HISTOCOMPATIBILITY ANTIGEN-ACTIVATED CYTOTOXIC T LYMPHOCYTES

I. Estimates of the Absolute Frequency of Killer Cells Generated In Vitro*

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Numerous studies of the immunobiology of cytotoxic thymus-derived (T) lymphocytes (CTL)[1] have provided detailed information on both the induction and detection of CTL activity directed against alloantigen-bearing target cells (1). These studies have depended heavily on the availability of rapid cytolytic assays which involve the release of radioactive ^{51}Cr from isotopically labeled target cells. While these methods have been employed to derive relative estimates of cytotoxic potency of various immune lymphocyte populations, this assay as currently used does not provide direct estimates of the absolute frequency of CTL in such populations.

In a previous study the frequency of killer cells in a population of lymphocytes from the draining lymph nodes of immunized animals was estimated to be approximately 1–2%. This figure was based on limiting dilution of immune lymphocytes and analysis of target cell survival with an electronic particle counter (2). There were certain weaknesses in that study: (*a*) it involved relatively long assay periods (48 h), making it difficult to rule out that new killer cells were generated in the cultures; (*b*) the assumption that a killer cell could affect only a single target cell, an assumption which if not valid leads to erroneously high estimates of CTL frequency; and (*c*) it could not be determined at the time whether killer cells were exclusively of thymic origin.

In the present study, we describe another procedure which combines limiting dilution and ^{51}Cr release for deriving a minimal estimate of the absolute frequency of CTL generated in mouse lymphocyte populations activated to alloantigens of the major histocompatibility complex in bulk mixed lymphocyte cultures (MLC), some details of the specificity of CTL, as well as an estimate of the number of target cells killed by a single CTL.

Materials and Methods

Mice Mice of both sexes, 2–5 mo of age, were used. B10.BR ($H\text{-}2^k$) were purchased from The Jackson Laboratory, Bar Harbor, Maine, while C3H/He ($H\text{-}2^k$), BALB/c ($H\text{-}2^d$), DBA/2 ($H\text{-}2^d$),

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[1] *Abbreviations used in this paper:* CTL, cytotoxic T lymphocytes; HPBSS-2 and HPBSS-5, Hanks' balanced salt solution supplemented with 2% and 5% fetal calf serum, respectively; MLC, mixed lymphocyte culture.

and C57BL/6 ($H\text{-}2^b$) were obtained from the Institute for Cancer Research, Fox Chase, Pa., and maintained in our colonies.

Media. Four different media were used. Before culture cells were washed in Hanks' balanced salt solution (powder; Microbiological Associates, Bethesda, Md.) buffered with 1 mM phosphate, and supplemented with 2% (vol/vol) fetal calf serum (lot no. 88373; Microbiological Associates) (HPBSS-2). Target cells were prepared and washed in the same medium but supplemented with 5% (vol/vol) fetal calf serum which had been heated for 2 h at 56°C (HPBSS-5). Culture medium was RPMI 1640 supplemented with 12 mM Hepes, 25% (vol/vol) fetal calf serum, penicillin-streptomycin (all from Microbiological Associates), 2 mM L-glutamine (BDH Chemicals Ltd., Poole, England), and 4×10^{-5} M 2-mercaptoethanol (Eastman Kodak Co., Rochester, N. Y.). The same medium was used for the cytotoxic assay, except that it contained no 2-mercaptoethanol and only 10% (vol/vol) fetal calf serum which had been heat inactivated.

In Vitro Immunization. Lymphocytes were stimulated with alloantigens in MLC prepared from spleens or, where indicated, from lymph nodes. These organs were removed aseptically into HPBSS-2 and gently teased with forceps. Clumps were allowed to settle and the cells remaining in suspension were washed once by centrifugation at 130 *g* for 8 min in HPBSS-2. Viable cells were assessed by phase microscopy using eosin. The cells to be used as stimulators were irradiated 1,750 R (110 R/min) with a ^{137}Cs source. Mixed cultures were conducted in Falcon no. 3012 flasks (Falcon Plastics, Div. of BioQuest, Oxnard, Calif.) with 10×10^6 responding and 8×10^6 stimulating cells in 4.5 ml culture medium (3). The cultures were incubated at 37°C in a humidified atmosphere of 8% CO_2 in air. Cells to be restimulated were collected after 10 or 11 days of culture and resuspended in fresh medium, and 1×10^6 viable cells were cultured with 8×10^6 fresh stimulator cells as described above.

Target Cells. The DBA/2 mastocytoma, P815, obtained from Dr. C. S. Henney, Johns Hopkins University, Baltimore, Md., and maintained in ascites form by weekly passage, was employed for cytotoxicity assays 4–7 days after transfer. The tumor cells were washed once in HPBSS-5 at 32 *g* for 6 min. For labeling, 10^7 cells were incubated in 0.3 ml of RPMI 1640 with 25% fetal calf serum and 1–2 mCi ^{51}Cr as sodium chromate (10 mCi/ml, approximately 250 mCi/mg Cr; New England Nuclear, Boston, Mass.) for 2 h at 37°C. Thereafter, the labeled target cells were washed twice at 100 *g* for 6 min in HPBSS-5 before use in the assay. In some experiments the target cells were incubated 0.5–1 h at 37°C before the last wash. Viable cells (usually >90%) were counted with eosin and diluted to 2×10^3 cells/ml.

Preliminary experiments indicated that the amount of ^{51}Cr taken up by the target cells was directly related to the concentration of the isotope and remained constant after 2 h of incubation. Each target cell contained 1.5–3 cpm releasable ^{51}Cr. The high concentration of ^{51}Cr did not appear to affect the spontaneous release or the ability of the target cells to be lysed; nor did the low concentration of target cells used in the cytotoxicity assay significantly affect spontaneous release values. In all experiments, spontaneous release ranged from 17.4 to 34.1% of the maximum release with a median of 22.6%.

Cytotoxicity Assay. Immune cells were collected from primary MLC on day 5 and from secondary cultures on day 3 after restimulation. The cells were suspended in assay medium and viable cells were assessed by phase-contrast microscopy using eosin dye. The assay was carried out in V-bottom microtiter plates (IS-MVC-96-TC; Linbro Scientific Company, New Haven, Conn.); each well contained 0.1 ml of the desired dilution of immune cells in assay medium and 0.05 ml of labeled target cells (100 cells added per well). Each immune cell dose was tested in a minimum of 24 wells, but usually in 48 or 96 wells. Target cells were also added to control wells containing medium alone, from which spontaneous release was determined, and to others containing 0.1 ml of 3.5% (wt/vol) cetrimide (Fisher Scientific Co., Fair Lawn, N. J.), from which maximum release values were determined.

Lids were then placed on the plates and they were spun at 20 *g* for 5 min after which they were placed for 6 h at 37°C in a humidified incubator with an atmosphere of 8% CO_2 in air. At the end of this incubation period, 0.05 ml of HPBSS-5 was added to each well with a Hamilton repeating syringe (Arthur H. Thomas Co., Philadelphia, Pa.), and the plates were centrifuged at 1,000 *g* for 8 min. A constant volume of supernate was removed from each well, and the amount of ^{51}Cr released into the medium was determined by counting for 2 or 4 min in a gamma spectrometer.

Since the time required for counting an experiment was usually 2–3 days, groups of tubes were counted in order of increasing expected ^{51}Cr release. Thereby the spontaneous release values are slightly higher in relation to the experimental values, and the sensitivity is somewhat lower in

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groups with most killing. No further correction for radioactive decay was applied.

In each experiment the cytotoxic activity of immune cells was also tested simultaneously in a standard assay containing 10^4 target cells and immune cell:target cell ratios ranging from 20:1 down to 0.08:1 in triplicate. Plots of percent specific ^{51}Cr release as a function of the dose of effector cells from each experiment were superimposable. Plateau levels of 60–100% specific ^{51}Cr release were reached in 6 h at a ratio of five immune cells per target cell.

Results

Detection of Cytotoxicity With Low Numbers of Immune Lymphocytes. Detection of cytotoxic activity caused by very low numbers of immune lymphocytes requires an assay sensitive enough to reveal the lysis of small numbers of target cells; i.e., the killing of as few target cells as possible should represent ^{51}Cr release values significantly above spontaneous release levels. With the labeling procedure adopted, as few as 100 target cells contained enough radioactivity to allow quantitation of released label with conventional gamma spectrometry, and under these conditions, the killing of 18 target cells in 6 h represented a value three standard deviations above the mean of spontaneous release.

Fig. 1 shows the effect of adding limiting dilutions of immune lymphocytes to 100 highly labeled target cells. With 10 immune cells, the overall frequency distribution of ^{51}Cr release is not significantly different from spontaneous release, thus setting an upper limit of 1 per 10 to the frequency of cytotoxic cells. However, in a few wells with 10 immune cells, the ^{51}Cr release was clearly different from the spontaneous release. With 30 immune cells, the overall frequency distribution differed significantly.

It is clear that as the number of cells increases, ^{51}Cr-release values also increase. However, rather than becoming a biphasic distribution with some wells clearly negative and others clearly positive, the whole distribution shifted toward higher ^{51}Cr release. This suggests that the number of target cells killed by each CTL varies over a wide range. As there was no clear demarcation between negative and positive wells, we arbitrarily defined as positive those wells in which ^{51}Cr release exceeded three standard deviations above the mean of the spontaneous release value. This is a conservative estimate which reduces the probability of false positives to less than 0.14%, and the killer cell frequency obtained must therefore be considered to be a minimal estimate.

Frequency of CTL in Immune Lymphocyte Populations. Semilogarithmic plots of the fraction of negative wells as a function of the dose of immune lymphocytes displayed a linear relationship with an intercept on the ordinate of 1.0 (Fig. 2). This is consistent with previous conclusions of others (2, 4–6) that cell-mediated cytotoxicity is the outcome of a single cell event. It follows from the Poisson distribution that the dose of cells which give 37% negative wells contains, on the average, a single CTL (7). MLC-activated *H-2*^{k} anti-*H-2*^{d} spleen cells contain a minimum of 1 CTL per 82–127 cells specific for *H-2*^{d} targets, whereas 17,000–40,000 similarly activated *H-2*^{d} anti-*H-2*^{k} cells are required to provide similar activity on *H-2*^{d} targets.

Table I summarizes all of our experiments of this type in which various strain combinations, spleen- and lymph node-responding cells, the effect of restimulation, and the killing of cross-reactive targets is compared. Part A shows that (*a*)

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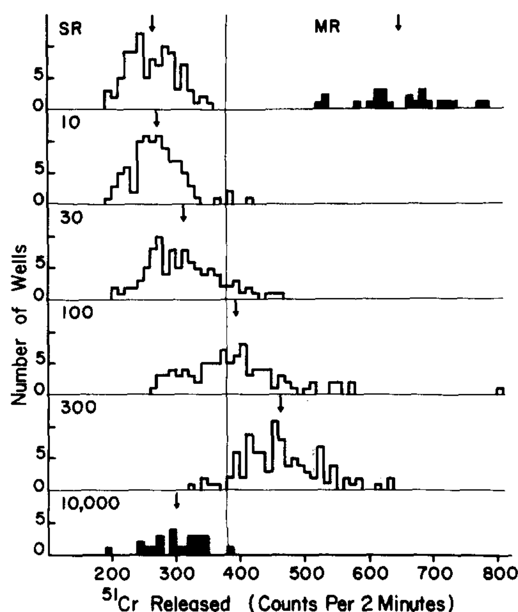

FIG. 1. Frequency distribution of ^{51}Cr release from wells with various numbers of immune lymphocytes added to 100 P815 target cells. Spontaneous release (SR) with medium alone and maximum release (MR) in the presence of cetrimide are indicated in the top panel. Second to fifth panels, C3H anti-DBA/2; bottom panel, DBA/2 anti-C3H. The mean of each distribution is indicated by an arrow. The vertical line indicates three standard deviations above the mean of spontaneous release values; wells plotted to the right of this line are considered positive, while wells plotted to the left are considered negative.

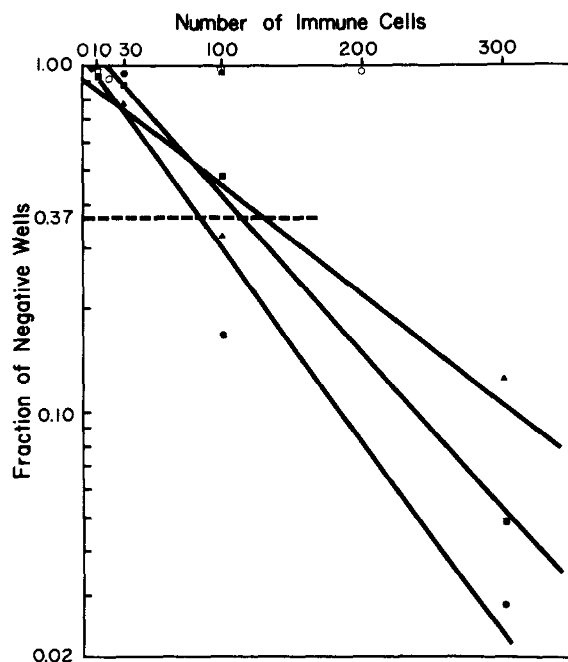

FIG. 2. Results of Fig. 1 and two similar experiments plotted as the fraction of negative wells vs. the dose of immune lymphocytes. C3H anti-DBA/2 (■); B10.BR anti-BALB/c (▲, ●); DBA/2 anti-C3H (□); and BALB/c anti-B10.BR (△, ○), all tested on 100 P815 target cells. The lines were fitted by the least squares method.

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TABLE I

	A. CTL frequency in immune lymphocyte populations							B. Average rate of killing (no. of targets/CTL hour)		
Exp.	Responder	Stimulator	No. CTL per 100 cells	Intercept	n*	r^2‡	Cell yield§	10^2 IC‖, 10^2 TC	10^3 IC, 10^2 TC	1.25×10^4 IC, 10^4 TC
							%			
1	B10.BR spleen	BALB/c spleen	1.17	1.065	4	0.950	51	3.25		3.65
	BALB/c	B10.BR	0.006	0.876	4	0.816	54			
2	B10.BR	BALB/c	0.79	0.911	4	0.946	74	4.09		5.62
	BALB/c	B10.BR	0.003	0.965	4	0.783	50			
3	C3H	DBA/2	0.88	1.187	4	0.995	67	4.24		4.99
	DBA/2	C3H	0.005	1.033	4	0.262	41			
	BALB/c	DBA/2	0.003	1.061	4	0.067	40			
4	C3H anti-DBA/2¶	DBA/2	1.07	1.298	5	0.982	242	3.56		4.91
5	C3H anti-DBA/2	DBA/2	1.08	1.146	4	0.964	350	3.34		5.59
	"	C3H	0.16	1.087	3	0.995	125	1.92	2.62	6.44
6	C3H LN	DBA/2 spleen	0.72	1.292	4	0.983	128	3.41		3.52
	"	C57BL/6	0.07	1.068	5	0.953	118	–	4.51	11.65
7	C3H LN	DBA/2	0.70	1.424	4	0.933	117	1.80		2.15
	"	C57BL/6	0.07	0.989	6	0.994	158	8.60	3.69	8.63

* n, number of points on the regression line.
‡ r^2, coefficient of determination.
§ Number of immune cells recovered from the cultures as a percentage of the initial number of responding cells.
‖ IC, immune cells; TC, target cells.
¶ Immune cells were primed in vitro for 10 (exp. 4) or 11 days (exp. 5); the cells were tested 3 days after restimulation (see Materials and Methods).

the frequency of CTL in all of the specifically activated immune lymphocyte populations tested ranged from 0.7 to 1.2%; (*b*) there was no difference in the frequency of CTL in activated lymph node and splenic lymphocyte populations, although the cell yield was twofold higher using lymph node lymphocytes as responding cells; (*c*) despite a higher yield and an earlier response in restimulated cultures, the frequency of CTL was the same as that generated in primary cultures; and (*d*)*H-2^k* anti-*H-2^b* lymphocytes, having normal activity on specific targets (data not shown) were only one-tenth as active on cross-reactive *H-2^d* targets in comparison to *H-2^k* anti-*H-2^d* immune cells. It must be re-emphasized that these CTL frequencies are minimal estimates; it is possible that the frequency of cross-reactive CTL is higher, and that each has less cytolytic activity.

Rate of Killing in Cell-Mediated Cytotoxicity. The rate of ^{51}Cr release proved to be constant over the 6-h assay period at any given dose of immune cells. Given an estimate of the minimal frequency of CTL it is therefore possible to derive a maximal estimate of the average rate of target cell killing (i.e., counts per minute above spontaneous release/counts per minute per target cell/estimated number of CTL per well/hours of culture). As shown in Table II, the average rates of killing were comparable in the assay systems which employed 10^2 and 10^4 target cells. The rapid decrease in the rate of killing as the number of immune cells increases probably reflects a decreased frequency of meaningful collisions between effector and target cells caused by increasing numbers of "bystander" lymphocytes.

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TABLE II
Average Rate of Killing at Different Doses of Immune Cells

10^2 Target cells		10^4 Target cells	
No. of immune cells	Targets killed/CTL/hour	No. of immune cells	Targets killed/CTL/hour
10	2.10	7.8×10^2	19.31
30	5.13	31.3×10^2	10.98
100	4.24	125×10^2	4.99
300	2.19	500×10^2	1.62
1,000	0.67	$2{,}000 \times 10^2$	0.42

Results of experiment shown in Figs. 1 and 2 in which there was an average of 1 CTL per 113.4 immune cells.

Table I, part B shows the results from all our experiments, giving the rates of killing at ratios of 1 immune cell per target cell or 1 CTL per 100 target cells. The killing rates vary over the range 1.8 to 11.65 targets/CTL/hour with a median of 4 targets/CTL/hour, and they are comparable for lymph node and spleen cells, as well as for restimulated cells.

Discussion

The present study combines the sensitivities of a limiting dilution procedure for immune cells and of ^{51}Cr release from highly labeled target cells to derive an estimate of CTL frequency in a population of mouse lymphocytes activated to H-2 alloantigens in bulk MLC. Using this assay procedure, we showed that (*a*) between 0.7 and 1.2% of surviving cells in an MLC-activated population are cytolytically active (Fig. 2, Table I), and (*b*) from this CTL frequency estimate, the maximal rate of target cell killing at immune cell:target cell ratios of 1:1 could be calculated to be 4 targets/CTL/hour (Tables I and II). These conclusions rest on the single assumption that significant ^{51}Cr release can be caused by a single killer cell. This assumption could not be verified experimentally since the frequency distribution was not biphasic, showing clearly positive and negative wells (Fig. 1). Therefore, it must be re-emphasized that the estimates of CTL frequency provided are minimal; clearly more CTL could be present, each causing less ^{51}Cr release.

Our estimate that 1% of an MLC-activated lymphocyte population are killer cells is consistent with previous studies of CTL frequencies. Golstein and Blomgren (8) demonstrated that as few as 10^3 highly purified T lymphocytes from an educated thymus cell population displayed significant cytotoxic activity. Moreover, with a recently developed plaque assay for cytotoxic cells it was found that between 0.05 and 2% of the cells were active in alloimmune lymphocyte preparations (9). Wilson (2) and Henney (4), employing the assumption that cytolytically active cells undergo only one round killing, suggested that the frequency of killer cells present in lymphocyte populations obtained from an immune animal may be as high as 1–4%. Several studies have recently appeared in which immune lymphocytes are allowed to interact only once with target cells and form complexes, and the CTL frequency is then estimated from the frequency of complexes and the resulting number of target cells killed. The

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estimates of the frequency of CTL range from 0.7 to 2.2% (10) to 6 to 10% (11) in the spleen and from 6 (6) to 35% (11) among peritoneal exudate lymphocytes from immune donors.

CTL frequencies obtained with MLC-activated lymphocyte populations do not necessarily reflect the total activity that can be obtained with other procedures of sensitization. Thus the very high frequency of CTL among peritoneal exudate lymphocytes probably reflects both the comparative efficiency of in vivo sensitization and the selective migration of activated cells to a peritoneal site of inflammation.

Knowledge of the absolute frequency of CTL in a given immune lymphocyte population is important in establishing two other parameters of cell-mediated cytotoxicity. Firstly, the average number of target cells killed by a single CTL during the assay period can be calculated. The killing rate thus obtained (Tables I and II) is consistent with estimates provided from direct observations (5). Secondly, from the absolute frequency of CTL and of precursors of CTL, it is possible to obtain estimates of average clone size after activation of a single precursor, as described in the accompanying paper (12).

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Summary

A sensitive procedure employing limiting dilution of activated lymphocytes and ^{51}Cr release from highly labeled target cells was used to derive minimal estimates of the absolute frequency of cytotoxic T lymphocytes (CTL) present in a population of mouse lymphocytes activated to alloantigens of the major histocompatibility complex in bulk mixed lymphocyte cultures. From this figure (0.7–1.2%), the maximal rate of target cell killing could be calculated to be approximately 4 targets/CTL/hour.

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