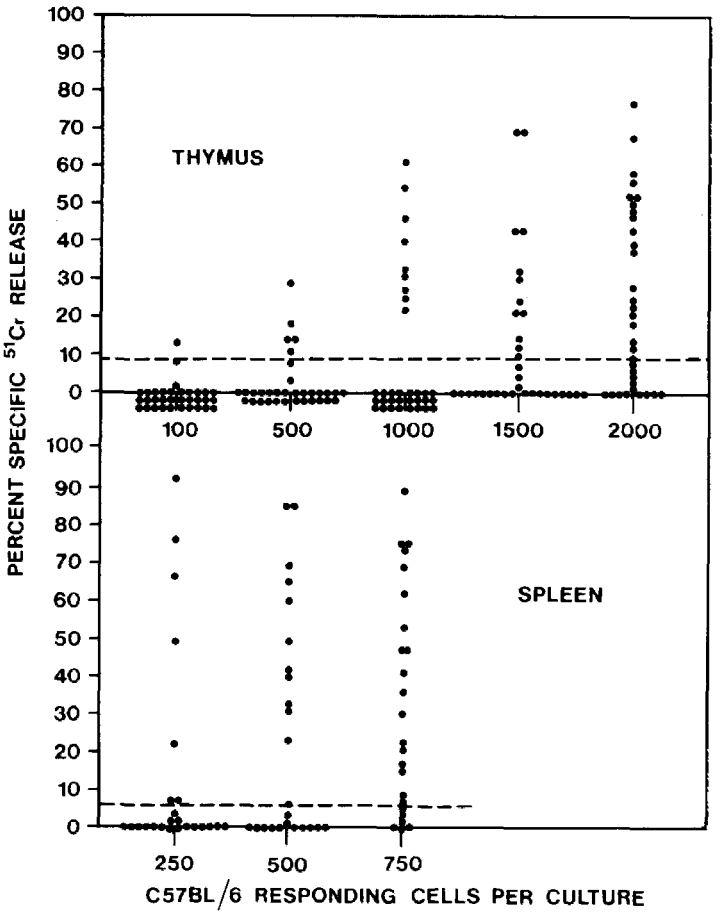

Fig. 1. Cytolytic activity of individual micro-MLC in representative limiting dilution analyses of thymus and spleen. The indicated number of normal C57BL/6 responding cells and 1×10^6 irradiated (2000 rads) allogeneic DBA/2 spleen cells were cultured for 7 days in the presence of 50% (v/v) 2° MLC SN and 10% (v/v) FBS. Each microculture was then assayed individually against ^{51}Cr labelled P-815 mastocytoma (DBA/2) target cells. The dotted line represents the lower limit of microculture CTL activity accepted as positive; this limit was defined by 3 SD above the mean spontaneous ^{51}Cr release for control cultures containing irradiated DBA/2 cells, 2° MLC SN, and FBS in the absence of C57BL/6 responding cells. For the thymus experiment, spontaneous release was 141 cpm (14% of the total release). For the spleen experiment, spontaneous release was 138 cpm (13%).

fitted by the least-squares method. Fig. 2 shows linear regression curves for the results presented in Fig. 1. The CTL-P frequencies were determined by the slopes of the linear regression curves. For these representative determinations, a CTL-P frequency of 0.41 per 10^3 cells (1/2441), an a-value of 106, and an r^2-value of 0.93 were determined for thymus, and a CTL-P frequency

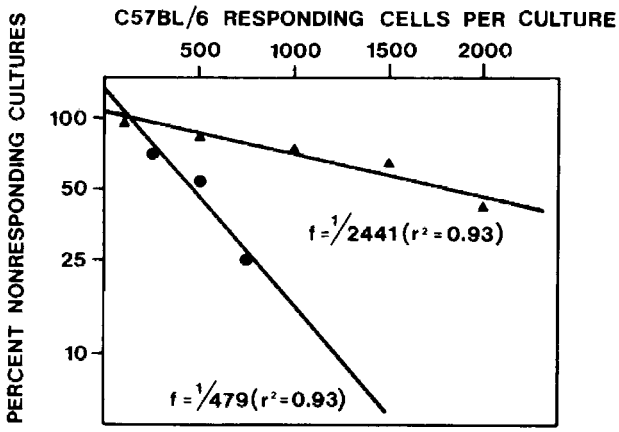

Fig. 2. Determination of minimal estimates of CTL-P frequencies in thymus and spleen. Data from Fig. 1 were fitted by the least-squares method to the linearized Poisson equation, $\ln y = -fx + \ln a$, where y is the percentage of non-responding microcultures per group, x is the number of C57BL/6 responding cells per microculture, f is the CTL-P frequency equal to the negative of the slope, and a is the y-axis intercept. r^2 is the coefficient of determination; (▲) thymus, $a = 106$; (●) spleen, $a = 130$.

of $2.1/10^3$ (1/479), an a-value of 130, and an r^2-value of 0.93 for spleen. Table I summarizes 41 separate CTL-P frequency determinations for thymus and various other lymphoid tissues. For these experiments, the mean a-value was 99 and the mean r^2-value was 0.92. The mean CTL-P frequency of 11 separate determinations for thymus was $0.68/10^3$ (1/1467) with SD $\pm 0.22/10^3$ and a range from $0.28/10^3$ to $1.08/10^3$. In comparison, the mean CTL-P frequency for spleen was $2.6/10^3$ (1/380); for NWC purified spleen,

TABLE I
Frequency of CTL-P in thymus and other lymphoid tissues. [a]

Source of CTL-P (number of determinations)	Mean CTL-P [b] frequency	Range [c]	Reciprocal of mean
Thymus [11]	0.68 ± 0.22	0.28— 1.08	1467
Spleen [19]	2.6 ± 1.3	1.0 — 5.9	380
NWC purified spleen [4]	5.3 ± 3.3	1.9 — 9.8	188
Peripheral blood [4]	5.5 ± 3.3	1.0 — 8.7	180
Lymph node [3]	14.7 ± 9.4	6.8 —25.0	68

[a] Limiting dilution analyses were performed as described in Fig. 1 and CTL-P frequencies were calculated as explained in Fig. 2. For the 41 determinations summarized here, the mean a-value is 99, the mean r^2-value is 0.92, and the mean number of experimental points per determination is 3.4.
[b] Results are expressed as the mean ± 1 SD of the number of CTL-P per 10^3 cells.
[c] Minimum and maximum CTL-P frequencies per 10^3 cells.

$5.3/10^3$ (1/188); for peripheral blood, $5.5/10^3$ (1/180); and for lymph node, $15/10^3$ (1/68).

Effect of Cor treatment on CTL-P frequency in thymus and spleen

Mice were injected i.p. with a single dose of aqueous hydrocortisone acetate at 100 mg/kg 2 days before sacrifice. Limiting dilution analyses were then performed on pools of 3 thymuses or spleens per determination for both Cor treated mice and normal control mice. Fig. 3 shows the linear regression curves for a representative experiment. Table II summarizes the results from this and 2 other similar experiments. Cor treatment produced a mean 22-fold increase in the CTL-P frequency of treated thymus compared to normal thymus. In absolute terms, a mean CTL-P recovery of 87% was obtained despite a reduction in the mean number of total cells to only 4% of normal. In contrast, Cor treatment did not significantly change the CTL-P frequency in treated spleen compared to normal spleen. It did, however, decrease the mean number of total cells to 37% of normal. Thus, Cor treatment produced a drastic reduction in total thymus cells and only a relatively minor reduction in thymus CTL-P whereas the same Cor treatment produced a similar reduction in both total spleen cells and spleen CTL-P.

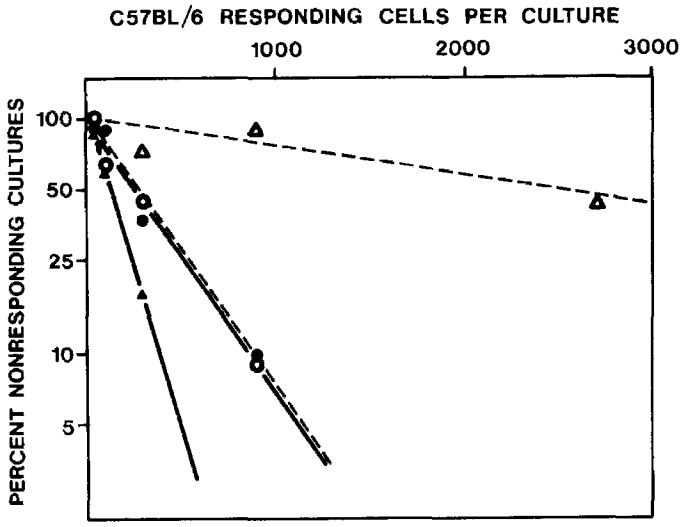

Fig. 3. Linear regression curves (see Fig. 2) for normal and Cor treated thymus and spleen. Limiting dilution analyses (see Fig. 1) were performed on pools of 3 thymuses or spleens per determination. For the Cor treatment, each mouse received a single i.p. injection of 100 mg/kg aqueous hydrocortisone acetate and was sacrificed 2 days later. (△) normal thymus, $f = 1/3599$, $a = 99$, and $r^2 = 0.84$; (▲) Cor treated thymus, $f = 1/167$, $a = 111$, and $r^2 = 1.00$; (○) normal spleen, $f = 1/373$, $a = 99$, and $r^2 = 0.99$; (●) Cor treated spleen, $f = 1/364$, $a = 102$, and $r^2 = 0.98$.

TABLE II

Effect of cortisone treatment on CTL-P frequency in thymus and spleen. [a]

Source of CTL-P	CTL-P frequency per 10^3 cells		Recovery (% untreated)	
	Untreated	Treated	Total cells	CTL-P
Thymus	0.47 (0.72, 0.28, 0.41)	10.42 (18.52, 5.99, 6.76)	4 (5, 3, 4)	87 (129, 65, 66)
Spleen	2.43 (2.17, 2.68, ND)	2.58 (2.42, 2.75, ND)	37 (35, 38, ND)	39 (39, 39, ND)

[a] Values listed are the mean of and individual results from 3 separate Cor treatment experiments similar to and including the one described in Fig. 3. For these 10 determinations, the mean a-value is 105, the mean r^2-value is 0.93, and the mean number of experimental points per determination is 4.4.
ND = not done.

TABLE III

Effect of cyclophosphamide treatment on CTL-P frequency in thymus and spleen. [a]

Cyclophosphamide dose	Source of CTL-P	CTL-P frequency per 10^3 cells		Recovery (% untreated)	
		Untreated	Treated	Total cells	CTL-P
100 mg/kg	Thymus	0.80	0.58	82	59
	Spleen	1.39	1.81	69	90
300 mg/kg	Thymus	0.82	0.64	20	16
	Spleen	2.09	0.16	10	1

[a] 2 Cy treatment experiments (one at each dose indicated) were performed analogously to the Cor treatment experiments (see Fig. 3 and Table II) with the substitution of cyclophosphamide saline for aqueous hydrocortisone acetate. For these determinations, the mean a-value is 85, the mean r^2-value is 0.83, and the mean number of experimental points per determination is 3.9.

Effect of Cy treatment on CTL-P frequency in thymus and spleen

Table III summarizes the results from 2 in vivo Cy treatment experiments. These experiments were performed in a manner entirely analogous to the Cor treatment experiments. Cy at 100 mg/kg did not significantly reduce the number of either total cells or CTL-P, nor did it significantly affect CTL-P frequencies in either thymus or spleen. Cy at 300 mg/kg, however, did clearly reduce the number of both total cells and CTL-P. For thymus, the reduction was similar with a total cell recovery of 20% and a CTL-P recovery of 16%, whereas for spleen, the reduction was more specific with a total cell recovery of 10% and a CTL-P recovery of only 1%. As a result, Cy treatment decreased the CTL-P frequency in spleen by more than 10-fold without significantly affecting the CTL-P frequency in thymus.

DISCUSSION

We have examined the CTL-P frequencies of C57BL/6 (H-2^b) thymus cells activated by DBA/2 (H-2^d) spleen cells in primary micro-MLC and assayed against P-815 mastocytoma (H-2^d) target cells. We obtained a mean CTL-P frequency of 1/1467. Lee [11] has reported the only other frequency determination for thymic CTL-P. His frequency of 1/7000 was obtained, however, in a system using an H-2^k anti-H-2^d strain combination. Although direct comparison between these frequencies is therefore not possible, we do note that the ratio of our mean thymic to mean splenic CTL-P frequencies was much higher than was the ratio of his respective frequencies. Lindahl and Wilson [12] using C57BL/6 anti-DBA/2 micro-MLC, and Teh et al. [13] using C57BL/6 nu/+ anti-(C57BL/6 × DBA/2)F_1 micro-MLC, have reported

frequency determinations for H-2^b anti-H-2^d CTL-P in tissues other than thymus. For example, compared to our mean frequency of 1/68 for lymph node, they obtained maximum frequencies of 1/677 and 1/571, respectively. As another example, compared to our mean frequency of 1/380 for spleen, Teh et al. [13] obtained a maximum frequency of 1/2300. Our mean frequencies are thus significantly higher than those of other investigators for similar strain combinations suggesting that our assay may have a higher plating efficiency.

These frequencies were calculated by an application of Poisson statistics based upon the assumption that the CTL-P is the sole limiting factor in the assay. The theory predicts that a semilogarithmic line plot of the percentage of non-responding cultures per group versus the number of responding cells per culture should intersect the y-axis at 100 if limiting dilution conditions have been established. Since we obtained a mean a-value of 99 (with SD limits at 76 and 128) from 45 separate frequency determinations, we accepted this method as valid for the calculation of CTL-P frequencies in our assay. We assume that the observed variation in a-values was due simply to random statistical fluctuations which serve to emphasize the importance of correctly determining CTL-P frequencies by the slope of the linear regression curve rather than by the x-value corresponding to $y = 37$. A more complete discussion of this problem is found in the Appendix. It is possible that the 2° MLC SN used in our micro-MLC is responsible for the single cell event nature of the limiting dilution assay as well as the very high frequencies mentioned above.

It is of interest to consider the relative magnitude of experimentally observed CTL-P frequencies with respect to those which would be expected on the basis of T lymphocyte frequency distributions in lymphoid tissues of the normal mouse. Since spleen lymphocytes are comprised of 30—35% T lymphocytes, lymph node 65—70%, and peripheral blood 70% [14], a 2-fold higher CTL-P frequency would be expected for peripheral blood and lymph node relative to spleen. Furthermore, since NWC purification enriches the frequency of T lymphocytes in spleen by 1.5—2-fold [10], a correspondingly higher CTL-P would likewise be anticipated. Although we did in fact observe the expected 2-fold higher frequencies in peripheral blood and NWC purified spleen, we observed almost a 6-fold higher CTL-P frequency in lymph node. It is thus apparent that the proportion of CTL-P within the total T lymphocyte population may vary from one tissue to another.

In addition to examining the CTL-P frequencies in normal cell populations, we also investigated the effect of in vivo drug treatments on CTL-P frequencies in thymus and spleen. We found that Cor treatment dramatically increased the CTL-P frequency in thymus despite a drastic reduction in the number of total thymus cells. Although the same Cor treatment also reduced the number of total spleen cells, it did not affect the CTL-P frequency in spleen. These results corroborate those of Lee [11] who also examined the effect of Cor treatment on CTL-P frequencies in thymus and spleen. Our

results are also in agreement with earlier reports of increased CTL activity in Cor treated thymus [15—17]. Furthermore, we note that the mean CTL-P frequency we obtained for Cor treated thymus (1/96) was very similar to the mean frequencies we obtained for lymph node (1/68) and for peripheral blood (1/180). Consequently, these results support the view that Cor resistant thymocytes constitute part, if not all, of the immunocompetent lymphocyte subpopulation of the thymus [3—5].

In contrast to the results obtained with Cor, Cy (at 300 mg/kg) significantly decreased the CTL-P frequency in spleen without affecting that in thymus. A lower dose of Cy (100 mg/kg) produced no effect. Although no other CTL-P frequencies in Cy treated lymphoid tissues have been reported, our results are compatible with those of Milton et al. [18], who observed decreased CTL activity in Cy treated (300 mg/kg) spleen following in vivo sensitization against allogeneic tumor cells. Conflicting results have been obtained by other investigators using lower doses of Cy (48—200 mg/kg) [19—22]. Future investigations should therefore focus attention upon a range of Cy doses.

Due to its high level of sensitivity, our limiting dilution analysis system has also been effectively used to investigate the ontogeny of CTL-P in the thymus of neonatal mice (M.B. Widmer et al., in prep.). With proper modifications for the measurement of other in vitro functional activities and in conjunction with other experimental methods such as physical separation techniques [4,5], this system for the determination of precursor frequencies should provide an effective tool for further investigations of T lymphocyte subpopulations and their developmental pathways.

APPENDIX

Calculation of CTL-P frequencies

Generally, the logarithm of the percentage of non-responding cultures (y) has been plotted versus the number of responding cells (x) and the CTL-P frequency has been accepted as the x-value corresponding to $y = 37$. It should be noted, however, that frequencies determined in this manner are mathematically valid only if the y-axis intercept is the theoretically correct value of 100. This problem has often been avoided by drawing the experimental curves directly through the theoretical y-axis intercept without the use of linear regression analysis. This method of plotting the data, though, may at times strongly bias the results. We have therefore followed the notable exception of Lindahl and Wilson [12]: the theoretical y-axis intercept has not been used in the calculations (except as explained below) and all frequencies have been mathematically determined by the linearized Poisson equation, $\ln y = -fx + \ln a$, where y and x are as defined above, f is the CTL-P frequency equal to the negative of the slope, and a is the y-axis intercept. Experimental x- and y-values were fitted to this equation by the

least-squares method. Results were reported as the 3 values determined by linear regression analysis: the frequency f, the y-axis intercept a, and the coefficient of determination r^2. The true decimal number frequencies and not their reciprocals were used to calculate frequency averages. It should be noted that averaging the reciprocals produces a mathematically false result. Also, the natural logarithms of the a-values and not the a-values themselves were used to calculate a-value averages, since the variable subjected to linear regression analysis is ln y and not y itself.

Approximation of CTL-P frequencies for saturation curves

All positive saturation points, i.e. experimental points with a y-value of 0% non-responding cultures, were excluded from the calculations, whereas all negative points, i.e. experimental points with a y-value of 100% non-responding cultures, were included in the calculations. As a consequence, of a total of 50 determinations reported in this article, 45 were calculated from curves fitted to 2—6 experimental points per determination with a mean and SD of 3.8 ± 1.1. For the remaining 5 determinations in which only 1 experimental point was left after the exclusion of saturation points, minimal estimates were obtained from the line drawn between the single experimental point and the theoretical y-axis intercept of 0,100. This approximation method was chosen as the best for 2 reasons: (1) the mean a-value for the 45 determinations without the theoretical 0,100 point was 99 (with SD limits at 76 and 128) therefore making the theoretical validity of the approximation reasonable, and (2) an alternative approximation method based on the nearest saturation point produced far greater variability in resultant CTL-P frequency, therefore making the chosen approximation method the most consistent one. The a-values of 100 for the 5 determinations based on the theoretical point approximation were included in the calculations of the various a-value averages reported throughout the text since their respective frequencies were dependent upon these a-values. In no case, however, was any artificial r^2-value assigned to any determination based on only 2 points.

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REFERENCES

[1] Greaves, M.F., Owen, J.J.T. and Raff, M.C. (1974) T and B lymphocytes: Origins, properties and roles in immune responses. Excerpta Medica, Amsterdam.

[2] Cantor, H. and Boyse, E. (1977) Regulation of the immune response by T cell subclasses. Contemp. Top. Immunobiol. 7, 47.

[3] Stutman, O. (1977) Two main features of T cell development: thymus traffic and postthymic maturation. Contemp. Top. Immunobiol. 7, 1.
[4] Shortman, K., von Boehmer, H., Lip, J. and Hopper, K. (1975) Subpopulations of T lymphocytes. Physical separation, functional specialisation and differentiation pathways of sub-sets of thymocytes and thymus-dependent peripheral lymphocytes. Transplant. Rev. 25, 163.
[5] Droege, W. and Zucker, R. (1975) Lymphocyte subpopulations in the thymus. Transplant. Rev. 25, 3.
[6] Ryser, J.E. and MacDonald, H.R. (1979) Limiting dilution analysis of alloantigen-reactive T lymphocytes. I. Comparison of precursor frequencies for proliferative and cytolytic responses. J. Immunol. 122, 1691.
[7] Miller, R.G., Teh, H.-S., Harley, E. and Phillips, R.A. (1977) Quantitative studies of the activation of cytotoxic lymphocyte precursor cells. Immunol. Rev. 35, 38.
[8] Ryser, J.E., Cerottini, J.-C. and Brunner, K.T. (1978) Generation of cytolytic T lymphocytes in vitro IX. Induction of secondary CTL responses in primary long-term MLC by supernatants from secondary MLC. J. Immunol. 120, 370.
[9] Cerottini, J.-C., Engers, H.D., MacDonald, H.R. and Brunner, K.T. (1974) Generation of cytotoxic T lymphocytes in vitro. I. Response of normal and immune mouse spleen cells in mixed leukocyte cultures. J. Exp. Med. 140, 703.
[10] Julius, M.H., Simpson, E. and Herzenberg, L.A. (1973) A rapid method for the isolation of functional thymus-derived murine lymphocytes. Eur. J. Immunol. 3, 645.
[11] Lee, K.C. (1977) Cortisone as a probe for cell interactions in the generation of cytotoxic T cells. I. Effect on helper cells, cytotoxic T cell precursors, and accessory cells. J. Immunol. 119, 1836.
[12] Lindahl, K.F. and Wilson, D.B. (1977) Histocompatibility antigen-activated cytotoxic T lymphocytes. II. Estimates of the frequency and specificity of precursors. J. Exp. Med. 145, 508.
[13] Teh, H.-S., Harley, E., Phillips, R.A. and Miller, R.G. (1977) Quantitative studies on the precursors of cytotoxic lymphocytes. I. Characterization of a clonal assay and determination of the size of clones derived from single precursors. J. Immunol. 118, 1049.
[14] Raff, M.C. and Owen, J.J.T. (1971) Thymus-derived lymphocytes: their distribution and role in the development of peripheral lymphoid tissues of the mouse. Eur. J. Immunol. 1, 27.
[15] Brunner, K.T., Nordin, A.A. and Cerottini, J.-C. (1971) In vitro studies of sensitized lymphocytes and alloantibody-forming cells in mouse allograft immunity. In: Cellular interactions in the immune response, p. 220. Karger, Basel.
[16] Wagner, H., Harris, A.W. and Feldman, M. (1972) Cell-mediated immune responses in vitro: II. The role of thymus and thymus-derived lymphocytes. Cell Immunol. 4, 39.
[17] Altman, A. and Cohen, I.R. (1975) Heterogeneity in the development of cytotoxic T lymphocytes in vitro revealed by sensitivity to hydrocortisone. J. Exp. Med. 142, 790.
[18] Milton, J.D., Carpenter, C.B. and Addison, I.E. (1976) Depressed T cell reactivity and suppressor activity of lymphoid cells from cyclophosphamide-treated mice. Cell Immunol. 24, 308.
[19] Fass, L. and Fefer, A. (1972) The application of an in vitro cytotoxicity test to studies of the effects of drugs on the cellular immune response in mice. I. Primary response. J. Immunol. 109, 749.
[20] Tagart, V. (1977) Cytotoxicity in picryl chloride-painted mice is enhanced by pretreatment with cyclophosphamide. Transplantation 23, 287.
[21] Röllinghoff, M., Starzinski-Powitz, A., Pfizenmaier, K. and Wagner, H. (1977) Cyclophosphamide-sensitive T lymphocytes suppress the in vivo generation of antigen-specific cytotoxic T lymphocytes. J. Exp. Med. 145, 455.
[22] Gerber, M., Andress, D., Pioch, Y., Radal, M. and Serrou, B. (1978) Effect of cyclophosphamide and methylprednisolone on in vitro cellular immune response to allogeneic tumor cells. Correlation with in vivo rejection. Transplantation 26, 142.