T-T CELL INTERACTIONS DURING IN VITRO CYTOTOXIC T LYMPHOCYTE RESPONSES

V. Precursor Frequencies and Specificity of Alloreactive Helper T Cells*

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The in vitro induction of antigen-specific cytotoxic T lymphocytes (CTL)[1] requires the collaboration between functionally dintinct T cell subpopulations (1–5). Studies at the population level have revealed that upon antigen-specific activation, helper T lymphocytes (HTL) release nonspecific mediators of T help such as interleukin 2 (Il-2), which in turn promote clonal expansion and maturation of antigen-triggered precursors of CTL (CTLp) into cytotoxic effector cells (6–11). Consequently, the efficiency of generating a T cell response depends upon the precursor frequencies of both the regulatory and effector T cells reactive to the antigen in question.

The understanding of the relative roles of functionally distinct T cell subsets during the induction of CTL requires investigations of both the quantitative representation and the specificity repertoire of the respective T cell subset within the responder population. For this type of analysis, however, experimentation at the clonal level rather than at the population level is mandatory. In this regard, the frequency analysis of CTLp and specificity analysis of cloned lines of CTL has provided new information on the expression and development of the repertoire of alloreactive, self-major histocompatibility complex- (MHC) restricted and allo-MHC-restricted antigen-specific sets of CTLp (12–15). However, information is sparse on the frequency and specificity of precursors of helper T lymphocytes (HTLp) required for differentiation and proliferation of CTLp (16). This communication records our results on limiting dilution analysis to enumerate the precursor frequencies of helper T cells for specific antigen responses in splenic T cells. We present evidence for an absolute requirement of alloreactive Lyt-1^+ HTL or their soluble products for the generation of CTL from the pool of Lyt-123^+ and Lyt-23^+ T cells. Furthermore, we establish the frequency distribution of HTL reactive to different cell surface antigens coded for by the MHC and Mls locus. Finally, we define the antigen specificity of HTL at the clonal level.

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[1] *Abbreviations used in this paper:* C, complement; CTL, cytotoxic T lymphocyte; CTLp, precursor of CTL; CTLL, cloned T lymphocyte line; FCS, fetal calf serum; Il-1, interleukin 1; Il-2, interleukin 2; HTL, helper T lymphocyte; HTLp, precursor of HTL; MLC, mixed leukocyte culture; Mls, M-locus; 2°MLC, secondary MLC; 2°CTL, CTL derived from 2°MLC.

Materials and Methods

Mice. All mice were obtained from OLAC Ltd., Shaw's Farm, Blackthorn, England, and were used at 4–8 wk of age.

Media. An equal mixture of RPMI 1640 and Click's medium (Seromed, München,) was used as culture medium. It was supplemented with 10 mM Hepes, 10 mM glutamin, 5×10^{-5} M 2-mercaptoethanol, 0.15% $NaHCO_3$, 100 U/ml penicillin, 100 ng/ml streptomycin, and 10% of selected batches of heat-inactivated fetal calf serum (FCS). When indicated, 0.5% mouse serum was used instead of FCS. The medium used for the preparation of cell suspensions consisted of Hepes buffered Hanks' balanced salt solution (HBSS) containing 5% FCS.

Purification of T Cells. Enrichment for T cells was carried out according to the method described by Mage et al. (17). Briefly, the spleen cells were allowed to adhere to plastic dishes coated with purified goat anti-mouse IgG antibodies. The nonadherent (sIg^-) fraction was carefully pipetted off and referred to as T cells.

Antibody and Complement Treatment of Lymphocytes. Monoclonal anti-mouse Thy-1.2, anti-mouse Lyt-2.2, and anti-mouse Lyt-1.2 antisera were purchased from NEN Chemical GmbH, Dreieich, Federal Republic of Germany. Nontoxic rabbit serum was the source of complement. T cells were first incubated for 30 min at 4°C with the respective antiserum. Complement (C) was added in a final dilution of 1:16, and the cell suspension was incubated for 45 min at 37°C.

Positive Selection of Lyt-2^+ Cells. Separation of Lyt-2^+ and Lyt-2^- lymphocytes was done according to the method described by Mage et al. (18). Briefly, splenic T cells (sIg^-) were incubated with monoclonal anti-mouse Lyt-2.2 antiserum for 40 min at 4°C. The cells were washed, resuspended, and placed in petri dishes coated with goat anti-mouse IgG antibodies. The nonadherent cells were removed and referred to as Lyt-2^- cells. Thereafter, the plates were washed four times with phosphate-buffered saline, and the attached cells were removed by vigorous pipetting of HBSS medium onto the cell layer. These cells were referred to as Lyt-2^+ cells.

Stimulation of Cells. The stimulator cells were treated twice with monoclonal anti-mouse Thy-1.2 plus C to avoid the production of helper activity by irradiated T lymphocytes. Depletion of Il-2-producing T cells from the stimulator cells was demonstrated by their inability to produce Il-2 in response to 1 μg/ml concanavalin A (Con A).

Limiting Dilution Cultures

ESTIMATION OF CTLp FREQUENCIES. Replicates of 32–96 cultures were set up in V-shaped microtiter plates (Greiner, Nürtingen, Federal Republic of Germany). Limiting numbers of responder lymphocytes were stimulated with 5×10^5 irradiated (2,000 rad) allogeneic spleen cells in the presence of 20% (vol/vol) supernatant of Con A-activated spleen cells as a source for Il-2. On day 6, each microculture was assayed for cytotoxicity: after removal of culture medium, 150 μl fresh medium containing 10^3 ^{51}Cr-labeled P815 ($H\text{-}2^d$) tumor targets was added. The percent of specific ^{51}Cr release after 4 h of incubation was calculated from the formula described (11). Cultures were considered positive provided they gave a ^{51}Cr release exceeding the mean spontaneous release by at least three standard deviations.

ESTIMATION OF HTLp FREQUENCIES. 32–96 replicates of microcultures were set up containing graded numbers of responding cells as a source for HTLp and constant numbers (2,000) of syngeneic Lyt-2^+ spleen cells as a source of CTLp. Each culture was stimulated with 5×10^5 irradiated (2,000 rad) allogeneic T cell-depleted spleen cells. After 6 d, each culture was assayed for cytotoxicity as described. Cultures were considered positive provided they gave a ^{51}Cr release exceeding the mean of negative controls by at least three standard deviations.

ESTIMATION OF FREQUENCIES OF HTLp PRODUCING SOLUBLE HELPER FACTORS. In a first step, microcultures were set up in V-shaped 96-well culture trays in a volume of 200 μl per well. 32–96 replicates of limiting numbers of responding lymphoid cells were incubated with 5×10^5 T cell-depleted, x-irradiated (2,000 rad) stimulator spleen cells at 37°C in a water saturated air with 5% CO_2 in gas phase for 7 d. Thereafter, the microculture plates were centrifuged for 10 min at 5,000 rpm, and the culture medium was shaken off. Then, 150 μl culture medium containing fresh stimulating cells was added. After 2 d, the microtiter plates were centrifuged, and 100 μl of the secondary mixed leukocyte culture (2°MLC) supernatants was transferred to U-shaped microtiter plates by a multichannel pipette and assayed for helper activity.

Biological Assays for Soluble Helper Activity. Il-2 levels of individual culture supernatants were assayed by:

(*a*) PROLIFERATION ASSAY. According to the method described by Gillis et al. (19), 100 μl culture medium containing 10^4 cells of an IL-2-dependent cloned CTL-line (CTLL), (clone 3/E2, a kind gift of Dr. T. Huenig, Würzburg) was added to each individual supernatant. After 48 h of incubation, the cultures were pulsed with 1 μCi of [^{3}H]thymidine for 16 h. The samples were collected with a semiautomatic cell harvester, and the counts per minute were recorded.

(*b*) CYTOTOXICITY ASSAY. 10^4 C57Bl/6-derived CTL obtained after repeated in vitro stimulation with BALB/c spleen cells (2° CTL) (7) were added in a volume of 100 μl culture medium to the supernatants. Cytolytic activity was determined at day 4 in a ^{51}Cr release assay as described. For both test systems, the cultures were considered positive when they exhibited (*a*) [^{3}H]thymidine uptake or (*b*) ^{51}Cr release exceeding the mean of the respective negative controls by at least three standard deviations.

Helper activity of individual culture supernatants was assayed by (*a*) generation of primary CTL responses in Lyt-2$^+$ spleen cells. Positively selected Lyt-2$^+$ lymphocytes were used as a source for a CTLp indicator population lacking helper T cell activity (see Results). They were stimulated with 5×10^5 x-irradiated T cell-depleted allogeneic spleen cells in the presence of individual 2°MLC supernatants. After 6 d of culture, each micro-MLC was assayed for cytolytic activity; or (*b*) generation of primary CTL responses in peanut agglutinin-positive (PNA$^+$) thymocytes: 2×10^5 PNA$^+$ thymocytes purified by cell affinity chromatography according to the method of Irlé et al. (20) were taken as source for CTLp and stimulated with 5×10^5 x-irradiated T cell-depleted allogeneic spleen cells. The generation of cytolytic activity in the presence of individual 2°MLC supernatants was assessed at day 6 of culture. For both test systems, the cultures were considered positive provided they exhibited cytolytic activity exceeding the mean of the respective negative controls by at least three standard deviations.

Statistical Analysis. Calculation of frequencies from limiting dilution data was based on the Poisson distribution. Fractions of negative microcultures were plotted against the responding cell numbers. Using the least squares method (21), a regression line of best fit to the experimental values was determined. Frequencies were then calculated from the slope of the regression lines. In addition, we analyzed all data with the χ^2 minimization method according to Taswell (22). The frequency data shown in this paper were selected for regression lines with r^2 values above 0.90, and y-axis intercepts between 0.90 and 1.20 as calculated by the least squares method. In all these cases, the additional analysis by the χ^2 minimization method revealed close convergence of frequency estimates.

Results

Limiting Dilution Analysis of Lyt-2$^+$ and Lyt-2$^-$ T Cells: CTLp and HTLp Frequencies. It was shown previously (5, 7, 23, 24) that in primary MLC the CTL derive from the Lyt-123$^+$ and Lyt-23$^+$ (Lyt-2$^+$) pool of T cells, whereas HTL express the Lyt-1$^+$ (Lyt-2$^-$) phenotype. Provided these two functional subsets can be experimentally separated on the basis of their Lyt phenotype expression, it should be possible to determine the frequency of CTLp in the Lyt-2$^+$ fraction and the frequency of HTLp in the Lyt-2$^-$ fraction by admixing nonlimiting numbers of the respective counterpart. In Fig. 1, data are presented that indicate the feasibility of this approach to measure CTLp and HTLp frequencies. The Lyt-2$^+$ and Lyt-2$^-$ T cell populations were obtained by the panning technique described by Mage et al. (18). In Fig. 1A, the results of a representative limiting dilution analysis for CTLp in a primary C57BL/6 anti-BALB/c MLC are depicted. In the presence of supernatant derived from Con A-activated spleen cells as source for the helper T cell product Il-2, the CTLp frequency within positively selected Lyt-2$^+$ T cells was 1/213, whereas that in negatively selected Lyt-2$^-$ T cells turned out to be 1/15,000. However, in the absence of exogenously added Il-2, the Lyt-2$^+$ responder population was virtually unable to generate cytotoxic

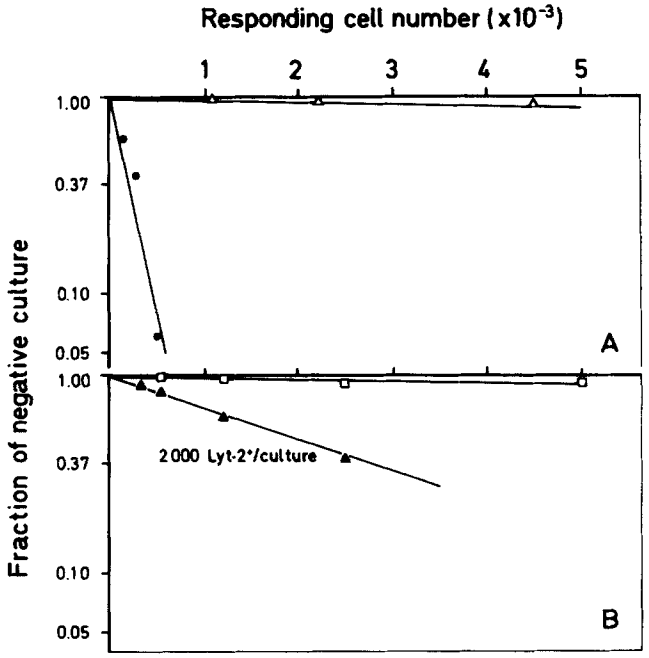


FIG. 1. CTLp and HTLp frequencies in Lyt-2$^+$ and Lyt-2$^-$ T cells. (A) To determine CTLp frequencies, limiting numbers of C57BL/6-derived Lyt-2$^+$ and Lyt-2$^-$ T cells were cultured with 5 × 10^6 BALB/c T cell-depleted x-irradiated stimulating spleen cells in the presence of exogenously added Il-2. After 6 d, the microcultures were tested for cytolytic activity towards ^{51}Cr labeled P815 (H-2^d) tumor target cells. △, Lyt-2$^-$, f-1/15.370; ●, Lyt-2$^+$, f-1/213. (B) To determine the HTLp frequencies, limiting numbers of C57BL/6-derived Lyt-2$^+$ and Lyt-2$^-$ responding cells were cultured with alloantigen in the absence of Il-2. As source for HTLp, Lyt-2$^-$ T cells were titrated into microcultures containing 2,000 C57BL/6 Lyt-2$^+$ cells as CTLp indicator population and 5 × 10^5 BALB/c T-depleted stimulating cells. □, Lyt-2$^+$, f-1/46.357; ▲, Lyt-2$^-$, f-1/2.509.

effector cells, as judged by a >200-fold decrease in the apparent frequency (Fig. 1 B). These data indicate that both the separation procedure was effective and the generation of CTL from the Lyt-2$^+$ subset of CTLp requires T help that is mediated by cells of the Lyt-2$^-$ phenotype (Fig. 1 B).

To determine frequencies of HTLp, a prerequisite is to provide nonlimiting numbers of CTLp to the cultures such that the HTLp is the only limiting cell type for the generation of cytolytic activity. Consequently, we set up replicates of cultures each containing allogeneic T cell-depleted stimulating cells and nonlimiting numbers of Lyt-2$^+$ C57BL/6 T cells as a source of CTLp and added graded numbers of C57BL/6 Lyt-2$^-$ T cells as a source of T cell help. As depicted in Fig. 1 B, a HTLp frequency of 1/2,500 was determined in Lyt-2$^-$ T cells. When the fraction of cytolytically negative cultures was plotted against the responding cell dose, the experimental points fit a straight line passing the y-axis near the 1.0 value.

Because application of the Poisson distribution revealed single hit conditions, it was concluded that the induction of Lyt-2$^+$-derived CTL responses was dependent on one single type of Lyt-2$^-$ (Lyt-1$^+$) HTL, the frequency of which was about 5- to 10-fold lower than that of alloreactive CTLp. Furthermore, the data also implied that Lyt-123$^+$ T cells, at least under the conditions used, did not give rise to functional active HTL by themselves (Fig. 1 B). The data given in Table I indicate that the

frequencies of HTLp were dependent on the alloantigens used. Only ~10% of C57BL/6 mouse-derived HTLp reactive against fully allogeneic (H-2^d) stimulator cells were reactive towards H-$2D^d$-incompatible stimulator cells. A combination of H-2 plus Mls incompatibility increased the HTLp frequency approximately twofold. As expected, the corresponding frequencies within unseparated spleen cells or splenic T cells were lower than those within the Lyt-2^- subset.

Limiting Dilution Analysis of HTLp-producing Soluble Helper Factors. Since in primary CTL responses the requirements for helper T cells can be substituted for by soluble mediators rich in Il-2 (6–11), it was of interest to investigate whether the HTLp limiting for Il-2 production is also limiting for the overall helper activity. Therefore, a limiting dilution analysis of helper T cells able to produce soluble helper factors was performed. To this end, a two-step culture system was used. In a first step, replicates of microcultures containing limiting numbers of C57BL/6 mouse-derived spleen cells plus 5×10^5 T cell-depleted, irradiated DBA/2 stimulator cells were set up. After a 7-d culture period, during which alloantigen-sensitized responder cells underwent clonal expansion, the cultured cells were washed and restimulated. After 48 h, 100 μl of 2°MLC supernatant was withdrawn from each individual microculture and assayed in a second step for Il-2 activity as well as for helper activity. Il-2 activity in 2°MLC supernatants was tested by its ability to cause proliferation of a Il-2-dependent CTLL or by its ability to maintain cytolytic activity of alloantigen-primed CTL (2°CTL) (7). Helper activity was assessed by the capacity to generate primary CTL responses. Fig. 2 depicts a representative experiment in which either [^{3}H]thymidine incorporation of CTLL or the lytic activity of 2°CTL as induced by supernatants from individual wells is plotted against the initial responding cell number. Obviously, in the case of 2°CTL, the percentage of cytolytically positive microcultures was dependent on the number of responder cells seeded in the first step culture. Similar results were obtained in terms of [^{3}H]thymidine incorporation using a Il-2-dependent CTLL (Fig. 2).

When the logarithms of the fractions of negative cultures of both assays were

TABLE I

*HTLp Frequencies in Different Spleen Cell Populations**

Strain	Responding cell population	Stimulating cells	Alloantigens	Reciprocal of frequency
C57BL/6	Spleen	BALB/c	H-2^d + minor H	6,950
	Splenic T‡			4,490
	Lyt-2^-‡			2,510
C57BL/6	Spleen	DBA/2	H-2^d + Mls^a + minor H	4,120
	Splenic T			2,480
	Lyt-2^-			1,470
B10.HTG	Spleen	B10.D2	H-$2D^d$	60,300
	Splenic T			38,100
	Lyt-2^-			22,700

* Serial dilutions of the indicated responder cells were cultured together with 2,000 syngeneic Lyt-2^+ T cells and 5×10^5 T cell-depleted stimulator cells. HTLp frequencies were calculated as detailed in Materials and Methods.

‡ Splenic T cells and Lyt-2^- T cells were prepared by the adherence technique as described in Materials and Methods.

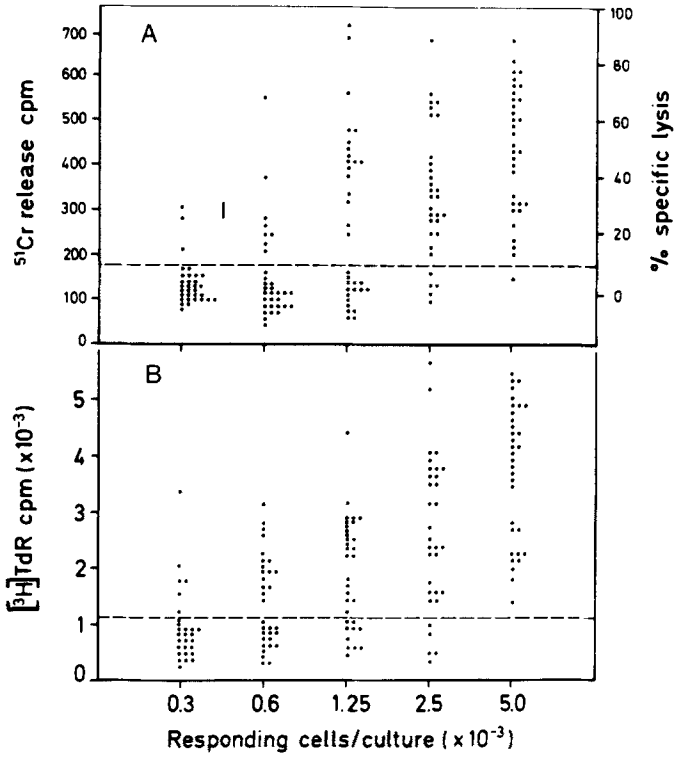


FIG. 2. Il-2 levels in 2°MLC supernatants, generated by limiting numbers of responder cells. Limiting numbers of C57BL/6-derived spleen cells were cultured with 5 × 10^5 T cell-depleted DBA/2-stimulating spleen cells for 7 d. The cultures were restimulated with fresh stimulating cells, and after 48 h, 2°MLC supernatants were removed from each microculture. Il-2 activity was tested on either 2°CTL (A) or CTLL (B). Each point represents the specific ^{51}Cr release mediated by 2°CTL or [^{3}H]thymidine incorporation of CTLL induced by individual 2°MLC supernatants. The dotted lines correspond to the mean of negative controls plus three standard deviations.

plotted against the dose of responding cells in the first step culture, the experimental values followed the zero order term of the Poisson equation and closely fit a straight line with y-intercepts near the 1.0 value. Based on the zero order term of the Poisson equation, the data suggested single hit conditions and indicated that only one cell type was limiting for Il-2 production. As can be seen in Fig. 3 A, the HTLp frequencies, as calculated from the slope of the regression lines derived from two independent assay systems, were in close agreement to each other (1/1.185 vs. 1/1.576).

In parallel to the frequency analysis of HTLp limiting the Il-2 production, culture supernatants derived from the same first-step limiting dilution culture were also assayed for helper activity required for the induction of primary CTL responses. Individual 2°MLC supernatants were added to replicates of primary MLC containing ~10 CTLp per well reactive to H-2^d alloantigens. In the primary indicator, MLC either 2,000 positively selected Lyt-2^+ splenic T cells or 2 × 10^5 PNA$^+$ Lyt-123^+ thymocytes (25) were stimulated with 5 × 10^5 T cell-depleted H-2^d spleen cells. As depicted in Fig. 3 B, single hit conditions were revealed in both instances. The calculated frequencies of HTLp able to provide help for Lyt-2^+ T cell and PNA$^+$ thymocyte-derived primary CTL responses were 1/2,250 and 1/4,200, respectively.

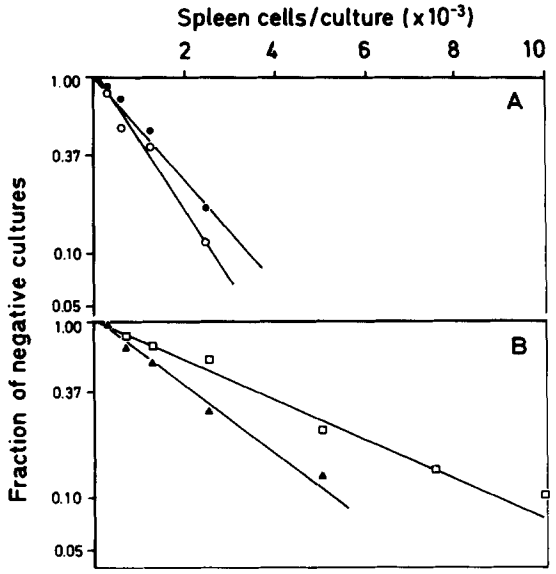


FIG. 3. HTLp frequencies as detected by different biological assay systems. Micro-MLC with limiting numbers of C57BL/6 responding cells and DBA/2-stimulating cells were set up as described in the legend to Fig. 2. (A) 2°MLC supernatants were tested on either 2°CTL, (●, f-1/1576) or CTLL (○, f-1/1185). (B) 2°MLC supernatants were tested for their capability to induce primary CTL responses in 2,000 C57BL/6-derived Lyt-2$^+$ T cells (▲, f-1/2258) or 2 × 10^5 PNA$^+$ thymocytes (□, f-1/4245).

As such, these values were approximately two- to threefold lower compared with those established for HTLp limiting the Il-2 production.

Frequencies and Lyt Phenotype of HTLp Reactive to Different Cell Surface Alloantigens. The experimental design in which limiting numbers of responding lymphoid cells were set up in a first step, followed by the assessment of Il-2 activity produced after restimulation in a second step (Fig. 3), was subsequently applied to enumerate the frequencies of Il-2-producing T cells reactive towards a variety of alloantigens. From the results summarized in Table II, it becomes apparent that the frequency of HTLp reactive to non-H-2 determinants encoded for by the Mls locus is high (1/500). Within the H-2 complex, H-2I region-encoded alloantigens stimulated approximately 10-fold more HTLp than did H-2K and H-2D-encoded alloantigens. It turned out that HTLp responding either to fully allogeneic stimulator cells or only to D region-incompatible stimulator cells expressed the Lyt-1$^+$ phenotype (Table III). The frequency of HTLp within the Lyt-23$^+$ T cell subset (Table III) was found to be negligible.

Specificity Analysis of Alloreactive HTLp. The next set of experiments addressed the antigen specificity of alloreactive HTLp. C57BL/6 spleen cells were sensitized under limiting dilution conditions towards T cell-depleted semiallogeneic (C57BL/6 × BALB/c)F_1 stimulator cells. After a 7 d culture period, each of the microcultures was split, washed, and restimulated with either T cell-depleted irradiated BALB/c (H-2^d), A.SW (H-2^s), or C57BL/6 cells. After 48 h, the culture supernatants of the individual microcultures were assayed for Il-2 activity using 2°CTL as indicator cells. The data obtained (Fig. 4) indicate that only after antigen-specific restimulation could high frequencies (1/4,500) of HTLp cells be detected. Restimulation with third-party

TABLE II

Frequencies of HTLp Limiting for Il-2 Production in Different Strain Combinations *

Responding spleen cells	Stimulating cells	Alloantigens	Number of determinations	Reciprocal of mean frequency (range)
C57BL/6	BALB/c	H-2^d + minor H	22	4,300 (2,910–9,191)
	DBA/2	H-2^d + Mls^a + minor H	13	1,800 (1,337–2,067)
B10	B10.D2	H-2^d	5	4,100 (2,502–6,981)
	DBA/2	H-2^d + Mls^a + minor H	3	1,900 (1,733–2,228)
	B10.BR	H-2^k	4	7,300 (6,919–9,556)
	AKR/Cum	H-2^k + Mls^a + minor H	3	2,800 (2,417–3,161)
B10.HTG	B10.D2	H-$2D^d$	3	42,000 (28,315–60,512)
B10.AQR	B10.A	H-$2K^k$	3	56,000 (40,607–72,557)
B10.AQR	B10.T (6R)	H-$2I^q$	3	9,300 (6,886–12,315)
BALB/c	DBA/2	Mls^a + minor H	3	550 (523–589)

* 2°MLC supernatants generated by limiting numbers of responding cells were assayed on 2°CTL for Il-2 levels. HTLp frequencies were calculated as detailed in Materials and Methods.

TABLE III

Lyt-phenotype of HTLp *

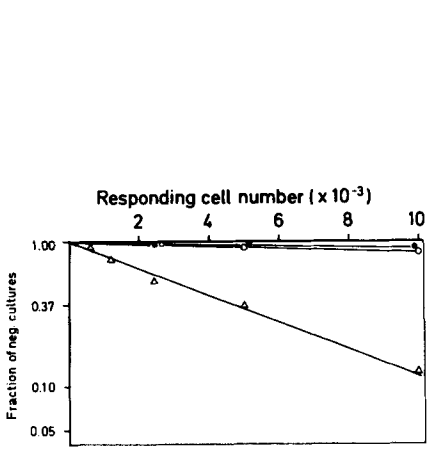
Responding cells	Treatment	Nucleated cell killing	Stimulating cells	Alloantigens	Reciprocal of frequency
		%			
C57BL/6	—	0	BALB/c	H-2^d + minor H	2.800
Splenic T‡	C	8			2.900
	Anti-Lyt-2.2 + C	43			1.800
	Anti-Lyt-2.2 + C	79			55.000
B10.HTG	—	0	B10.D2	H-$2D^d$	28.000
Splenic T	C	6			26.700
	Anti-Lyt-2.2 + C	46			18.000
	Anti-Lyt-1.2 + C	85			>100.000

* 2°MLC supernatants generated by limiting numbers of responding cells were assayed on 2°CTL for Il-2 levels. HTLp frequencies were calculated as detailed in Materials and Methods.

‡ Splenic T cells were prepared by the plastic dishes adherent techniques as described in Materials and Methods.

alloantigens or syngeneic cells yielded frequency values of ~1/60,000, suggesting that culture conditions did not induce substantial unspecific activation of HTLp, as could have occurred through mitogenic or antigenic components in FCS.

To ascertain the specificity of HTL at the single cell level, microcultures were established, each containing 1,000 C57BL/6 splenic responder cells and T cell-depleted BALB/c stimulator cells. In parallel, the HTLp frequency within the C57Bl/6 responder cells reactive towards H-2^d alloantigens was determined. From their frequency value it was deduced that, on average, at a responding cell dose of 1,000, >0.25 HTLp reactive towards H-2^d were present per microculture. Thus, the clone probability of each of the positive cultures was high (>88%) (13). We therefore considered them to be clones. After 7 d, the microcultures were split and restimulated either with the H-2^d alloantigens used for immunization or with third-party (H-2^s) alloantigens for 48 h. Subsequently, the supernatants of these cultures were tested for



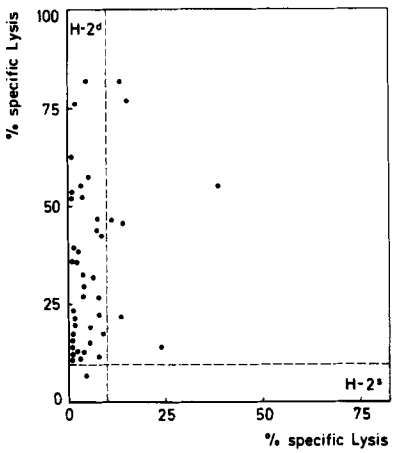


FIG. 4 (left). Specificity of H-2^d-induced C57Bl/6-derived HTL. Limiting numbers of C57BL/6 responding spleen cells were cultured with 5 × 10^5 T cell-depleted (C57BL/6 × BALB/c)F_1 stimulating cells. After 7 d, the cultures were split and restimulated with either (C57BL/6 × BALB/c)F_1- (△), A.SW- (○), or C57BL/6- (●) derived T cell-depleted spleen cells. 2°MLC supernatants were tested for Il-2 levels on 2°CTL. ●, C57BL/6, f-1/63.00; ○, ASW, f-1/59.00; △, (C57BL/6 × BALB/c)F_1, f-1/4.590.

FIG. 5 (right). Cross-reactivity of alloreactive HTL at the clonal level. 192 microcultures were set up containing 1,000 C57BL/6 spleen cells and 5 × 10^5 T cell-depleted BALB/c-stimulating cells. After 7 d, the cultures were split and restimulated with either BALB/c or A.SW-derived stimulating cells. For restimulation, 0.5% mouse serum was used in the culture medium. 2°MLC supernatants were assayed for Il-2 levels on 2°CTL. The dotted lines correspond to the mean of negative controls plus three standard deviations. Each point represents the specific ^{51}Cr release effected by 2°CTL, as induced by individual 2°MLC supernatants. n = 148.

TABLE IV

*Specificity Analysis of Helper T Cells at the Clonal Level**

1°MLC	Restimulating cells	Number of positive cultures/number tested	Percent specific	Percent cross-reactive
C57BL/6 α BALB/c	BALB/c	44/192 (46/192)‡	70.4 (76.0)	29.6 (24.0)
	BALB/k (H-2^k)	13/192 (11/192)		
	BALB/c	45/192 (56/192)	75.6 (83.9)	24.4 (16.1)
	A.SW (H-2^s)	11/192 (9/192)		
	BALB/c	36/192 (39/192)	83.3 (87.2)	16.7 (12.8)
	B10.G (H-2^q)	6/192 (5/192)		

* 10^3 C57BL/6 spleen cells were cultured with 5 × 10^5 T cell-depleted BALB/c-stimulating cells. After 7 d, the cultures were split and restimulated with the indicated T cell-depleted spleen cells. 2°MLC supernatants were assayed on 2°CTL for Il-2 levels.

‡ The values in brackets were obtained when 0.5% mouse serum was used in the culture medium for restimulation instead of 10% fetal calf serum.

Il-2 activity using 2°CTL as indicator cells. The reactivity pattern of single clones of HTL is shown in Fig. 5. Of the 44 positive clones in the group of 192 tested microcultures, 37 were H-2^d specific, while 7 clones reacted with both H-2^d and H-2^s. As can be seen from Table IV, the percentage of clones recognizing "third-party" alloantigens was dependent on the haplotype combinations used and ranged from 16% (H-2^q) to 29% (H-2^k). When the restimulation was performed in medium supplemented with 0.5% NMS instead of 10% FCS, the ratio of specific clones

increased (Table IV, values in brackets). These results indicated that, in the presence of FCS, the cross-reactivity pattern noted is in part the result of antigenic properties of the FCS batch used.

Discussion

A requirement of Lyt-1$^+$ HTL for the generation of alloreactive CTL is now well recognized (1–5). However, little information is available concerning their quantitative representation within nonsensitized responder T cells (17) or the specificity characteristics that helper T cells express at the clonal level. In the present study, we approached these questions by adopting the limiting dilution system for the quantitation of functionally active HTLp. Our results demonstrate three main points. First, a single type of HTLp limits the overall generation of helper activity and Il-2 production; second, within splenic responder T cells, this antigen-specific HTLp expresses the Lyt-1$^+$ phenotype and occurs at a 5- to 10-fold lower frequency compared with that of alloreactive CTLp; and third, ~80% of alloreactive HTL clones exhibit specificity for the H-2 gene products used for primary immunization, whereas ~20% are cross-reactive to third party alloantigens.

When limiting numbers of responder cells as source of HTLp were titrated in the presence of alloantigen on appropriate CTLp indicator cells, one single type of HTLp expressing the Lyt-1$^+$23$^-$ phenotype was found to be limiting for the generation of primary CTL responses (Fig. 1, Table I). Similar results were obtained by adding to the same alloantigen-stimulated CTLp indicator cells 2°MLC supernatants generated in individual microcultures under limiting dilution conditions (Fig. 3 B). These results are the first to establish that, in quantitative terms, soluble products derived from HTL mediate T help as efficiently as do HTLp themselves. Because almost equal frequency values were established in the two assay systems used (Fig. 3 B, Table I), it was concluded that in both instances one and the same HTLp represented the limiting cell type.

When 2°MLC supernatants generated under limiting dilution conditions were individually tested for their Il-2 contents, again, one single type of HTLp was found to be limiting for the Il-2 production. Although the Il-2 assay system revealed twofold higher frequency values compared with those obtained by testing the 2°MLC supernatants for helper activity, the HTLp limiting for Il-2 production seems to be identical with the one limiting the generation of primary CTL responses. This reasoning is based on the following considerations: provided the lower frequencies were to be attributed to a second HTLp involved in the production of soluble helper factors distinct from Il-2, the ratio between these two distinct HTLp would be ~1:2. Consequently, multihit curves should become apparent (26). Because we did not observe multihit curves, we conclude that the differences in frequencies reflect differences in sensitivity of the various assay systems used. In this context, some aspects of the recently formulated interleukin model (27), as basis of T-T cell interactions in primary CTL responses, needs to be mentioned. There is, for example, experimental evidence that Lyt-1$^+$ HTLp are in fact functionally heterogeneous (28), in the sense that Lyt-1$^+$ inducer T cells control Il-1 production by antigen-presenting macrophages (27, 29). Il-1, in turn, is required as a second signal for the antigen-specific activation of the Il-2-producing Lyt-1$^+$ HTLp (30, 31). If T-T cell interactions within the Lyt-1$^+$ subset were necessary for Il-2 production, the frequency values established here could

reflect the quantitative representation of either the Lyt-1$^+$-inducer T cells or the Lyt-1$^+$ Il-2-producing HTLp within the responder population. In any case, because in each of the three assay systems described one single HTLp turned out to be limiting and occurred in similar frequencies, we conclude that in all three instances one and the same HTLp was detected. It should be mentioned that none of our limiting dilution assays revealed multiphasic Poisson distribution curves as reported by Simon et al. (32) for alloreactive HTLp. It could be that multiphasic curves are confined to systems in which HTLp are polyclonally induced by Con A, and also that functionally distinct HTLp become limiting, some of which might be subject to suppressive mechanisms (32).

Interestingly, most, if not all, of the alloreactive HTLp-limiting Il-2 production expressed the Lyt-1$^+$ phenotype, even when stimulated against H-2D region-encoded alloantigen (Tables I and II). These data provide direct evidence that the repertoire of Lyt-1$^+$ HTLp includes reactivity towards K and D region-encoded (class I) alloantigens. As such, they do not support the conclusion that stimulation by class I alloantigens induces Lyt-1$^-$23$^+$ T cells to produce Il-2 (33). On the other hand, the quantitative representation of class II (I region)-reactive Lyt-1$^+$ HTLp exceeded approximately tenfold that of class I (K, D region)-reactive Lyt-1$^+$ HTLp (Table II). The low frequency of, for example, anti-H-2D region-reactive HTLp in splenic lymphocytes (Tables II and III) most likely explains the inefficiency of inducing in vitro CTL responses towards D region-incompatible stimulator cells, even though the frequency of antigen-reactive CTLp is high (34).

Our frequency values of HTLp is response to distinct alloantigens, as coded for by the H-2 complex of Mls locus, revealed a similar distribution pattern compared with that observed by Lutz et al. (16). Thus, strong Mls^a alloantigens stimulated more HTLp (1/500) than did H-2 alloantigens (Table II). Surprisingly, the frequency of HTLp responding to Mls^a determinants in the context of self-MHC was higher than those responding to Mls^a in the context with allogeneic MHC products (Table II). A straightforward explanation would be that BALB/c mice have higher numbers of HTLp responding to Mls^a determinants than do C57Bl/6 mice. Alternatively, Mls^a antigens might be recognized in an H-2-restricted manner (35) rather than H-2 independently (36). In the first instance, a higher frequency of HTLp responding to self plus Mls^a were to be expected.

Specificity analysis of clones of CTL induced in primary MLC revealed, first, that a stable specificity pattern existed during clonal expansion and, second, the existence of specific, cross-reactive, and heteroclytic CTL clones (13). Upon investigation of the antigen specificity of HTLp at the clonal level, the majority (~80%) of alloantigen-activated HTL clones proved to be specific to determinants expressed on alloantigens used for primary sensitization. A minority, ~10–20% of clones, were cross-reactive in the sense that they also produced Il-2 when restimulated with third-party alloantigens (Table IV). Even though some of the cross-reactive clones could, in fact, not be true clones (the clone probability was ~88%), the apparent cross-reactivity of the remaining clones could be caused by either specific recognition of shared (public) determinants on alloantigens or because of cross-reactive antigen receptors on HTL. These data parallel other observations concerning the cross-reactivity of alloreactive proliferative and cytolytic T cells (13, 37).

Recently it has been demonstrated, at the clonal level, that the sets of alloreactive

and H-2-restricted antigen-specific cytolytic and proliferative T cells do overlap (12, 15, 34, 38). In fact, alloantigen and H-2-restricted antigen recognition might not be mediated by functionally different T cell subsets, but might reflect recognition by cross-reactive T cells. To analyze the relationship between H-2-restricted and alloreactive sets of HTL, we are currently extending our limiting dilution analysis of TNP and virus-specific, H-2-restricted HTL.

Summary

We assessed the quantitative representation and specificity of alloreactive helper T lymphocytes (HTL) within murine spleen cells by three different limiting dilution systems. For the induction of primary cytolytic T lymphocyte (CTL) responses towards alloantigens, a Lyt-1^+23^- HTL precursor (HTLp) could be defined, which occurred at frequencies of 1/2.000–1/50,000, depending on the alloantigen in question. The HTLp limiting for interleukin-2 (Il-2) production also expressed the Lyt-1^+ phenotype and occurred in similar frequencies. This cell type was concluded to be the limiting HTLp for the overall helper activity required for the induction of primary CTL responses.

HTLp reactive to Mls^a-encoded antigens occurred at higher frequencies (1/500) than those reactive towards whole allogeneic H-2 haplotypes (1/4,000–1/7,000). Within the H-2 complex, I region-encoded alloantigens activated ~10 times more HTLp than did H-2K or H-2D regions.

When alloreactive HTL were tested for antigen specificity at the clonal level, ~80% of the HTL clones proved to be specific to the alloantigen used for immunization, whereas ~20% reacted also towards third-party alloantigens. The data are discussed with respect to putative T-T interactions within the helper T cell population and the precision of alloantigen recognition by HTL.

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