CLONAL ANALYSIS OF CYTOLYTIC T LYMPHOCYTE SPECIFICITY

I. Phenotypically Distinct Sets of Clones as the Cellular Basis of Cross-Reactivity to Alloantigens

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Cytolytic T lymphocytes (CTL)[1] recognize a wide variety of antigens cross-reacting with those against which the immune population was originally sensitized (1). Definitive analysis of this cross-reactivity requires examination of the killing specificity exhibited by CTL at the single-cell level rather than at the population level. Such an examination would enable the investigator to determine whether cross-reactivity is the result of (*a*) a homogeneous population of killer cells with each cell possessing multiple specificities, (*b*) a heterogeneous population of killer cells with each cell possesing a single specificity, or (*c*) a more complex combination of both of these possibilities. Although attempts have been made to investigate cross-reactivity by cold target-cell inhibition of CTL in immune populations (2), this method cannot distinguish between the alternative models for the cellular basis of cross-reactivity. Furthermore, despite the availability of a micromanipulation method theoretically capable of determining the specificity of individual CTL (3), it is not readily amenable to the examination of large numbers of cells.

Investigators interested in the cellular basis of cross-reactivity have therefore been obligated to turn from the formal examination of individual CTL to the more practical examination of clones of CTL. These CTL clones can be generated under limiting-dilution conditions in mixed leukocyte microculture (micro-MLC) systems that have been recently developed by several groups (4–7). These systems provide an efficient method for the routine examination of large numbers of clones sufficiently active to be divided for assays against two or more target cells. Thus they are highly amenable to the analysis of specificity and cross-reactivity. However, clones generated in these systems are only theoretically derived from single CTL precursor cells (CTL-P) and are only believed to remain homogeneous throughout clonal expansion. Because these unproven assumptions can severely limit the interpretation of many results, we decided to test them and the consequent validity of this general method for the analysis of specificity and cross-reactivity.

With these considerations in mind, we took advantage of the extremely high frequency (up to 25%) of CTL-P in primary mixed-leukocyte culture (MLC) popu-

[1] *Abbreviations used in this paper:* CTL, cytolytic T lymphocyte(s); CTL-P, CTL precursor cell(s); DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; micro-MLC, mixed-leukocyte microculture(s); MLC, mixed-leukocyte culture(s); SN, supernate(s); 1°, primary; 2°, secondary.

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lations (8, 9) to use a responding-cell dose of 1 cell/well in micro-MLC of two different strain combinations chosen as a model of alloreactivity. We assayed these micro-MLC against specific and third-party target cells, selected individual clones, subcloned them, and assayed the subclones against the same target cells. In this report, we present results from these experiments to provide evidence for the short-term stability of the specificity phenotype of selected clones during subcloning and, consequently, for the existence of three sets of CTL (specific, heteroclitic, and cross-reactive) as the cellular basis of cytolytic cross-reactivity in MLC populations assayed against two different target cells.

Materials and Methods

Mice. Adult mice of the inbred strains C57BL/6, DBA/2, and C3H/He were obtained from the animal colony maintained at the Swiss Institute for Experimental Cancer Research, Lausanne, Switzerland.

Preparation of Supernate from Secondary MLC. As detailed elsewhere (10), secondary (2°) MLC supernate (SN) was prepared by mixing 10×10^6 viable cells recovered from pools of 10- to 14-d-old primary C57BL/6 anti-DBA/2 MLC with 40×10^6 irradiated (2,000 rad) DBA/2 spleen cells in 10 ml of culture medium in small tissue culture flasks (25 cm^2; 1,461, Nunc, Roskilde, Denmark). Alternatively, it was prepared by mixing 30×10^6 MLC cells with 120×10^6 irradiated DBA/2 spleen cells in 30 ml of culture medium in large tissue culture flasks (75 cm^2; 3,024, Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, Calif.). SN were collected after 24 h of incubation and sterilized by filtration.

MLC. Cultures were established in Dulbecco's modified Eagle's medium (DMEM) supplemented as described previously (11) with additional amino acids, 10 mM Hepes, 5×10^{-5} M 2-mercaptoethanol, and 2% fetal bovine serum (FBS). Primary mass cultures were prepared as described previously (11) by mixing 25×10^6 responding C57BL/6 spleen leukocytes with 25×10^6 stimulating DBA/2 or C3H/He irradiated (2,000 rad) spleen leukocytes in 15 ml of culture medium in tissue culture flasks (1,461, Nunc). 2° microcultures for the cloning of CTL-P and tertiary microcultures for the subcloning of clones were prepared as described previously for primary microcultures (7). Briefly, limiting numbers of viable 5- to 14-d-old primary MLC or of 10- to 13-d-old 2° micro-MLC cells were mixed with 1×10^6 irradiated (2,000 rad) spleen leukocytes of the same strain as the original stimulating cells in culture medium further supplemented with 10% (vol/vol) FBS and 50% (vol/vol) 2° MLC SN in a final vol of 0.2 ml in round-bottomed microwells (Greiner, Nurtingen, Federal Republic of Germany). Plates were wrapped in aluminum foil to minimize evaporation. All cultures were maintained at 37°C in a water-saturated atmosphere of 5% CO_2 in air.

Target Cells. P815 mastocytoma cells of DBA/2 origin and AKRA lymphoma cells of AKR origin were maintained in culture and labeled with $Na_2\ ^{51}CrO_4$ as described previously (11). Labeled cells were washed three times and resuspended at a concentration of 15×10^3/ml in DMEM supplemented with 5% (vol/vol) FBS and 10 mM Hepes buffer.

Assay for Cytolytic Activity of micro-MLC. A modification of the ^{51}Cr-release assay (11) was used to determine CTL activity. After 7 d of culture, 0.06 ml of resuspended cells from each microculture was transferred to the corresponding microwells of each of two additional assay plates, leaving 0.08 ml of the microculture in the original culture plate for later use. 0.14 ml of ^{51}Cr-labeled target cells (P815 or AKRA) was then added to each pair of assay plates. The plates were centrifuged and then incubated for 3–6 h at 37°C. Cytolysis was assessed by counting the radioactivity of 0.10–0.15 ml of SN for 0.5–1.0 min in a well-type scintillation counter. For the determination of spontaneous release, control microcultures that contained irradiated stimulating cells, 2° MLC SN, and FBS in the absence of responding cells 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 (11).

Calculation of CTL-P Frequencies and Clone Probabilities. Minimal estimates of CTL-P frequencies in cell populations were calculated by analysis of the Poisson distribution relationship

between the number of responding leukocytes per microculture and the fraction of cytolytically negative microcultures per group. In particular, the zero-order term Poisson equation (12) was linearized to the form, $\ln y = -fx + \ln a$, where x is the number of responding leukocytes, y is the fraction of negative microcultures, f is the CTL-P frequency equal to the negative of the slope, and a is the y-axis intercept theoretically equal to 1. Experimental x- and y-values were fitted to this equation by the least squares method. Results were reported as the three 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 has been published elsewhere (13). The probability of each positive microculture being a clone derived from a single CTL-P was calculated as the ratio of the probability of 1 CTL-P/microculture to the probability of ≥1 CTL-P/microculture where both individual probabilities are calculated according to the Poisson equation for a given frequency and responding-cell dose. In particular, if

$$P_n(f,x) = \frac{(fx)^n \cdot \exp(-fx)}{n!},$$

where $P_n(f,x)$ is the probability of n CTL-P per microculture for a given frequency f of CTL-P and dose x of responding cells, then

$$P_{clone} = \frac{P_1(f,x)}{1 - P_0(f,x)} = \frac{fx \cdot \exp(-fx)}{1 - \exp(-fx)},$$

where P_{clone} is the desired probability of being a clone.

Results

Limiting Dilution Analysis of Populations Derived from Primary MLC: CTL-P Frequency and Microculture Clone Probability. Limiting numbers of viable responding leukocytes from primary C57BL/6 anti-DBA/2 MLC and 1×10^6 irradiated DBA/2 stimulating spleen leukocytes were cultured for 7 d in the presence of 50% 2° MLC SN and 10% FBS. Each microculture was then divided and assayed separately for CTL activity against both P815 (DBA/2) and AKRA (AKR) ^{51}Cr-labeled target cells. Micro-MLC were defined as positive when the ^{51}Cr release exceeded the mean spontaneous ^{51}Cr release by at least 3 SD. Table I presents the results from a representative limiting-dilution analysis of a primary (1°) C57BL/6 anti-DBA/2 MLC population. A dose-response effect can be clearly seen where the percentage of cytolytically positive microcultures ranged from 100% at a dose of 15 responding cells to 42% at 1 responding cell for activity against P815 target cells and from 50% at a dose of 15 to 21% at 1 for activity against AKRA. The mean specific ^{51}Cr release for all microcultures positive for P815 target cells was 47% where the minimum positive specific ^{51}Cr release was 9%, whereas for AKRA these values were 17 and 5%, respectively. The logarithm of the fraction of cytolytically negative microcultures was then plotted against the dose of responding cells according to Poisson statistics, and curves were fitted by the least squares method. Fig. 1 shows linear regression curves for the results presented in Table I. The CTL-P frequencies were determined by the slopes of the linear regression curves. For this representative 1° C57BL/6 anti-DBA/2 MLC population, a CTL-P frequency of 29.7% (1/3.4), an a value of 0.94, and an r^2 value of 0.96 were determined for reactivity against P815, whereas for reactivity against AKRA, these values were 2.96% (1/34), 0.84, and 0.81, respectively. The clone probability of each positive microculture was then calculated according to Poisson statistics as a function of the CTL-P frequency and the responding-cell dose. Table I presents the clone probability of microcultures at each dose of responding cells based

TABLE I

*Limiting Dilution Analysis of a Population Derived from 1° C57BL/6 Anti-DBA/2 MLC**

Responding-cell dose	Number of positive micro-MLC (mean percent specific ^{51}Cr release)		Clone probability‡	
	P815	AKRA	P815	AKRA
			%	
1	10 (20)	5 (8)	86	99
5	17 (47)	7 (25)	43	93
10	23 (46)	7 (20)	16	86
15	24 (59)	12 (14)	5	79

* Groups of 24 microcultures that contained the indicated number of viable responding cells from 1° C57BL/6 anti-DBA/2 MLC and 1×10^6 irradiated DBA/2 stimulating spleen cells were established for 7 d in the presence of 50% 2° MLC SN and 10% FBS. Each microculture was then divided and assayed separately for CTL activity against both specific P815 mastocytoma (DBA/2) and third-party AKRA lymphoma (AKR) target cells. Micro-MLC were defined as positive when the ^{51}Cr release exceeded the mean spontaneous ^{51}Cr release (determined in the absence of responding cells) by at least 3 SD. For P815, the spontaneous release was 102 cpm (10% of the maximum release) and the minimum positive specific release was 9%. For AKRA, the spontaneous release was 148 cpm (8% of the maximum release) and the minimum positive specific release was 5%.

‡ The probability that microcultures were clones was calculated (Materials and Methods) according to Poisson statistics as a function of the P815- and AKRA-reactive CTL-P frequencies (assuming an independent distribution) for this population (determined in Fig. 1) and of the indicated responding-cell dose.

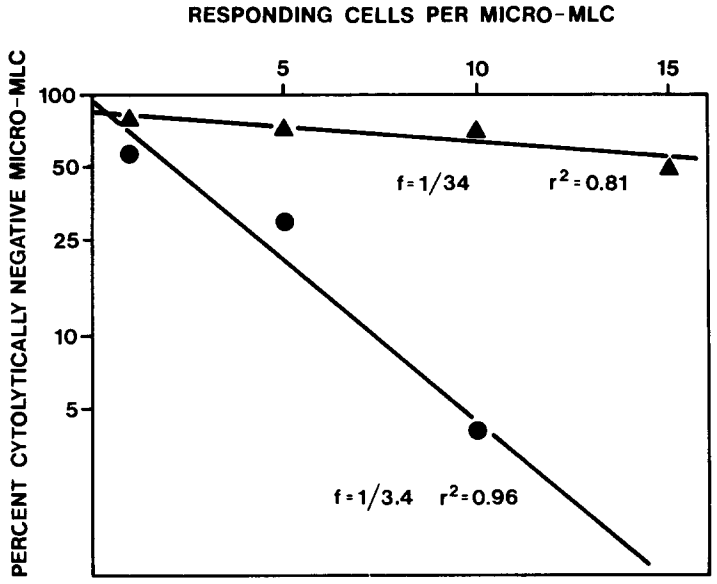

FIG. 1. P815- and AKRA-reactive CTL-P frequencies in 1° C57BL/6 anti-DBA/2 MLC. Data from Table I were fitted by the least squares method to the linearized Poisson equation, $\ln y = -fx + \ln a$, where x is the number of responding cells per micro-MLC, y is the fraction of cytolytically negative micro-MLC per group, 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. (●) P815, $a = 94$; (▲) AKRA, $a = 84$.

upon the CTL-P frequencies for reactivity against both P815 and AKRA as determined by the curves in Fig. 1. When based upon the frequency of 1/3.4 for P815 reactivity, the clone probability for microcultures from this 1° C57BL/6 anti-DBA/2 MLC population rapidly decreased from 86% at a responding-cell dose of 1 to 5% at a dose of 15, whereas when based upon the frequency of 1/34 for AKRA reactivity, it only decreased from 99% at 1 to 79% at 15.

Specificity Analysis of CTL Clones: Phenotype Distribution. In conjunction with the preparation and examination of small groups of microcultures for the determination of CTL-P frequencies as described above, a large group of microcultures was similarly and simultaneously established at a responding-cell dose of 1 and assayed for the determination of the specificity of each individual microculture. The CTL activity of each microculture against both P815 and AKRA was thus plotted on a two-dimensional graph of percent specific ^{51}Cr release (P815 vs. AKRA). Fig. 2 shows the activities of the 91 microcultures that were cytolytically positive for either one or both of the targets in a group of 288 microcultures derived from the same 1° C57BL/6 anti-DBA/2 MLC population for which CTL-P frequencies and clone probabilities were presented in Fig. 1 and Table I. Because the clone probability of microcultures derived from this population at a responding-cell dose of 1 was very high (86 and 99% when based, respectively, upon the P815- and AKRA-reactive CTL-P frequencies), we considered them to be clones. Henceforth labeled as such, these clones were then classified according to their specificity phenotype as positive for P815 and negative for AKRA ($P815^+AKRA^-$), negative for P815 and positive for AKRA ($P815^-AKRA^+$), or positive for both ($P815^+AKRA^+$), and the phenotype distribution was calculated. Thus, of the 91 positive clones in the group of 288 microcultures tested, 75% were $P815^+AKRA^-$ or specific with a mean specific ^{51}Cr release of 34%, 15% were $P815^-AKRA^+$ or heteroclitic with a mean specific ^{51}Cr release of 11%, and 10% were $P815^+AKRA^+$ or cross-reactive with a mean specific ^{51}Cr release of 40% on P815 and 15% on AKRA. The minimum positive specific ^{51}Cr release was the same as reported for Table I, namely, 9% for P815 and 5% for AKRA. This representative 1° C57BL/6 anti-DBA/2 MLC population, analyzed as demonstrated by Table I and Figs. 1 and 2, is listed as population II in Table II, which summarizes the phenotype distributions of clones from this and four other 1° C57BL/6 anti-DBA/2 MLC and two 1° C57BL/6 anti-C3H/He MLC populations. For the C57BL/6 anti-C3H/He populations, $P815^+AKRA^-$ becomes the heteroclitic phenotype and $P815^-AKRA^+$ becomes the specific phenotype because C57BL/6 anti-C3H/He constitutes the strain combination reciprocal to C57BL/6 anti-DBA/2 in the model system used for this investigation. For the five different 1° C57BL/6 anti-DBA/2 MLC populations, the overall phenotype distribution of the 211 positive clones among the 1,404 microcultures tested was 81% specific, 9% heteroclitic, and 9% cross-reactive; whereas for the two different C57BL/6 anti-C3H/He populations, the distribution of the 76 positive clones among the 360 microcultures tested was 80% specific, 13% heteroclitic, and 7% cross-reactive. A chi-square test with a 2×2 contingency table for the independent association of CTL-P reactive against P815 and AKRA resulted in rejection of the hypothesis at the 0.05% level of significance ($P << 0.0005$) for the 1,404 C57BL/6 anti-DBA/2 microcultures.

Specificity Analysis of CTL Subclones: Phenotype Distribution. After specificity analysis of CTL clones as described above, individual clones were selected for subcloning and

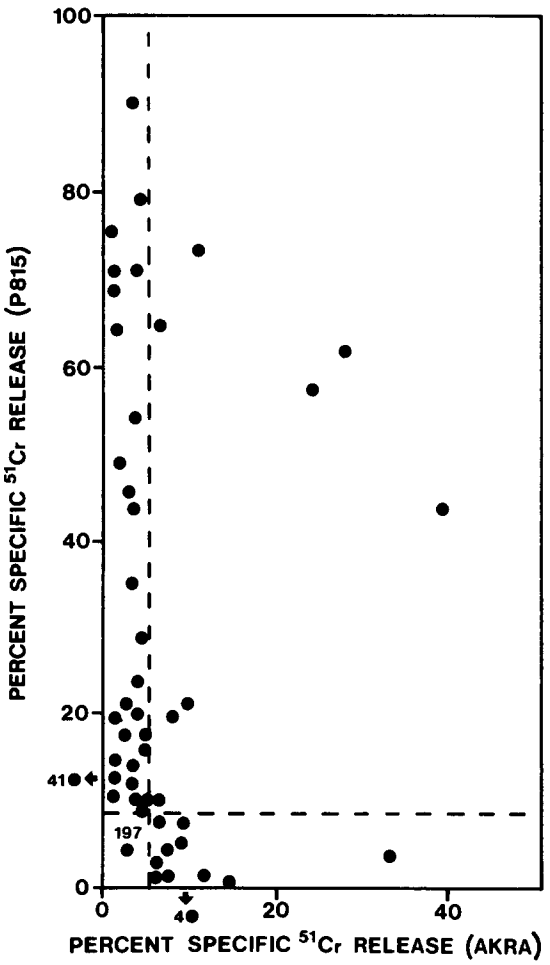

FIG. 2. Specificity of CTL clones derived from a 1° C57BL/6 anti-DBA/2 MLC population. A group of 288 micro-MLC was established and assayed similarly and simultaneously to those of Table I and derived from the same population as analyzed in Table I. Because the responding-cell dose of this group was 1 and the clone probability was therefore 86–99% (Table I), the 91 cytolytically positive microcultures were considered clones. Of these clones, 75% were P815$^+$AKRA$^-$ or specific with a mean specific ^{51}Cr release of 34%, 15% were P815$^-$AKRA$^+$ or heteroclitic with a mean specific ^{51}Cr release of 11%, and 10% were P815$^+$AKRA$^+$ or cross-reactive with a mean specific ^{51}Cr release of 40% on P815 and 15% on AKRA. The minimum positive specific ^{51}Cr release was the same as reported for Table I.

assaying by the same procedure by which they were generated and detected. Limiting numbers (3–12) of viable responding leukocytes from individual clones and 1×10^6 irradiated stimulating spleen leukocytes of the same strain as the original stimulating cells were cultured for 7 d in the presence of 50% 2° MLC SN and 10% FBS. Each microculture was then divided and assayed separately for CTL activity against both P815 and AKRA target cells. Because these microcultures are derived from clones, they will henceforth be labeled as subclones despite the absence of a clone probability calculation analogous to that for the original clones. Specificity analysis was then completed in a manner similar to that done for the original clones. Thus, Fig. 3 shows

TABLE II

*Phenotype Distributions of CTL Clones Derived from 1° C57BL/6 Anti-DBA/2 and C57BL/6 Anti-C3H/He MLC Populations**

Responding population			Cytolytically positive clones				
1° MLC combination	Identification number	Total number/ number tested	Minimum positive percent specific ^{51}Cr release		Phenotype distribution‡ (mean percent specific ^{51}Cr release)		
			P815	AKRA	P815$^+$AKRA$^-$	P815$^-$AKRA$^+$	P815$^+$AKRA$^+$
			%				
C57BL/6 anti-DBA/2	I	14/72	8	4	Specific 71 (45)	Heteroclitic 21 (29)	Cross-reactive 7 (71; 24)
	II§	91/288	9	5	75 (34)	15 (11)	10 (40; 15)
	III	18/468	33	20	89 (43)	0	11 (41; 27)
	IV	43/288	19	17	86 (38)	5 (24)	9 (45; 44)
	V	45/288	24	15	89 (45)	2 (39)	9 (49; 41)
C57BL/6 anti-C3H/He	VI	6/72	4	7	Heteroclitic 50 (8)	Specific 33 (39)	Cross-reactive 17 (6; 8)
	VII	70/288	16	27	10 (35)	84 (44)	6 (42; 43)

* CTL clones from the indicated 1° MLC populations were generated, detected, and analyzed as explained in Table I and Fig. 2. All microcultures were established at a responding-cell dose of 1. The mean probability that the 211 cytolytically positive microcultures in the C57BL/6 anti-DBA/2 combination were clones was 88% (range: 86–95%) based on the P815-reactive CTL-P frequency and 99% (range: 97–100%) based on the AKRA-reactive CTL-P frequency. For the 76 cytolytically positive microcultures in the C57BL/6 anti-C3H/He combination, these probability values were 100% (97–100%) based on P815 and 95% (95–97%) based on AKRA.

‡ Expressed as percentage of the total number of cytolytically positive clones.

§ Activities of the individual clones derived from this population are plotted in Fig. 2.

the activities of cytolytically positive subclones derived from a specific clone, a heteroclitic clone, and a cross-reactive clone of C57BL/6 anti-DBA/2 origin, and Table III summarizes the phenotype distributions of subclones derived from these three clones (I-1, I-3, and I-4) and eight other clones also of C57BL/6 anti-DBA/2 origin. Examination of the phenotype distribution of subclones for each clone indicates that the majority of clones retain their specificity phenotype during the generation of subclones. For example, of 35 positive subclones among the 36 microcultures derived from the specific clone I-1, 100% remained specific with a mean specific ^{51}Cr release of 58%. Exceptions to this general result may be explained by a rare event as in the case of the 2% (1/41) of the subclones derived from the heteroclitic clone I-3, which became specific. They may also be explained by the deficiencies of the detection limits in the system as in the case of the 4 and 2% of the subclones derived from the cross-reactive clone I-4, which became, respectively, specific and heteroclitic with mean specific ^{51}Cr-release values of 6 and 3%. The minimum positive specific ^{51}Cr release values for these subclones were 5% on P815 and 3% on AKRA. Table IV summarizes the phenotype distributions of subclones derived from clones of C57BL/6 anti-C3H/He origin. Although the same general result was obtained as for subclones derived from clones of C57BL/6 anti-DBA/2 origin, the subclones derived from heteroclitic and cross-reactive clones of C57BL/6 anti-C3H/He origin exhibited less stability of

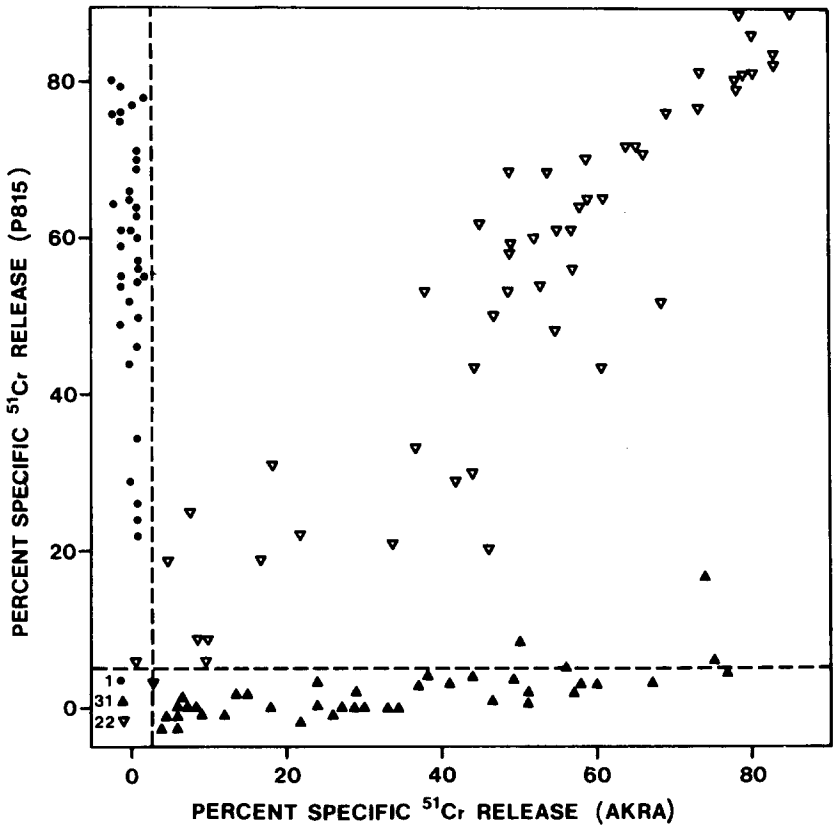

FIG. 3. Specificity of CTL subclones derived from specific, heteroclitic, and cross-reactive C57BL/6 anti-DBA/2 clones. Subclones were generated at a responding-cell dose of 3–12 under the same microculture conditions and detected by the same assay procedure as for the clones from which they were derived (Table I). (●) Subclones derived from a specific clone with a specific ^{51}Cr release of 70% on P815 and 0% on AKRA; (▲) subclones derived from a heteroclitic clone with a specific ^{51}Cr release of 0% on P815 and 63% on AKRA; (▽) subclones derived from a cross-reactive clone with a specific ^{51}Cr release of 71% on P815 and 24% on AKRA. The minimum positive specific ^{51}Cr release for these clones was 8% on P815 and 4% on AKRA. The minimum positive specific ^{51}Cr release for the subclones plotted here was 5% on P815 and 3% on AKRA.

specificity phenotype. A total of 37 clones was subcloned, of which only the 22 (59%) summarized in Tables III and IV generated at least 1 cytolytically positive subclone.

Size of Clones. After being divided on day 7 and immediately before subcloning on days 10–13, the above 37 clones were counted and found to have a mean size of 39 $\times$ 10^3 cells (486 $\times$ 10^3 cells/ml $\times$ 0.08 ml) with a 31 $\times$ 10^3 SD and a range from 5 $\times$ 10^3 to 166 $\times$ 10^3 cells. Five control microcultures that lacked responding cells (used for the determination of spontaneous ^{51}Cr-release values) were likewise counted and found to have a mean size of 16 $\times$ 10^3 cells. If a simple volume correction is made without an accompanying time correction for growth or death of the cells between the microculture split on day 7 and the cell count on days 10–13, these mean sizes become 97 $\times$ 10^3 and 41 $\times$ 10^3 cells, respectively. If the control microculture size is then subtracted from the clone size, the resultant estimate of the actual clone size is

TABLE III

*Phenotype Distributions of CTL Subclones Derived from Specific, Heteroclitic, and Cross-Reactive C57BL/6 Anti-DBA/2 Clones**

Original clone				Cytolytically positive subclones			
Phenotype	Identification number	Percent specific ^{51}Cr release		Total number/ number tested	Phenotype distribution‡ (mean percent specific ^{51}Cr release)		
		P815	AKRA		$P815^+$ $AKRA^-$ specific	$P815^-$ $AKRA^+$ heteroclitic	$P815^+AKRA^+$ cross-reactive
		%					
$P815^+AKRA^-$ specific	II-1	79	4	42/48	100 (71)	0	0
	II-2	90	3	48/60	100 (67)	0	0
	I-1§	70	0	35/36	100 (58)	0	0
	II-3	75	0	67/84	100 (44)	0	0
	II-4	69	0	1/84	100 (32)	0	0
	II-5	72	0	1/36	100 (32)	0	0
	II-6	71	1	1/60	100 (21)	0	0
	I-2	86	0	22/72	64 (19)	5 (3)	32 (54; 6)
$P815^-AKRA^+$ heteroclitic	I-3§	0	63	41/72	2 (30)	88 (30)	10 (9; 64)
$P815^+AKRA^+$ cross-reactive	II-7	43	39	2/36	0	0	100 (27; 32)
	I-4§	71	24	52/72	4 (6)	2 (3)	94 (55; 52)

* Subclones were generated and detected as explained in Fig. 3 and were then classified according to their phenotype. The roman and arabic numerals of each clone identification number represent, respectively, the population from which the clone was derived (Table II) and the particular clone itself. The minimum positive specific ^{51}Cr-release values for the clones are listed in Table II. For subclones derived from clones of population I origin, these values were 5% on P815 and 3% on AKRA; and for subclones derived from clones of population II origin, they were 12% on P815 and 16% on AKRA.

‡ Expressed as percentage of the total number of cytolytically positive subclones.

§ Activities of the individual subclones derived from these clones are plotted in Fig. 3.

56×10^3 cells corrected for the presence of stimulating cells. This estimated clone size corresponds to ~16 divisions with a doubling time of 11 h for the 7-d incubation.

Discussion

We have investigated the cytolytic cross-reactivity observed in 1° allogeneic MLC populations assayed against two different target cells and concluded that three phenotypically distinct sets of CTL clones constitute its cellular basis. In two different strain combinations (C57BL/6 anti-DBA/2 and C57BL/6 anti-C3H/He) assayed against both specific and third-party target cells (P815 and AKRA), we observed specific, heteroclitic, and cross-reactive micro-MLC with a mean frequency of 81, 11, and 8%, respectively. These results are consistent with the work of Lindahl and Wilson (6), who first demonstrated the existence of specific, heteroclitic, and cross-reactive micro-MLC in an allogeneic model system (C3H/He anti-C57BL/6 and C3H/He anti-DBA/2 assayed against EL4 and P815) and suggested that their presence constitutes the cellular basis of cross-reactivity.

Because it is possible that the existence of cross-reactive micro-MLC may be a result of the independent association of two precursor cells with unique specificities rather

TABLE IV

*Phenotype Distributions of CTL Subclones Derived from Specific, Heteroclitic, and Cross-Reactive C57BL/6 Anti-C3H/He Clones**

Original clone				Cytolytically positive subclones			
Phenotype	Identification number	Percent specific ^{51}Cr release		Total number/number tested	Phenotype distribution‡ (mean percent specific ^{51}Cr release)		
		P815	AKRA		P815$^+$ AKRA$^-$ heteroclitic	P815$^-$ AKRA$^+$ specific	P815$^+$ AKRA$^+$ cross-reactive
		%					
P815$^+$AKRA$^-$ heteroclitic	VI-1	16	6	71/84	32 (20)	0	68 (31; 15)
P815$^-$AKRA$^+$ specific	VII-1	0	100	51/84	0	100 (40)	0
	VII-2	0	56	32/48	0	100 (38)	0
	VII-3	1	56	28/48	0	100 (35)	0
	VII-4	0	81	8/96	0	100 (19)	0
	VI-2	2	26	12/84	0	100 (18)	0
	VII-5	0	57	1/48	0	100 (15)	0
	VII-6	0	57	1/48	0	100 (15)	0
	VI-3	3	52	10/84	0	90 (10)	10 (7; 76)
P815$^+$AKRA$^+$ cross-reactive	VII-7	51	42	37/84	0	22 (20)	78 (46; 46)
	VI-4	6	8	4/84	0	100 (11)	0

* Subclones were generated and detected as explained in Fig. 3 (with the substitution of C3H/He for DBA/2 as the irradiated stimulating cells) and were then classified according to their phenotype. The roman and arabic numerals of each clone identification number represent, respectively, the population from which the clone was derived (Table II) and the particular clone itself. The minimum positive specific ^{51}Cr-release values for the clones are listed in Table II. For subclones derived from clones of population VI origin, these values were 4% on both P815 and AKRA; and for subclones derived from clones of population VII origin, they were 29% on P815 and 13% on AKRA.

‡ Expressed as percentage of the total number of cytolytically positive subclones.

than the presence of a single precursor cell with cross-reacting specificities as suggested by Lindahl and Wilson (6), we applied the chi-square test for the independent association of frequencies to the results obtained above in the C57BL/6 anti-DBA/2 strain combination. This test resulted in rejection of the hypothesis of independent association at the 0.05% level of significance ($P << 0.0005$). Thus, our results are not in agreement with those of Teh et al. (14–16), who concluded that the hypothesis of independent association was valid for their allogeneic model system (RNC anti-B6D2F$_1$ assayed against EL4 and P815). Whereas these two conflicting interpretations can be ascribed to a failure to apply appropriate statistical tests, it is also possible that any differences in results, if real, may be a result of the basic difference between the two types of allogeneic model systems (single haplotype-stimulating cells vs. double-haplotype F$_1$ hybrid-stimulating cells). In this context, we note that evidence both for and against the hypothesis of independent association of CTL-P with different reactivities has been obtained by several other groups with a variety of systems (17–24). These discrepancies may derive directly from the presence or absence of true clonal generation in any particular micro-MLC system. Thus, they serve to demonstrate the significance of the theoretical deficiencies inherent in these systems and the

necessity of imposing limitations upon interpretations of results until the assumption of clonal generation is proven for each individual system.

We sought, therefore, to confirm by more direct means the validity of this general method of specificity and cross-reactivity analysis in our micro-MLC system. Direct confirmation of the validity of any limiting dilution system and of the interpretation of results obtained from it necessitates verification of the two critical assumptions upon which it is based, namely, that each micro-MLC constitutes a clone derived from a single CTL-P and that the specificity of all CTL in each clone remains homogeneous throughout the clone's expansion. Thus, we established micro-MLC at an actual responding-cell dose of 1 cell/well rather than a responding-cell dose calculated to result in 1 CTL-P/well, calculated the probability (based upon the CTL-P frequencies) that these micro-MLC were clones, and then subcloned the clones to determine whether or not their specificity phenotype was stable.

We generated 287 cytolytically positive micro-MLC with the specificity phenotype distributions discussed above and mean clone probabilities of 90 and 99% when based, respectively, upon the frequencies of CTL-P reactive against the specific and third-party target cells. We successfully subcloned 22 clones, of which 20 retained and 2 changed their specificity phenotypes. We discounted these two exceptions (clones VI-1 and VI-4) to the general finding of phenotype stability because the CTL activities of these two clones were very low and close to the detection limits of the assay. Furthermore, the phenotype changes of these two clones consisted of what can be considered variations to and from the cross-reactive phenotype rather than a complete change between the specific and heteroclitic phenotypes. Finally, the mean clone probabilities for this group of 22 clones (93 and 99%, as explained above) indicates that ~1 or 2 of them should in fact not be true clones. Therefore, to the extent to which the 20 clones represented the behavior in general of CTL clones induced in micro-MLC, we concluded that the specificity phenotype of the clones remained stable for at least the time required to generate and detect subclones.

We emphasize that the conclusion of three operationally distinct and phenotypically stable sets of CTL remains valid only as defined by the cytolytic activity of clones derived from the primary allogeneic MLC populations tested, assayed against the two target cells tested, and for the time period tested. True specificities, absolute phenotype stability, and maximal numbers of distinct sets of CTL can only be determined by more sophisticated analyses with a large variety of target cells tested for extended periods of time. Despite these qualifications, we do note that the majority of work on the specificity phenotype of long-term CTL lines (25) supports our conclusion of short-term stability and the consequent validation of the method used for the clonal analysis of specificity and cross-reactivity.

In addition to the main issues discussed above, we were also interested in aspects of the clones relevant to their further amplification and applications. We thus estimated the size of clones to be $\sim 6 \times 10^4$ cells as determined by direct counts corrected for the presence of stimulating cells and the day 7 microculture volume split. This value corresponds to ~16 cell doublings during the 7 d of culture. With other methods, Teh et al. (5) estimated 10 cell doublings, also for a 7-d culture period, whereas Lindahl and Wilson (6) estimated a minimum of 3–4 cell doublings for a 6-d culture period. These differences could be because of differences in methods of clone size estimation. For example, Teh et al. (5) considered only CTL rather than all cells of the clone.

However, it is also possible that differences in clone sizes may be attributed to the influence of the different T lymphocyte growth factors used in each micro-MLC system. We observed, furthermore, that the mere existence of a relatively large clone (6×10^4 cells) was not sufficient to ensure successful subcloning because the subcloning efficiency was only 59% (22/37). These questions of clone size and plating efficiency (for the maintenance or subcloning of clones) should be investigated further. More knowledge about these practical aspects of the growth of CTL clones could accelerate the development of their use in other areas such as idiotype analysis. The generation of clones of defined specificity in micro-MLC systems would then ultimately prove to be most productive for the further elucidation of the acquisition and maintenance of the CTL repertoire as well as the physical mechanisms of CTL induction by antigen and target cell destruction by CTL.

Summary

The cellular basis of the cytolytic cross-reactivity observed in primary allogeneic (C56BL/6 anti-DBA/2 and C57BL/6 anti-C3H/He) mixed-leukocyte cultures (MLC) was investigated by analysis of the specificity of clonal progeny derived from individual cytolytic T lymphocyte (CTL) precursor cells (CTL-P) contained within these populations. A sensitive mixed-leukocyte microculture (micro-MLC) technique was used with limiting dilution analysis by Poisson statistics to determine the frequency of CTL-P reactive against both specific and third-party (P815 and AKRA) target cells, to calculate the probability that each micro-MLC was a clone derived from a single CTL-P, and to examine the specificity of each micro-MLC assayed separately against both target cells. A total of 287 phenotypically specific, heteroclitic, and cross-reactive micro-MLC from the 2 different strain combinations were observed with a relative frequency of 81, 11, and 8%, respectively, and were calculated to have mean clone probabilities of 90 and 99% when based, respectively, upon the frequencies of CTL-P reactive against the specific and third-party target cells. These clones were estimated to have an approximate size of 6×10^4 cells, which corresponded to roughly 16 cell doublings during the 7 d of culture. 22 clones were successfully subcloned and in virtually every case, the subclones retained the specificity phenotype of the original clone from which they were derived. These results provide direct evidence for three phenotypically distinct sets of CTL as the cellular basis of cross-reactivity in MLC populations assayed against two different target cells.

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References

1. Möller, G. 1976. Specificity of effector T lymphocytes. *Immunol. Rev.* 29.
2. Müllbacker, A., and R. V. Blanden. 1979. Cross-reactivity patterns of murine cytotoxic T lymphocytes. *Cell. Immunol.* **43**:70.
3. Zagury, D., J. Bernard, N. Thiernesse, M. Feldman, and G. Berke. 1975. Isolation and characterization of individual functionally reactive cytotoxic T lymphocytes. Conjugation, killing, and recycling at the single cell level. *Eur. J. Immunol.* **5**:818.
4. Skinner, M. A., and J. Marbrook. 1976. An estimation of the frequency of precursor cells which generate cytotoxic lymphocytes. *J. Exp. Med.* **143**:1562.
5. Teh, H.-S., E. Harley, R. A. Phillips, and R. G. Miller. 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.

6. Lindahl, K. F., and D. B. Wilson. 1977. Histocompatibility antigen-activated cytotoxic T lymphocytes. II. Estimates of the frequency and specificity of precursors. *J. Exp. Med.* **145:** 508.
7. Ryser, J.-E., and H. R. MacDonald. 1979. Limiting dilution analysis of alloantigen-reactive T lymphocytes. I. Comparison of precursor frequencies for proliferative and cytolytic responses. *J. Immunol.* **122:**1691.
8. Ryser, J.-E., and H. R. MacDonald. 1979. Limiting dilution analysis of alloantigen-reactive T lymphocytes. III. Effect of priming on precursor frequencies. *J. Immunol.* **123:**128.
9. Maryanski, J. L., H. R. MacDonald, and J.-C. Cerottini. 1980. Limiting dilution analysis of alloantigen-reactive T lymphocytes. IV. High frequency of cytolytic T lymphocyte precursor cells in MLC blasts separated by velocity sedimentation. *J. Immunol.* **124:**42.
10. Ryser, J.-E., J.-C. Cerottini, and K. T. Brunner. 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.
11. Cerottini, J.-C., H. D. Engers, H. R. MacDonald, and K. T. Brunner. 1974. Generation of cytolytic T lymphocytes in vitro. I. Response of normal and immune mouse spleen cells in mixed leukocyte cultures. *J. Exp. Med.* **140:**703.
12. Miller, R. G., H.-S. Teh, E. Harley, and R. A. Phillips. 1977. Quantitative studies on the activation of cytotoxic lymphocyte precursor cells. *Immunol. Rev.* **35:**38.
13. Taswell, C., H. R. MacDonald, and J.-C. Cerottini. 1979. Limiting dilution analysis of alloantigen-reactive T lymphocytes. II. Effect of cortisone and cyclophosphamide on cytolytic T lymphocyte precursor frequencies in the thymus. *Thymus.* **1:**119.
14. Teh, H.-S., R. A. Phillips, and R. G. Miller. 1977. Quantitative studies on the precursors of cytotoxic lymphocytes. II. Specificity of precursors responsive to alloantigens and to concanavalin A. *J. Immunol.* **118:**1057.
15. Teh, H.-S., R. A. Phillips, and R. G. Miller. 1977. Quantitative studies on the precursors of cytotoxic lymphocytes. III. The lineage of memory cells. *J. Exp. Med.* **146:**1280.
16. Teh, H.-S., R. A. Phillips, and R. G. Miller. 1978. Quantitative studies on the precursors of cytotoxic lymphocytes. IV. Specificity and cross-reactivity of cytotoxic clones. *J. Immunol.* **120:**425.
17. Ching, L. M., J. Marbrook, and K. Z. Walker. 1977. Spontaneous clones of cytotoxic T cells in culture. II. Specificity of the response. *Cell. Immunol.* **31:**293.
18. Ching, L. M., K. Z. Walker, and J. Marbrook. 1977. Clones of cytotoxic lymphocytes in culture: the difference between stimulated and nonstimulated cytotoxic lymphocytes. *Eur. J. Immunol.* **7:**846.
19. Ching, L. M., K. Z. Walker, and J. Marbrook. 1977. Spontaneous clones of cytotoxic T cells in culture. III. Discriminatory lysis of pairs of syngeneic blasts induced by different mitogens. *Eur. J. Immunol.* **7:**850.
20. Komatsu, Y., Y. Nawa, A. R. Bellamy, and J. Marbrook. 1978. Clones of cytotoxic lymphocytes can recognize uninfected cells in a primary response against influenza virus. *Nature (Lond.).* **274:**802.
21. Ching, L. M., and J. Marbrook. 1979. The clonal analysis of cytotoxic lymphocytes against 2,4,6-trinitrophenyl (TNP)-modified cells. *Eur. J. Immunol.* **9:**22.
22. Teh, H.-S., R. A. Phillips, and R. G. Miller. 1978. Quantitative studies on the precursors of cytotoxic lymphocytes. V. The cellular basis for the cross-reactivity of TNP-specific clones. *J. Immunol.* **121:**1711.
23. Teh, H.-S. 1979. Frequency estimations of cytotoxic precursors to trinitrophenyl-modified alloantigens and determination of the degree of cross-reactivity between allodeterminants and trinitrophenyl-modified self determinants. *Immunogenetics.* **8:**99.

24. Brunner, K. T., H. R. MacDonald, and J.-C. Cerottini. 1980. Antigenic specificity of the cytolytic T lymphocyte response to murine sarcoma virus induced tumors. II. Analysis of the clonal progeny of CTL precursors stimulated in vitro with syngeneic tumor cells. *J. Immunol.* **124**:1627.
25. Möller, G. 1980. T cell growth stimulating factors. *Immunol. Rev.* **51**.