HISTOCOMPATIBILITY ANTIGEN-ACTIVATED CYTOTOXIC T LYMPHOCYTES

II. Estimates of the Frequency and Specificity of Precursors*

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The disproportionate intensity of alloimmune reactions directed to antigenic determinants of the major histocompatibility complex (MHC)[1] in various species remains one of the provocative problems of current cellular immunology. In particular, numerous studies have established that from 1 to 10% of peripheral thymus-derived (T) lymphocytes are reactive to one or another of the MHC haplotypes (1–5). From recent studies of Cantor and Boyse (6), it is clear that different functional responses directed to different alloantigenic regions of the *H-2* mouse MHC are distributed in different lymphocyte subpopulations that are distinguishable by genetically determined Ly surface markers. It can be presumed from their studies that the bulk of proliferative activity in graft-vs.-host reactions and in mixed lymphocyte cultures (MLC) involving the high frequency of alloreactive cells probably reflects activities of the Ly 1+ subclass of peripheral T lymphocytes. The question therefore remains whether the population containing the progenitors of cytotoxic T lymphocytes, demonstrated to be of the Ly-23$^+$ subclass, has similarly high frequencies of alloreactive cells.

The present studies make use of micro-mixed lymphocyte cultures, conducted with limiting dilutions of responding mouse lymphocytes, to generate cytotoxic activity and to estimate the frequency of cytotoxic T-lymphocyte precursors (CTL.P) in the starting population. In addition, since this microculture system isolates single CTL.P and their clonal CTL progeny, we have also used this system to assess both the specificity and the clone size of CTL progeny derived from a single CTL.P.

Materials and Methods

Animals. Mice of both sexes, 1.5–6 mo of age, were used. DBA/2, BALB/c (both *H-2*^{d}), C3H/He (*H-2*^{k}), and C57BL/6 (*H-2*^{b}) were purchased from the Institute for Cancer Research, Fox Chase, Pa., while B10.BR, AKR (both *H-2*^{k}), and B10.D2 (*H-2*^{d}) were obtained from The Jackson Laboratory, Bar Harbor, Maine. Mice of strain A/Ss (*H-2*^{a}) were a gift from Dr. W. K. Silvers (University of Pennsylvania, Philadelphia, Pa.), and B10.A (3R) (*H-2*^{3R}) mice were a gift from Dr. F. H. Bach (University of Wisconsin, Madison, Wis.). BN rats were bred in our colonies and used at 2 mo of age.

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[1] *Abbreviations used in this paper:* Con A, concanavalin A; CTL, cytotoxic T lymphocyte; CTL.P, CTL precursors; LPS, lipopolysaccharide; MHC, major histocompatibility complex; MLC, mixed lymphocyte cultures.

Media. Washing media, culture media, and assay media were prepared as described in the accompanying paper (7). Conditioned medium was prepared in batches of 0.5–1 liter at a time. C3H or C57BL/6 responding lymph node cells (120–145 × 10^6) were cultured with X-irradiated DBA/2 spleen cells (90–100 × 10^6) in 50 ml culture medium in Falcon no. 3024 (Falcon Plastics, Div. of BioQuest, Oxnard, Calif.) or Corning no. 25110 flasks (Corning Glass Works, Science Products Div., Corning, N. Y.). The flasks were incubated lying down at 37°C in a moist atmosphere of 8% CO_2 in air. After 2 days of culture, the medium was collected, the cells were removed by centrifugation at 800 *g* for 10–15 min, and the supernate was sterilized by filtration through a 0.22 μm filter (Nalgene; A. H. Thomas, Philadelphia, Pa.). Conditioned medium was stored frozen in aliquots at −20°C and thawed once only just before use.

Preliminary experiments demonstrated that mixed cultures with low numbers of responding cells developed higher cytotoxic activity and more positive wells when the culture medium was supplemented with 50% (vol/vol) conditioned medium. Medium harvested on day 2 was superior to medium harvested on day 1, and medium from cultures supplemented with 25% (vol/vol) fetal calf serum was better than medium from serum-free cultures.

Mixed Cultures. Lymph node and spleen cell suspensions were prepared as described (7). Mixed cultures were conducted in V-bottom wells of microtiter plates (IS-MVC-96-TC; Linbro Scientific Co., New Haven, Conn.). To each well was added 1 × 10^6 irradiated spleen stimulator cells in 0.1 ml of fresh culture medium. At each cell dose 12–24 replicate cultures were set up. Xenogeneic cultures contained graded numbers of responding mouse lymph node cells and 3 × 10^5 irradiated BN rat lymph node cells as stimulators. Preliminary experiments indicated that these doses of stimulating cells lead to maximal development of cytotoxic activity. The plates were covered with sterile lids and incubated as described (7) for 6 days.

Mitogen Stimulation. Responding cells in 200 μl culture medium were stimulated with a final dilution of 30 μg/ml lipopolysaccharide (LPS, *Escherichia coli* 055:B5 W; Difco Laboratories, Detroit, Mich.) or 10 μg/ml concanavalin A (Con A; Calbiochem, La Jolla, Calif.). One-fifth μCi ^{3}H-thymidine (6.7 Ci/mmol; New England Nuclear, Boston, Mass.) was added to each well in 25 μl RPMI 1640 20 h before the cells were harvested onto glass fiber filters on day 3 of culture and processed for liquid scintillation counting.

Target Cells. The DBA/2 mastocytoma P815, the C57BL/6 lymphoma EL-4, both obtained from Dr. C. Henney (Johns Hopkins University, Baltimore, Md.), and a BN rat (*Ag-B*^{3}) lymphoma, obtained from Dr. F. Fitch (University of Chicago, Chicago, Ill.), were all maintained in ascites form by weekly passages. Another EL-4 line obtained from Dr. W. Biddison (Wistar Institute, Philadelphia, Pa.), was maintained in tissue culture. Target cells were prepared and labeled as described (7), except that lower doses of ^{51}Cr (250 μCi to 1 mCi) and shorter labeling periods (45 min to 2 h) were used. The labeled target cells were suspended at 10^5 viable cells/ml of assay medium. With these labeling conditions, 10^4 target cells always showed >540 cpm maximum release and between 12.4 and 69.6% spontaneous release with a median of 21.3%.

Cytotoxic Assay. After 6 days of culture, the plates were centrifuged for 5 min at 500 *g* and the culture medium was "flicked" off. The cell pellets were resuspended on a Vortex mixer, and 50 μl of assay medium was added to each well. 10,000 labeled target cells were added in 100 μl assay medium to each well, and the cultures were centrifuged, incubated for 6 h, and the amount of ^{51}Cr released into the supernate measured, all as previously described (7).

To determine the clone size of a CTL.P, the contents of individual culture wells were suspended in 100 μl assay medium and serial twofold dilutions made into neighboring wells containing 50 μl assay medium. Target cells were then added to all wells. For the specificity studies, the cells of individual wells were again resuspended in 100 μl assay medium, and 50 μl of the cell suspension added to each of two empty wells. Target cells were then added to the new wells.

TNP Modification. The surface of irradiated spleen-stimulating cells and of ^{51}Cr-labeled tumor target cells were modified with trinitrobenzene sulfonic acid (Eastman Kodak Co., Rochester, N. Y.) as described by Shearer (8).

Assessment of Proliferation and of Cytotoxic Activity. Proliferative activity, as evidenced by thymidine uptake, and cytotoxic activity, based on ^{51}Cr release, was measured in the same cultures as follows: 16–20 h before the cytotoxic assay, 0.1 μCi ^{14}C-thymidine (65 mCi/mmol, New England Nuclear) were added to each culture in 25 μl RPMI 1640. At the end of the culture period the radioactive medium was removed as described above, and the cultures were incubated for 1–2 h with 1,000-fold excess of cold thymidine in 0.1 ml RPMI 1640 medium to insure that free ^{14}C-thymidine would not be incorporated by the tumor target cells. The cells were then resuspended in

50 μl assay medium and the cytotoxic assay conducted as described before. After removal of the supernate for measurement of ^{51}Cr release from each culture, the cell pellets were harvested onto glass fiber filters and washed with distilled water. The air-dried filters were processed for liquid scintillation counting. Threshold settings on the beta spectrometer were adjusted so that ^{51}Cr counts were reduced to 4.9% of maximum activity (always less than 100 cpm), while 90% of ^{14}C activity remained.

Anti-Theta Treatment. Cells to be treated were incubated for 30 min at 4°C at 2.5×10^6 cells/ml of a 1:5 dilution of AKR anti-C3H serum in RPMI 1640 medium, washed once, and then incubated for 30 min at 37°C in the same volume of a 1:4 dilution of rabbit serum absorbed with agarose. Both were generous gifts of Doctors N. Klinman and E. Metcalf (University of Pennsylvania). After treatment the cells were washed once, counted, and cultured or tested in a cytotoxic assay.

Results

Development of Cytotoxic Activity in Cultures with Low Numbers of Responding Cells. In preliminary experiments a number of parameters were varied systematically to establish optimal conditions for generating killer cells in cultures having the lowest possible number of responding cells. These included day of assay, dose of stimulating cells, conditioned medium, assay period, target cell number, and the following conditions were settled upon: the microcultures were incubated for 6 days with 1×10^6 stimulating cells and 50% conditioned medium, while the cytotoxic assay was incubated for 6 h with 1×10^4 target cells.

With the culture system developed, it was sometimes possible to generate cytotoxic activity with as few as 300 responding lymph node cells (Fig. 1). ^{51}Cr release values in most of the wells having low numbers of responding cells corresponded to spontaneous release, while a few wells were clearly positive. We arbitrarily defined as positive wells in which the ^{51}Cr release exceeded three standard deviations above the mean of the spontaneous release values. As the number of responding cells increased, both the fraction of positive wells and the amount of ^{51}Cr released in each positive well increased.

Next, experiments were conducted to establish that the killer cells generated in this culture system were T cells: (*a*) Treatment of the responding cells with anti-theta serum and complement before they were cultured abolished, to the same extent as X irradiation, their ability to proliferate in mixed culture, to generate cytotoxic activity, and to respond to the T-cell mitogen Con A, whereas the response to the B-cell mitogen LPS remained normal (Table I). (*b*) In one experiment, a lymphocyte subpopulation enriched for T cells by nylon wool purification developed cytotoxic activity at lower numbers of responding cells than the untreated population. (*c*) In two experiments, treatment of a sensitized population with anti-theta and complement killed 74 and 81% of the cells and reduced the cytotoxic activity 84% in both cases.

Frequency of Cytotoxic T-Lymphocyte Precursors. Semilogarithmic plots of the frequency of wells displaying no cytotoxic activity as a function of the dose of responding cells demonstrated a linear relationship as seen in Fig. 2. From the slope of the lines a minimal estimate can be made of the frequency of the limiting unit (9), which we assume to be the cytotoxic T-lymphocyte precursor. For the combination C57BL/6 responding to DBA/2, shown in Fig. 2, we found CTL.P per 677–935 responding cells with a median of 1 CTL.P per 845 lymph node cells.

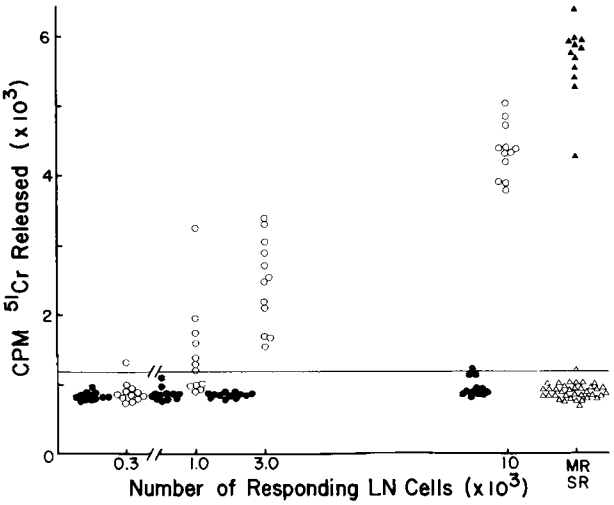

FIG. 1. Cytotoxic activity against P815 target cells in cultures with limiting numbers of responding C3H lymph node (LN) cells and irradiated stimulator cells from the spleens of DBA/2 (○) or C3H (●) mice. Maximum release (MR) (▲) is measured in wells with detergent and spontaneous release (SR) (△) in wells with medium alone. The horizontal line indicates the ^{51}Cr-release value corresponding to three standard deviations above the mean of the spontaneous release values.

TABLE I

Responses of 1 × 10⁴ C3H Lymph Node Cells To Mitogens and Allogeneic Cells

Day of assay	Stimulus	Assay	Responding cells treated with			
			–	1,750 rads	Complement (C')	Anti-theta + C'
3	None	^{3}H-thymidine uptake*	734 ± 95	88 ± 79	985 ± 83	553 ± 90
	LPS		4,062 ± 384	65 ± 13	3,967 ± 396	4,736 ± 591
	Con A		9,199 ± 1,103	1,350 ± 238	8,848 ± 1,353	1,045 ± 715
6	$C3H_x$	^{14}C-thymidine uptake*	316 ± 322	51 ± 13	284 ± 204	84 ± 40
		^{51}Cr release* ‡	154 ± 9	138 ± 9	139 ± 12	154 ± 16
	$DBA/2_x$	^{14}C-thymidine uptake§	5,426 ± 779	189 ± 101	5,487 ± 864	167 ± 47
		^{51}Cr release‡ §	356 ± 42	167 ± 67	339 ± 29	147 ± 9

* Mean and standard deviation of quadruplicate cultures

‡ ^{51}Cr release from 1 × 10⁴ P815 target cells, spontaneous release 134 1 ± 16 7 cpm (30 wells), maximum release 555 0 ± 48 0 cpm (6 wells)

§ Mean and standard deviation of eight replicate cultures

Since CTL reactive to *H-2K* and *H-2D* target antigens can be separated (10), it is likely that the CTL.P specific for *K*-end and *D*-end antigen also reside in different subpopulations. One would therefore expect a higher frequency of CTL.P contributing to responses directed to the whole *H-2* complex as compared to only the *K* end of *H-2*. Fig. 3 shows that this is the case.

A summary of the variety of combinations tested is shown in Fig. 4. The data indicate that: (*a*) The highest frequencies of CTL.P are found in allogeneic combinations differing with respect to the whole *H-2* complex and tested on the specific target, such as 4–10 CTL.P per 10⁴ C3H lymph node cells stimulated with DBA/2; (*b*) precursors activated to a particular *H-2* haplotype, and then tested for "cross-reactive" cytolytic activity against a different haplotype, were

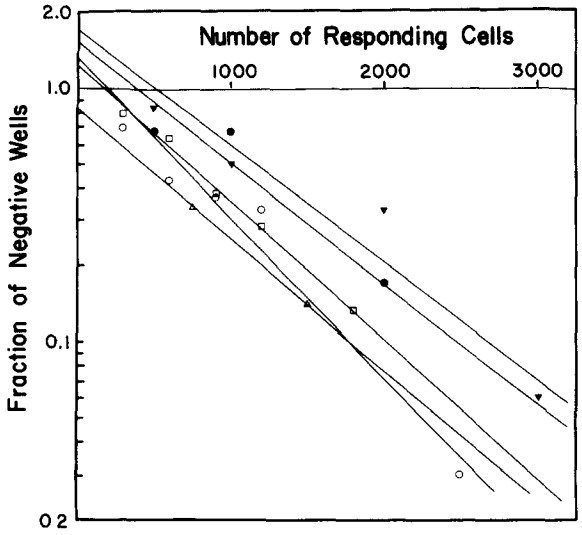

FIG 2. Results of five experiments, conducted at different times, testing the development of cytotoxic activity to P815 target cells in cultures with limiting numbers of C57BL/6 lymph node cells and with 10^6 irradiated DBA/2 spleen cells Each cell dose was tested in 12 (▼, ●), 30 (○), 60 (□), or 120 (△) wells, and all wells with ^{51}Cr-release values less than three standard deviations above the mean of the spontaneous release level were considered negative The lines were fitted by the least squares method.

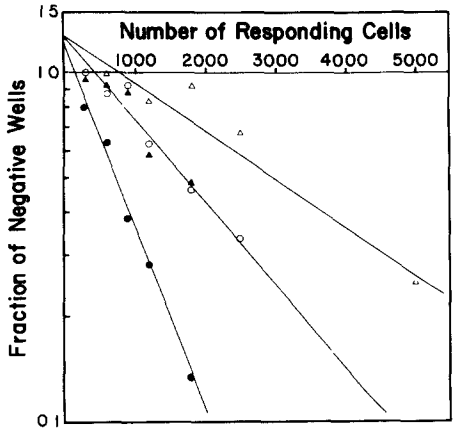

FIG 3. Development of cytotoxic activity to P815 target cells in cultures with limiting numbers of lymph node cells differing by a full or partial *H-2* haplotype from DBA/2 stimulator spleen cells. Responding cells of strains C57BL/6 ($H\text{-}2^b$) (●, 1 CTL P/816 cells), and C3H ($H\text{-}2^k$) (▲, 1 CTL.P/1,742 cells differed from the stimulator at all of the *H-2* complex, while responding cells of strains B10.A(3R) ($H\text{-}2^{i3}$) (○, 1 CTL.P/1,815 cells) and A ($H\text{-}2^a$) (△, 1 CTL.P/3,144 cells) differed only at the *K* end of the *H-2* complex. Both *i3* and *a* are recombinant haplotypes carrying *d* alleles at the *D* end, while at the *K* end *i3* carries *b* and *a* carries *k* alleles. The frequency of CTL.P indicated above is determined from the slope of the fitted regression lines.

present in considerably lower frequency, 0.2–1.5 per 10^4 cells; (*c*) mouse CTL.P reactive to BN rat cells were found at frequencies of 0.8–5 per 10^4 cells; and (*d*) the frequency of CTL.P reactive to trinitrophenyl (TNP)-modified syngeneic cells was 0.3–1.2 per 10^4.

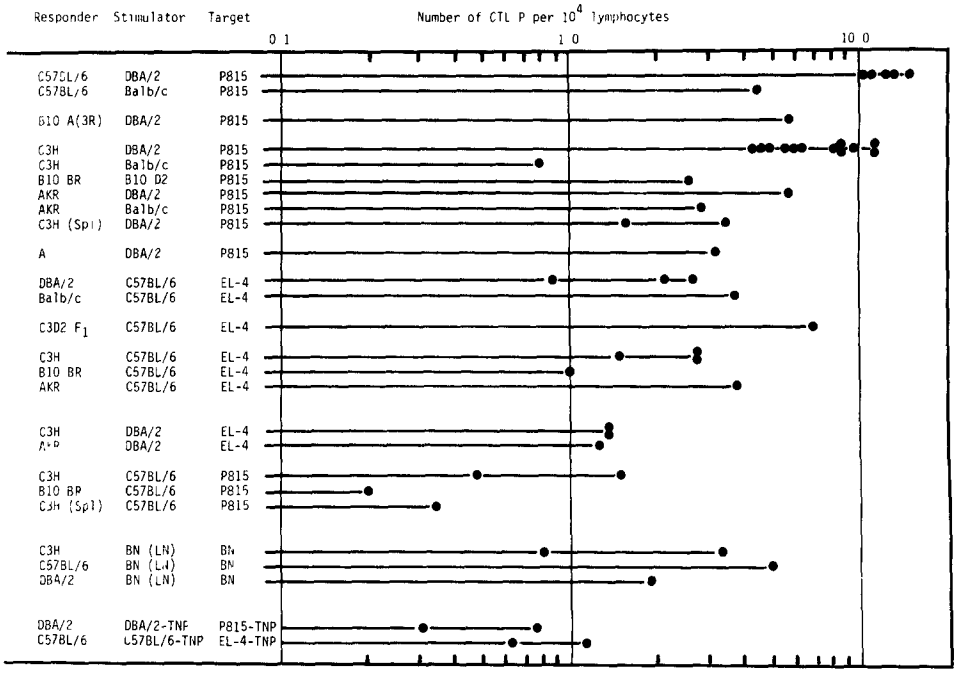

FIG 4. Summary of estimates of the frequency of CTL.P responding to a variety of allogeneic stimuli, to stimulation with xenogeneic cells, and to stimulation with TNP-modified syngeneic cells. Responder cells were of lymph node origin except where indicated. Stimulator cells were of splenic origin except for xenogeneic cultures.

In the 51 semilogarithmic plots used to derive the frequency estimates in Fig. 4, the intercepts with the ordinate ranged from 0.7 to 3.45 with a median of 1.25 (95% confidence limits 1.15–1.33). An intercept higher than 1.0 may indicate that the generation of cytotoxic activity is not a single cell event.

Clone Size of the CTL.P. Minimal estimates of the number of progeny generated from a single CTL.P after activation with alloantigens were obtained from cultures initiated with numbers of responding cells sufficiently low that positive cultures could be expected to contain only one CTL.P. After 6 days of culture, cytotoxic assays were conducted on serial twofold dilutions of the cultures, and the last dilution shows CTL activity determined. From the data in Table II it appears that the descendants of a single CTL.P can be diluted 8- to 16-fold and still show demonstrable killing, indicating that a single CTL.P undergoes a minimum of three to four cell divisions.

A similar estimate of the "burst size" was derived from MLC conducted in bulk cultures with knowledge of the minimal frequency of CTL in an immune population, as determined in the accompanying paper (7), and from the present estimates of CTP.P frequency. In two such experiments with bulk cultures, the absolute number of killer cells in the sensitized lymphocyte population was 11.6- and 13.1-fold higher than the starting number of CTL.P, again suggesting three to four cell divisions.

Assessments of Proliferation and Cytotoxic Activity on the Same Responding Lymphocyte Population. Fig. 5 presents the results of a typical experiment demonstrating a strong positive correlation between the amount of proliferation

TABLE II

Dilution of Sensitized Cells from 24 Single Microcultures on the Day of Assay

Exp.	Combination	No. of wells expected* to have					No. of wells cytotoxic to P815 target cells when diluted down to:						
		0	1	2	3	4	No activity	1/2	1/4	1/8	1/16	1/32	1/64
		CTL·P per well											
1	1,000 C3H + DBA/2$_x$	21	2.8	0.19	–	–	21	1	–	2	–	–	–
	1,000 C57BL/6 + DBA/2$_x$	7	8.6	5.3	2.2	0.67	7	1	2	2	4	–	8
2	500 C57BL/6 + DBA/2$_x$	20	3.6	0.32	–	–	20	1	1	–	2	–	–
	1,000 C57BL/6 + DBA/2$_x$	18	5.2	0.76	0.07	–	18	–	–	6	–	–	–

* Calculated from the frequency of negative wells in that experiment using the Poisson distribution.

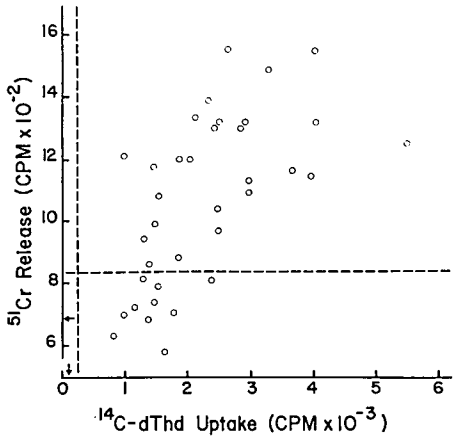

FIG. 5. ^{14}C-thymidine (^{14}Cl-dThd) uptake and ^{51}Cr release from P815 target cells in 36 microcultures of 1,200 C57BL/6 lymph node cells stimulated with 1 × 10^6 irradiated DBA/2 spleen cells. The arrow on the abscissa shows the mean ^{14}C-thymidine uptake in 12 cultures of 1,200 C57BL/6 lymph node cells stimulated with syngeneic spleen cells (103.3 ± 51.8 cpm), and the vertical dashed line is three standard deviations above this mean, our definition of a positive culture. The arrow on the ordinate shows the mean ^{51}Cr release in 36 cultures of P815 with medium alone (686.0 ± 49.3 cpm; maximum release in 12 cultures, 2,688.6 ± 165.4 cpm), and the horizontal dashed line is three standard deviations above this mean. There is a strong positive correlation between proliferation and cytotoxic activity (Spearman's rank correlation coefficient $r_s = 0.656$, $P < 0.001$).

(^{14}C-thymidine uptake) and the amount of cytotoxicity (^{51}Cr release) generated in the same culture well. Thus, at the level of response of a single CTL.P (*a*) all cultures showing cytotoxic activity also showed strong proliferative responses (stimulation indices 4.0), however, the amount of cytotoxicity varied for any given level of proliferation, and (*b*) some cultures showing good proliferative responses displayed no cytotoxic activity, a finding consistent with the conclusions of others that not all proliferating cells are killer cells (6, 11).

Table III compares the proliferative and cytotoxic responses at different dosages of C57BL/6 responding cells stimulated with an *H-2* difference (BALB/c) or with an additional strong *Mls* locus difference (DBA/2). Again, more proliferation was detected in wells that developed cytotoxicity than in those that did not. However, despite the strong proliferation caused by the *Mls* locus differ-

TABLE III

Proliferation of C57BL/6 Responding Lymph Node Cells and Development of Cytotoxic Activity to P815 Target Cells

No. of responding cells	Stimulating cells	^{14}C-thymidine uptake* (mean ± SD)		^{51}Cr release‡ (mean ± SD) in wells with cytoxic activity
		Wells with no cytoxic activity	Wells with cytoxic activity	
500	BALB/c$_x$	207 1 ± 204 5 (10)	465 0 ± 280 6 (8)	528 8 ± 144 5
	DBA/2$_x$	571 9 ± 303 6 (15)	1,332 7 ± 143 9 (3)	721 0 ± 96 8
1,000	BALB/c$_x$	212 8 ± 120 9 (11)	557 1 ± 310 2 (7)	571 1 ± 103 1
	DBA/2$_x$	822 7 ± 363 0 (9)	1,209 7 ± 331 4 (9)	550 9 ± 130 2
2,000	BALB/c$_x$	497 6 ± 153 3 (7)	816 8 ± 375 1 (11)	758 1 ± 306 3
	DBA/2$_x$	1,077 0 ± 419 9 (6)	1,718 9 ± 442 2 (12)	759 2 ± 145 4
3,000	BALB/c$_x$	460 0 ± 178 6 (4)	803 5 ± 194 3 (14)	614 4 ± 168 4
	DBA/2$_x$	875 0 (1)	1,767 6 ± 793 7 (17)	700 1 ± 186 9

* The mean ^{14}C-thymidine uptake in syngeneic control cultures ranged from 80 7 to 121 2 cpm

‡ Spontaneous ^{51}Cr release, 342 9 ± 30 3 cpm, maximum release, 1255 8 ± 33 1 cpm Wells in which the ^{51}Cr release exceeded 433 cpm were considered positive

§ Numbers in parentheses are the number of cultures

ence, known to augment generation of killer cells under some circumstances (12, 13), the amount of ^{51}Cr released in the wells was not influenced by the *Mls* locus. This finding demonstrates that in the present system the proliferative response to *H-2* antigens is sufficient to trigger differentiation of CTL.P and that the level of proliferation in these cultures is not the predominate limiting factor in the development of killers. At every responding cell dose, the mean ^{14}C-thymidine uptake was higher in the DBA/2 cultures with no cytotoxic activity than in the BALB/c cultures with a positive cytotoxic response.

Cellular Basis of Cross-Reactivity. Previous studies have demonstrated killing of third party targets by cytotoxic T lymphocytes (14, 15). Findings such as these raise the question whether cross-reactivity reflects the specificity and extent of differentiation of killer cells at the level of the population, or at the level of the single cell. Several possibilities can be considered: (*a*) all CTL display some activity against third party target cells, (*b*) only CTL highly active against specific targets are sufficiently differentiated to display some cross-reactive killing against third party targets, or (*c*) cross-reactive CTL represent a minority subpopulation active against third party target cells on the basis of shared antigenic determinants regardless of the level of activity to the specific target.

Analysis of killing activity of clonal progeny of single CTL.P against third party target cells provided an answer to this question. On the day of assay, the sensitized cell population in each well was divided in two. When each half was tested with the same target cells in replicate assays, they showed identical activity (Figs. 6*a* and *b*). However, when the sensitized cell population was similarly split and one half assayed with the specific target, and the other half with a third party target, it was apparent that most of the cultures developed cytotoxic activity against the specific target (Figs. 6*c* and *d*). A minority of the cultures, not necessarily showing the highest activity against the specific target, were cross-reactive and killed both target cells. Finally, some cultures

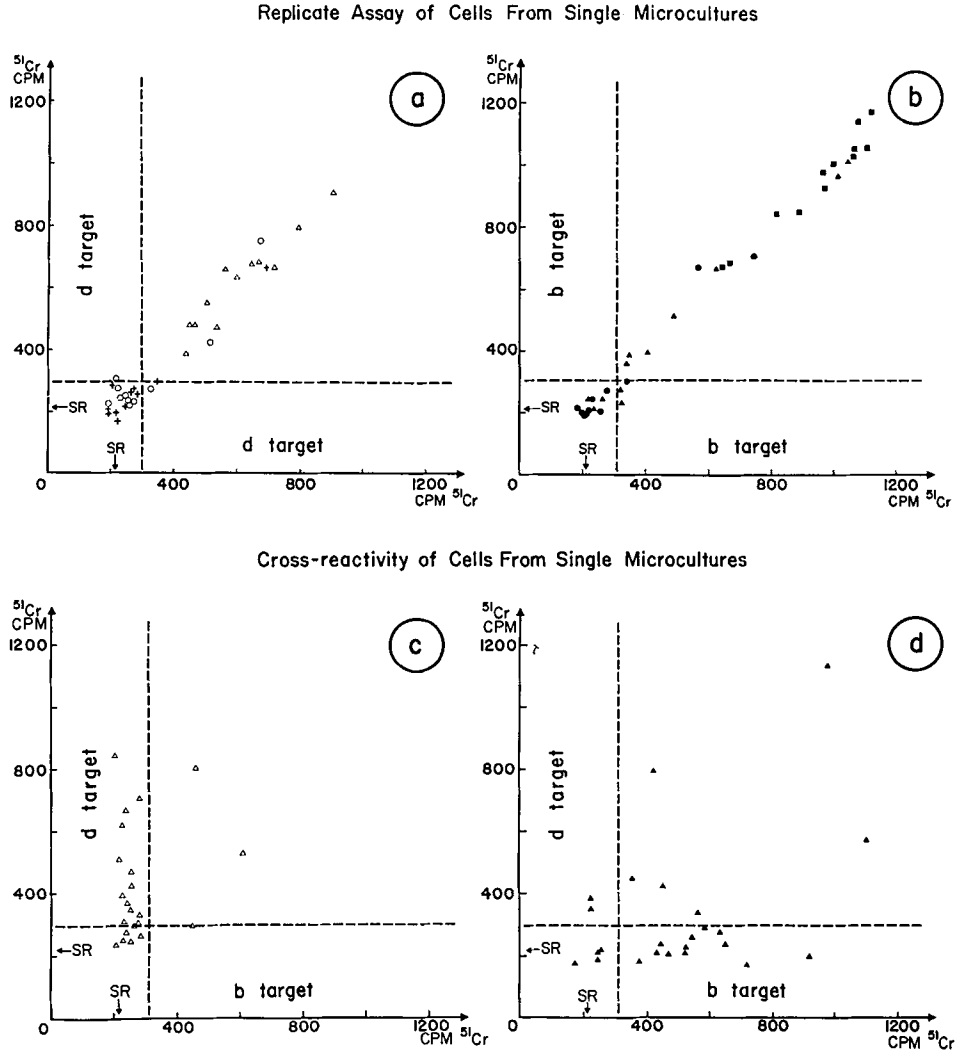

FIG 6. Comparison of the cytotoxic activity of each half of the sensitized cells from single microcultures, split on the day of assay. (*a*) Twelve cultures each with 3,000 (△), 1,000 (○), or 300 (+) C3H lymph node cells stimulated with 1×10^6 DBA/2 spleen cells, both halves assayed with 1×10^4 P815 target cells. (*b*) 12 cultures each with 10,000 (■), 3,000 (▲), or 1,000 (●) C3H lymph node cells stimulated with 1×10^6 C57BL/6 spleen cells, both halves assayed with 1×10^4 EL-4 target cells. (*c*) and (*d*) 24 cultures each of 3,000 C3H lymph node cells activated with 1×10^6 DBA/2 (△) or C57BL/6 (▲) spleen cells, half of each culture assayed with EL-4 target cells (abscissa) and half with P815 (ordinate). The spontaneous release for the P815 targets was 212.8 ± 26.8 cpm, the maximum release was 1,442.2 ± 40.9 cpm; spontaneous release for the EL-4 targets was 204.8 ± 34.4 cpm, maximum release was 1,425 1 ± 63.7 cpm The dashed horizontal and vertical lines are three standard deviations above and to the right of the mean of the spontaneous release values for the respective targets, our definition of positive cytotoxic activity

exhibited apparent heteroclicity in that they did not kill target cells of the original priming strain, but were active against other targets.

Discussion

Several studies have compared the relative potential of various lymphoid cell populations for the development of cytotoxic activity (see reference 16). The present study provides direct minimal estimates of the absolute frequency among mouse lymph node cells of killer cell precursors for a variety of antigens. It was found that on the average as many as 1 in 845 C57BL/6 lymph node cells had the potential to become killer cells against DBA/2 target cells (Fig. 2), and 1 in 1,400 C3H lymph node cells had the same potential (Fig. 4). Consistent with these data, Skinner and Marbrook recently estimated by a similar procedure that 1 in 1,700 CBA spleen cells were precursors of killer cells against DBA/2 (17). CTL.P reactive to xenogeneic BN rat target cells or to TNP-modified self were present in considerably lower frequencies (Fig. 4).

The killer cells and their precursors studied here were shown to be T lymphocyte (Table I). The studies of Cantor and Boyse have shown that among peripheral T lymphocytes of C57BL/6 mice, the CTL.P reside exclusively in the Ly-1^-, 2^+, 3^+ subpopulation, which constitutes 5–10% of the total T-cell population (6). From the number of Ly-1^-, 2^+, 3^+ cells contained in the above mentioned numbers of lymph node cells, it follows that all of the C57BL/6 lymph node cells which have the potential to become killers, at least 2.2% are reactive to *H-2*^{d}, and assuming the total pool of CTL.P in C3H mice is of the same size, a minimum of 1.3% of CTL.P in C3H lymph node cells are specific for *H-2*^{d}. Comparing the cytotoxic activity of Con A-activated *H-2*^{b} or *H-2*^{k} spleen cells towards P815 (*H-2*^{d}) target cells in the absence and presence of phytohemagglutinin, Bevan et al. similarly estimated that of all killer cells activated by Con A, 1.7–3.5% were specifically reactive to *H-2*^{d} (18).

It has recently been shown that suppressor cells and their precursors are found in the same Ly-2^+, 3^+ subpopulation as CTL.P (19, 20), and that mature suppressor cells can be distinguished from CTL on the basis of Ia antigens expressed on their surface (21). If the suppressor cell precursors are similarly distinct from the CTL.P, the total size of the CTL.P pool would be even smaller than assumed above, and the proportion of that pool specific for a given allogeneic major histocompatibility complex haplotype even larger than the 1–2% calculated above.

Although apparently high, the present estimates of CTL.P frequencies are minimal estimates, since several factors, such as availability of ancillary cells, plating efficiency, and sensitivity of the assay, may have contributed to make the estimates lower than the true frequencies. The frequency determined was that of the limiting unit, which was assumed to be the CTL.P. Several studies have shown that cells other than CTL.P have a facilitating, if not obligatory, role in the development of killer cells. These include nonspecific cells such as macrophages (22), a syngeneic non-T-cell found in spleens of nude mice (23), and specifically activated amplifier (24, 25) or proliferating helper cells (26). Consistent with these reports, we found an intercept higher than 1.0 in the semilogarithmic plots of fraction negative wells vs. cell dose (Figs. 2 and 3), indicating

that the generation of killer cells is not a single cell event; the fact that the average intercept was only slightly higher than 1.0, we take to indicate that the other cell types required are present in higher frequency than the limiting unit, of which only one cell is required. From earlier estimates, it appears that precursors of proliferating cells are present in 10- to 100-fold higher frequencies than the limiting unit measured here (1–5), and indeed we had direct evidence in the strongest combinations that proliferation was not the limiting factor (Table III). Some of the nonspecific cells may have been provided by the many stimulating cells, and seeing how the frequencies determined depend on the haplotype combination and on the antigenic differences (Figs. 3 and 4), we find it most likely that the limiting unit is antigen specific.

It is not clear whether all specific CTL.P present were activated and developed into killers, but the efficiency of activation appeared to be independent of cell numbers, as evidenced by the straight lines in the semilogarithmic plots of fraction-negative wells vs. cell dose (Figs. 2 and 3), and by the finding of similar clone sizes in microcultures and in bulk MLC, where no responder cells would be limiting. The use of conditioned medium increased both the number of positive wells and the amount of ^{51}Cr release in these wells; it may have worked by increasing the activation efficiency and may be in addition to the clone size. We do not know whether this effect was brought about by nutrients, processed antigen, allogeneic effect factor (27), T-derived medium factor (28), or by yet other mechanisms.

The sensitivity of the assay does not appear to be as critical in the determination of precursor frequencies as in the study of CTL frequencies (7), since each precursor develops into a clone of killer cells, with which the assay is done rather than a single cell. However, in the determination of clone size the sensitivity of the assay again becomes limiting. Since the later stages of development of the killer cells occurs under deteriorating culture conditions, the minimum of three to four cell divisions may not be fully representative of the potential for division of a CTL.P under more optimal conditions. Despite these reservations, our studies directly demonstrated that a CTL.P does divide after activation (Fig. 5), whereas previous studies had shown only that cell division in the cultures is necessary for development of primary CTLs (29–31), and that the dividing cells are antigen-specific (32). Our finding of a minimum of three to four cell divisions is in reasonable agreement with the report that the replication occurs on days 2–4 of cultures with maximal cytotoxic activity on day 5 (30).

Previous studies have shown that killing of cross-reactive target cells can be effectively blocked by cold target cells from the specific stimulator strain (14, 33), a finding that indicates that cross-reactivity is a specific phenomenon based on recognition of shared antigens. The experiments presented here (Fig. 6) show that the reactivity to these shared antigenic determinants resides in a minority subpopulation among the CTL.P reactive to the specific stimulator; cross-reactivity to a given third party target is not due to the generation of "superkillers" or a property of all CTL.P.

Some clones lysed only third party target cells, not the specific stimulator. A similar heteroclicity was observed in the previous study using blocking with unlabeled target cells (Table III in reference 14), and we see at present three

possible explanations of this phenomenon: (*a*) true heteroclicity as known for antibodies, indicating different antigenic requirements for activation and killing, (*b*) nonspecific activation and differentiation of CTL reactive only to third party antigens, or (*c*) since the stimulator cells and target cells were from different sources in this study, spleen and tumor cell lines, it is possible that some determinants present on the stimulatory cells were also present on third party target cells but absent on specific target cells. Although the last possibility appears unlikely, considering that "heteroclicity" was also found using LPS target cells and in a variety of strain combinations (14), we nevertheless attempted to rule it out by using stimulatory and target cells from the same source. However, in such a study the tumor cells failed to stimulate the generation of killer cells under any of the conditions tested, a not entirely unexpected finding (34).

A finding of some interest is the reproducible, almost twofold, difference in the frequency among C56BL/6 and C3H lymph node cell populations of CTL.P reactive to DBA/2 MHC alloantigens. This finding may reflect differences between the two responding strains in the size of the precursor pool, the number of target antigens presented to the responding cells, or some genetic control mechanism over the level of precursor frequency. In this context, the studies of Bevan et al. (18) have demonstrated an approximate twofold difference in the frequency of CTL from $H\text{-}2^k$ and $H\text{-}2^b$ mice responsive to $H\text{-}2^d$ target alloantigens.

If cross-reactivity depends on a subpopulation of cells recognizing shared antigenic determinants, the reaction should be of equal strength whether activated by one or the other of the cross-reactive cells. The one-way cross-reactivity which sometimes occurs (14, 35) can then be understood only if one takes into account that the frequencies of CTL.P reactive in different combinations differ. The C3H cells which react to both DBA/2 and C57BL/6 thus constitute a larger fraction of the pool of C3H cells reactive to C56BL/6 than of the cells reactive to DBA/2, and it may therefore appear as if activation with C57BL/6 leads to more cross-reaction than activation with DBA/2.

Summary

Using limiting dilutions of responding cells in mouse mixed leukocyte cultures, we obtained direct estimates of the minimum frequency of precursors of cytotoxic T lymphocytes (CTL.P) for a variety of antigens. Depending on the strain combination, there were as many as 4–15 CTL.P reactive to DBA/2 among 10^4 lymph node cells. Taking into account that only 5–10% of peripheral T lymphocytes have the potential to develop into cytotoxic T lymphocytes (CTLs) (6), this implies that at least 1–2% of all CTL.P are responsive to any given *H-2* haplotype difference. Precursors of cytotoxic cells thus have the same high frequency of cells reactive to alloantigens of the major histocompatibility complex as found among proliferating cells in graft-vs.-host reactions and mixed lymphocyte interactions. The frequencies of CTL.P reactive to xenoantigens (rat) or trinitrophenyl-modified self were less than half the frequency of alloreactive CTL.P. A minority of the CTL.P specific for one *H-2* haplotype were also reactive to a third party *H-2* haplotype, presumably on the basis of recognition

of shared determinants. By dilution of sensitized cells from single microcultures, it was shown that a single CTL.P undergoes a minimum of three to four cell divisions and generates at least 8–16 CTLs after antigenic activation.

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