RECOGNITION OF POLYMORPHIC H-2 DOMAINS BY T LYMPHOCYTES

I. Functional Role of Different H-2 Domains for the Generation of Alloreactive Cytotoxic T Lymphocytes and Determination of Precursor Frequencies

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H-2 antigens encoded by the K and the D end of the major histocompatibility complex (MHC)[1] are the primary target structures for alloreactive cytolytic T lymphocytes (CTL) (1–3). However, little is known about the repertoire of H-2-specific CTL and about which parts and epitopes of the H-2 antigens are recognized by CTL.

Recently (4) we were able to identify with a panel of monoclonal antibodies (mAb) two spatially separated domains on the H-2K^k molecule that harbor the alloantigenic sites defined by mAb (see Fig. 1). Using target inhibition of H-2K^k-specific CTL generated in a bulk culture system, we found that antibodies to one domain (cluster B) exerted a much stronger inhibitory effect than antibodies to antigenic determinants in cluster B, indicating a preponderance of CTL with specificity for cluster B (5). The presence of each individual mAb during the effector phase resulted in only partial blockade of the heterogeneous CTL population, whereas a mixture of all mAb could almost completely inhibit target cell lysis. These results suggest that the CTL are multiclonal and heterogeneous and that different epitopes on the H-2K^k antigen are recognized by different CTL clonotypes.

To verify this conclusion in the present report the fine specificity and repertoire of CTL clones were investigated. CTL clones were generated in a limiting dilution system that allows rapid analysis of hundreds of clones. Our results demonstrate the existence of at least three different CTL subpopulations against the H-2K^k molecule differing not only in their precursor frequencies but also in their repertoire. Thus the majority of clones in a high frequent subpopulation recognize almost exclusively determinants of cluster A whereas the less frequent population is predominantly specific for cluster B. The generation and expression of these CTL subpopulations appears to be controlled by as yet unknown regulatory mechanisms.

Materials and Methods

Mice. DBA/2 (H-2^d) and A/J (H-2^a) mice were purchased from Miles Yeda, Rehovot, Israel. B10.D2 (H-2^d) and B10.A (H-2^a) mice were supplied by the German Cancer Research

[1] *Abbreviations used in this paper:* Con A, concanavalin A; CTL, cytotoxic T lymphocyte; CTL-P, CTL precursor; H-2, murine major histocompatibility complex; LPS, lipopolysaccharide; mc, monoclonal; mAb, monoclonal antibody; MHC, major histocompatibility complex; TCGF, T cell growth factor.

TABLE I

Properties of mc BALB/c Anti-H-2^k Antibodies

Hybridoma	Ig class	Haplotypes	Determinant*	Cluster*
H100-5/28	IgG2a	K^k, r, q (D^k)	m3	A
H100-27/55	IgG2a	K^k, r (D^k)	m4	A
H116-22/7	IgG2a	K^k, f, q, s, r, b	m1	A
H100-30/33	IgG2b	K^k, s, r, b, q (D^k)	m5	B
H142-23/3	IgG2b	K^k, K^b, q, r, s	m9	B
H142-45/2‡	IgM	K^k, K^b, f, q, r, s	m10	B

All hybridomas have been described in detail (8).

* See Figure 1. The assignment to clusters is described in reference 4.

‡ H142-45/2 has been erroneously mentioned to belong to the IgG2b class (8).

Center animal breeding unit, Heidelberg, Germany. Within a given experiment only male or female mice in the age range of 6–16 wk were used.

Media. Spleen cell suspensions and target cells were prepared and washed in RPMI 1640 supplemented with 5% heat-inactivated (56°C, 1 h) fetal calf serum. CTL microcultures were set up in RPMI 1640 containing 10 mM HEPES, 5×10^{-5}M 2-mercaptoethanol, 1% penicillin-streptomycin, 2 mM L-glutamine, and 10% heat-inactivated fetal calf serum.

For the preparation of T cell growth factor (TCGF), Sprague-Dawley rat spleen cells were cultivated at 5×10^6/ml in 70 ml culture medium including 5 μg/ml concanavalin A (Con A) (6). After 24 h incubation supernates containing TCGF were collected by centrifugation, aliquoted, and stored at −70°C. The efficiency of each batch was tested by its ability to support T cell proliferation in a costimulator assay according to Paetkau et al. (7). Optimal results were obtained between 1:10 and 1:20 dilutions of TCGF. Conditioned medium for the experiments reported here was supplemented with optimal concentrations of Con A supernate.

Antibodies. Ascites fluids of six monoclonal BALB/c anti-H-2K^k antibodies were used. They have been described in detail previously (8) and their most important properties are summarized in Table I. Of these, m3, m4, and m1 define cluster A (see Fig. 1) whereas m5, m9, and m10 belong to cluster B on the H-2K^k molecule (4). Alloantiserum reactive to H-2K^k was raised in BALB/c mice by hyperimmunization with A/J spleen cells.

Limiting Dilution System for Generation of Alloreactive CTL Clones. Responding spleen cells (DBA/2 or sometimes B10.D2) were stimulated in vitro with alloantigen (A/J splenocytes or in some experiments B10.A) in round-bottomed microtiter trays (Linbro Chemical Co., Hamden, Conn.). Each well contained graded numbers of responder cells ranging from 40 to 10,000 cells/well and 1×10^6 irradiated spleen cells as stimulators (2,200 rad from a CO^{60} source) in 200 μl conditioned medium (containing 5–10% TCGF). For each cell dose, 32–96 replicate cultures were set up. The plates were cultured for 7 d at 37°C in a humidified incubator containing 5% CO_2 in air.

Target Cells. The C3H lymphoma line L929 and the A/J T cell line A115 (provided by Dr. Fathman, Mayo Clinic, Rochester, Minn.) were maintained in tissue culture. Lipopolysaccharide (LPS) blasts were prepared by incubation of 2×10^6/ml spleen cells in 6 ml of culture

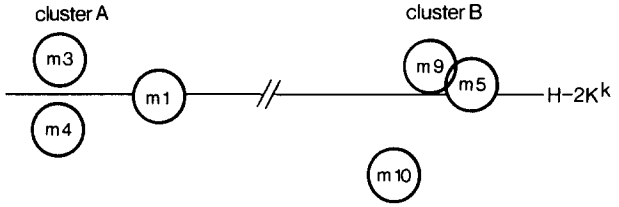


FIG. 1. Schematic diagram of the topographical arrangement of H-2K^k epitopes. Previous competitive binding studies with six monoclonal anti-H-2K^k antibodies showed that antigenic determinants are located in two spatially separated clusters on the H-2K^k molecules (4).

medium supplemented with 20 μg/ml LPS in Falcon flasks (Falcon Labware, Oxnard, Calif.) for 3 d.

Cytotoxicity Assay and Target Inhibition. At the end of the 7-d culture period the microcultures were divided into two equal parts and transferred to corresponding microwells. After centrifugation of the plates the culture medium was flicked off. The cell pellets were resuspended in 200 μl of assay medium containing 1×10^4 ^{51}Cr-labeled H-2K^k target cells per milliliter. For target inhibition by antibody, one set of the microwells received target cells that had been preincubated at a density of 1×10^5 cells/ml with an appropriate amount of monoclonal antibody at 37°C for 45–60 min. The cultures were centrifuged at 200 *g* for 5 min and then incubated for 4–5 h at 37°C. Cytolytic activity of each microwell was estimated by determination of released ^{51}Cr in 0.1 ml supernate. Control microcultures initiated in the absence of responder cells were assayed in the same manner for evaluation of spontaneous ^{51}Cr release. Wells were scored as responding when the ^{51}Cr release exceeded the mean spontaneous ^{51}Cr release by >3 SD.

Calculation of Limiting Dilution Results. The limiting dilution assay can be analyzed according to the Poisson probability distribution (9). The zero order term of the Poisson equation is linearized to the form $\ln y = -fx + \ln a$. Thus, the semilogarithmic plot of the proportion of nonresponding wells vs. the responder cells per culture should display a linear relationship. Providing single hit kinetics, the *y*-axis intercept (a) equals 1.0, the negative slope (−f) resembles the frequency of the CTL precursor (CTL-P). Experimental *x* and *y* values were fitted by the least squares method. For multiphasic curves only those values belonging to a linear part of the curve were used for calculation.

Frequencies of CTL inhibitable by mAb were determined in an analogous manner. In this case microcultures in which inhibition could be observed contained the relevant CTL-precursor. Thus, as positive wells only those displaying inhibition of their cytolytic activity due to the presence of mAb were scored, whereas the fraction of nonresponding cultures also included those wells that contained CTL activity but were not blocked by mAb. These calculations allow an estimate of the CTL-P directed against those determinants that are sterically covered by a particular mAb.

Results

Frequencies of H-2K^k-specific Alloreactive Cytotoxic T Lymphocyte Precursors. Results of limiting dilution analysis of H-2K^k-specific CTL precursors are presented in semilogarithmic plots with the fraction of cultures with no cytotoxic activity expressed as a function of responder cell dose. In a large set of limiting dilution experiments we consistently observed three distinct patterns of Poisson plots, one of which was triphasic, and the other two different biphasic types of curves. Typical examples are shown in Fig. 2. It can be seen from Fig.2 a that the experimental values display a triphasic distribution with three sets of points each fitting to a straight line. All lines cross the ordinate at ~1.0, suggesting that only one cell type is limiting. We assume the limiting units to be three different types of CTL precursors.

Input of low cell numbers resulted in the first linear part of the curve representing a high frequent type of precursor cell. At responder cell concentrations >600 cells/culture, the proportion of positive wells decreases with increasing cell dose, suggesting the influence of a suppressive mechanism on the frequent cell population. Cultivation of 1,000–4,000 cells/well resulted in a second linear increase of responding cultures, suggesting the existence of another CTL population. This second CTL subset also became suppressed at higher cell input (4,000–6,000 cells/well), which allowed the demonstration of a third CTL population with low frequency.

According to the Poisson distribution, cell doses that give 37% negative wells contain a single CTL precursor. Thus, 1 out of ~400 alloactivated H-2^d anti-H-2^a spleen cells was found to be a precursor of the high frequent killer cell subpopulation

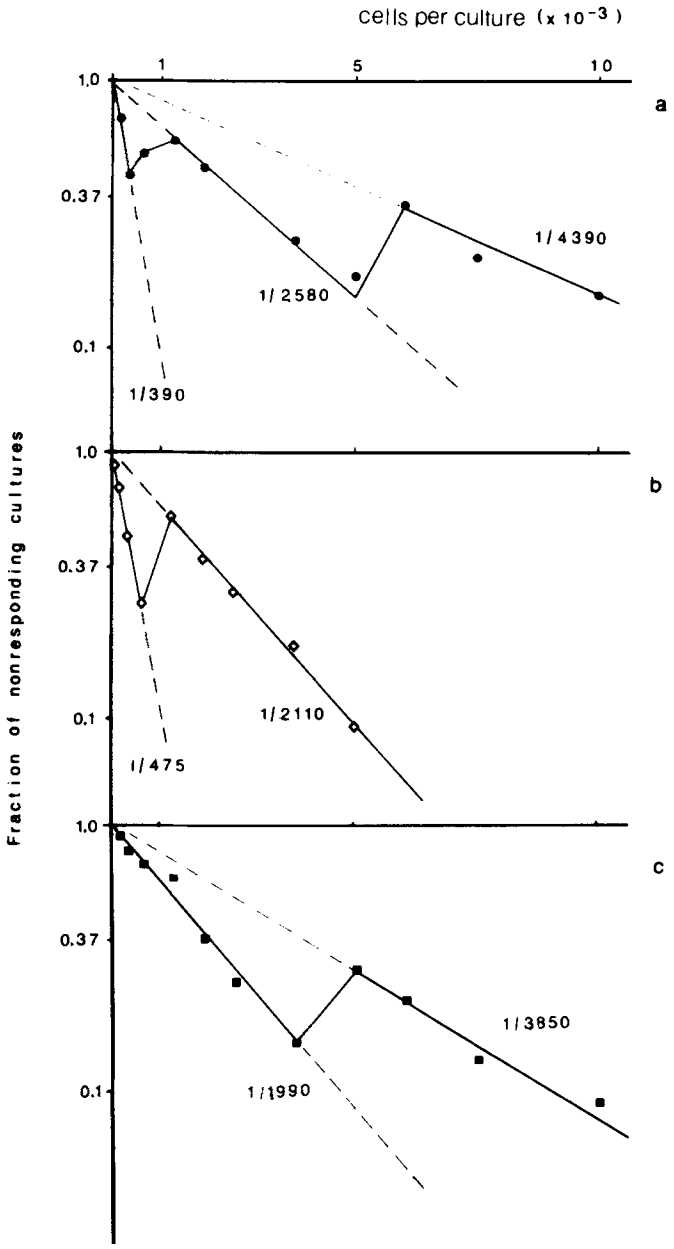


FIG. 2. Different H-2K^k-specific CTL precursor subpopulations revealed by limiting dilution analysis. 32–96 microcultures with DBA/2 responder splenocytes (50–10,000/well) were sensitized with 1×10^6 A/J stimulator splenocytes for 7 d in the presence of TCGF. Each well was tested for cytolytic activity against ^{51}Cr-labeled L929 targets in a 4 h assay. Cultures were scored positive when the ^{51}Cr release exceeded the spontaneous release by at least 3 SD. Fig. 2a, b, and c show representative types of different multiphasic Poisson distribution curves obtained in a large number of experiments. In Fig. 2a the frequencies of precursor subpopulations I, II, and III can be seen. In experiment 2b only CTL_I and CTL_{II} were found, while in experiment 2c only CTL_{II} and CTL_{III} could be observed.

(CTL-P_I) whereas the frequency of the second subset (CTL-P_{II}) is in the range of 1/1,200–1/3,000. The precursor frequency of the thir population (CTL-P_{III}) is in the range of 1/3,000–1/5,000. In most experiments only two populations could be demonstrated in the Poisson analysis. Fig. 2 b represents one type of biphasic curve. From the two linear parts of the curve precursor frequencies can be determined that are comparable with the estimations for CTL-P_I and CTL-P_{II} (see Table II). Plotting of the ^{51}Cr release of each single microculture in type 2 b biphasic Poisson curves against the responder cell number reveals a triphasic pattern reminiscent of the one shown in Fig. 2 a. Thus a marked decrease of cytolytic activity can be observed at cell doses between ~4,000–7,000, indicating that the CTL-P_{II} population is also subject to suppression in these experiments (data not presented). At higher cell doses the cytolytic activity increases, again suggesting the existence of a third set of CTL-P, probably identical to CTL-P_{III} as described in Fig. 2 a. Because suppression does not completely suppress cytolytic activity it cannot be seen in a Poisson plot, which scores only positive and negative events.

Poisson analysis of some other experiments resulted in a biphasic distribution with frequency determinations of ~1/1,600 and 1/4,000, respectively, as shown in Fig. 2 c. These frequency estimations and the pattern of fine specificity described below suggest that in those experiments only the second and third subpopulation were detected, whereas the first population was missing. It should be mentioned that in some rare cases the experimental values fitted only to one linear regression line. The reasons for the variations of our limiting dilution experiments are not clear. Patterns and CTL precursor frequencies as determined in a representative number of experiments are summarized in Table II.

Target Inhibition by Monoclonal (mc) Anti-H-2 of Clonal CTL Generated in a Limiting Dilution System. To investigate on a clonal level which H-2 epitopes or domains are recognized by CTL, target inhibition with mc anti-H-2 was performed in the limiting system. For determination of ^{51}Cr release in the presence and in the absence of mAb the microcultures had to be split into two equal parts. Since accuracy is essential for

TABLE II

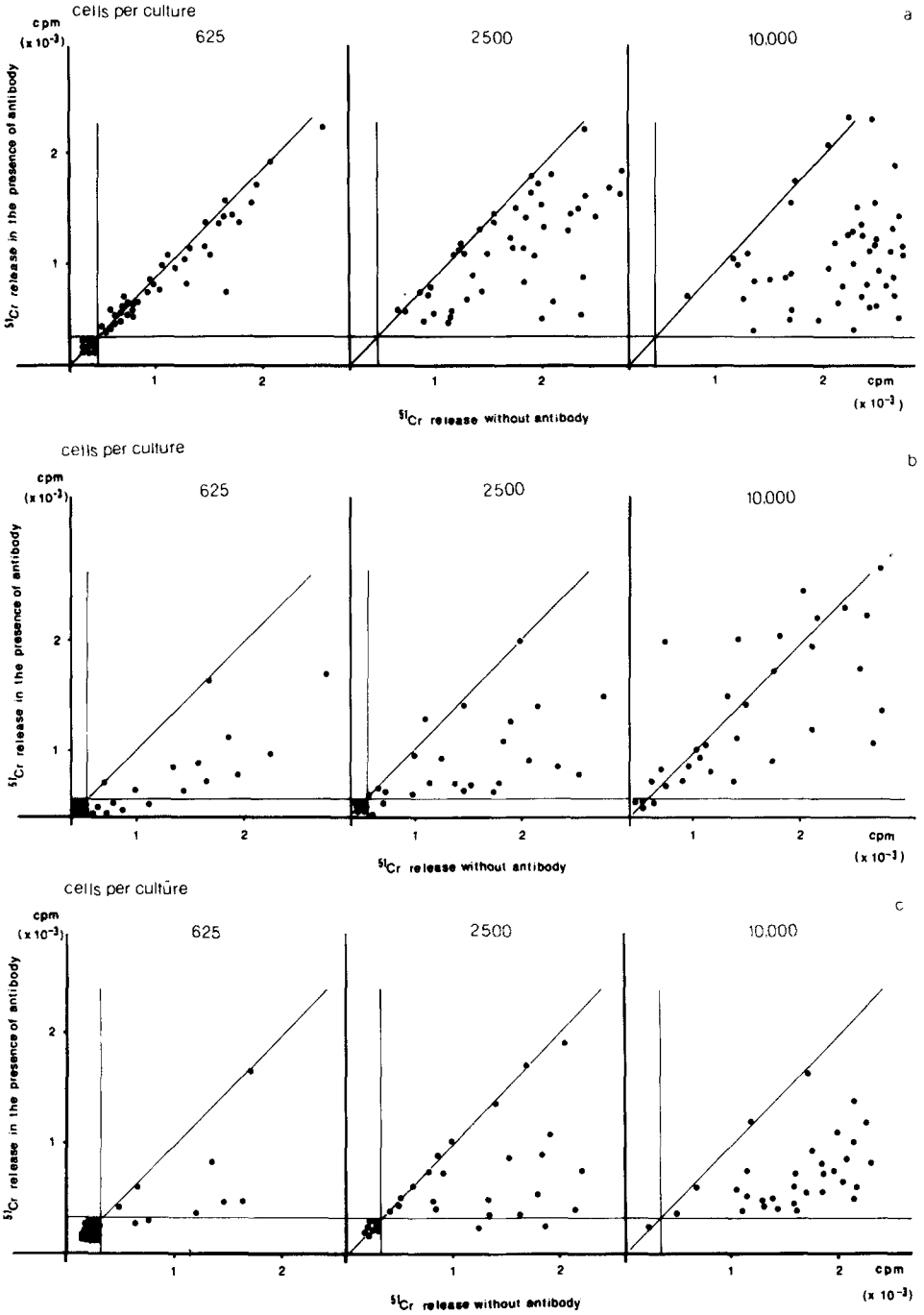
Summary of Limiting Dilution Experiments Demonstrating Different Subsets of $H\text{-}2^d$ Anti-H-2K CTL-P*

Type of limiting dilution curve*	Number of experiments	Range of frequencies		
		CTL_I	CTL_{II}	CTL_{III}
Triphasic (2 a)	5	1/320-1/400	1/2,500-1/3,060	1/4,300-1/5,400
Diphasic (2 b)	19	1/310-1/520	1/1,230-1/2,590	-‡
Diphasic (2 c)	28	-§	1/1,090-1/2,310	1/2,980-1/4,900

* Prototypes are shown in Fig. 2 a, b, and c. In some additional (5) experiments only a straight line was observed, however, in these cases a dot plot (chromium release plotted over responder cell input) revealed several subsets.

‡ CTL_{III} was not found in the Poisson distribution curves, but could be seen in dot plots.

§ Population CTL_I was not observed.



these studies, it was established in orientating experiments that the splitting procedure resulted in the same amount of chromium release (±10%) in both aliquots when tested independently against the same target cells. By using different H-2K^k target cells (A115 tumor cells and B10.A LPS blasts), as well as B10.A and B10.D2 LPS blasts for

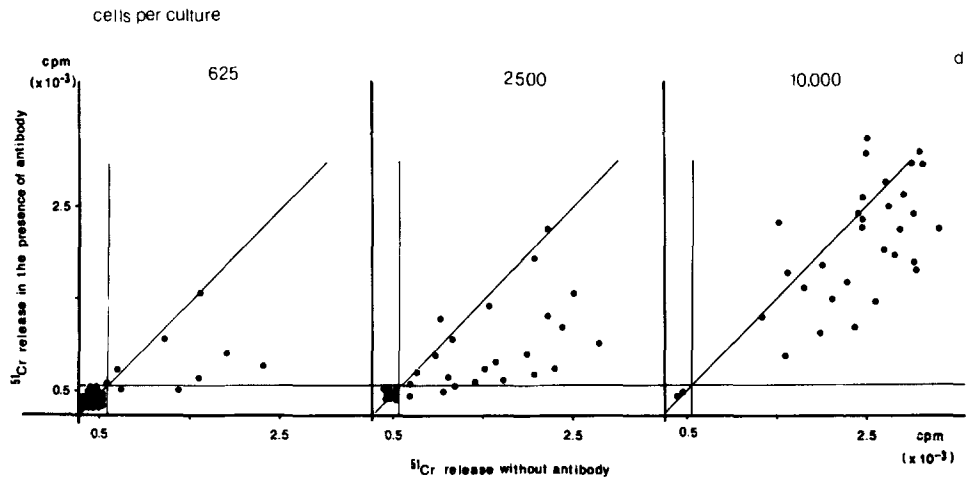


FIG. 3. Target inhibition by mc anti-H-2K^k of CTL clones in H-2K^k-specific subpopulations. CTL clones generated by limiting dilution were split into two aliquots and cytolytic activity was determined against H-2^k targets in the absence and presence of mc anti-H-2K^k. ^{51}Cr release in the presence of mAb is plotted over ^{51}Cr release in the absence of antibody. Inhibited wells appear below the diagonal. Fig. 3a and b demonstrate target inhibition in an experiment of the biphasic type shown in Fig. 2b. 625 cells/well represent CTL$_I$, 2,500 cells/well represent CTL$_{II}$, and 10,000 cells/well represent CTL$_{III}$. CTL$_{III}$ is not revealed by the Poisson analysis, but by the absolute chromium release values. 3a displays inhibitions with H142-45, a representative of cluster B; 3b, inhibition by H100-27/55, belonging to cluster A; Fig. 3c and d show target inhibition in an experiment of the biphasic type shown in Fig. 2c. Since the frequent population CTL$_I$ is missing in this experiment, input doses of 625 and 2,500 cells/well represent CTL$_{II}$ whereas 10,000 cells/well represents CTL$_{III}$. Fig. 3c indicates inhibition by H142-45 (cluster B) and Fig. 3d, inhibition by H100-27/55 (cluster A).

cold target inhibition, it was also verified that all CTL clones generated in the DBA/2 anti-A/J limiting dilution system were indeed specific for the H-2K^k molecule. Potential K cell activity was also excluded by assaying anti-H-2K^b CTL on H-2K^k targets in the presence of mc anti-H-2K^k. The optimal concentration for target inhibition with various mc anti-H-2K^k was determined by blocking of K^k-specific CTL generated in a bulk culture. In general targets were preincubated with mAb ascites fluid at dilutions of 1:50–1:100 (5).

For target inhibition of limiting dilution clones, microcultures were split and both aliquots simultaneously tested for killing ability on chromium-labeled L929 tumor targets that had been preincubated with either medium alone or with an optimal amount of mAb ascites fluid. Representative results of those assays for two mAb belonging to the two different clusters are depicted in a two-dimensional plot in which the chromium release in the presence of antibody is plotted against release without antibody (Fig. 3a–d). Three characteristic input doses of 625, 2,500, and 10,000 cells/culture were chosen which represent the three different subpopulations CTL$_I$, CTL$_{II}$ and CTL$_{III}$, respectively, as described in Fig. 2. In this plot microcultures in which CTL effector function was blocked by mAb are represented by symbols located below the diagonal, and cytolytic wells not affected by mAb are located on the diagonal. In cases of partial inhibition, wells were classified as inhibited when the chromium release was diminished by >25% compared with the control.

It is obvious from Fig. 3 that the antibodies exhibit striking differential target

inhibition effects on the three different CTL subpopulations. Thus, antibody H142-45/2 (anti-m10; cluster B) efficiently prevents target cell lysis at high responder concentrations representing CTL_{III} (Fig. 3 a, right panel). At intermediate cell numbers (2,500, middle panel), representative of CTL_{II}, the effect is less pronounced. On the other hand, the first and most frequent CTL subpopulation, represented by an input dose of 625 cells/well, is not blocked by the same antibody (Fig. 3 a, left panel).

In contrast are the inhibition results with mAb H100-27/55 (anti-m4), representative of cluster A. The data are depicted in Fig. 3 b. The majority of CTL clones generated with low responder cell numbers, accordingly progenitors of $CTL\text{-}P_I$, are blocked by sufficient antibody concentrations, whereas only a small proportion of CTL effector cells developed from $CTL\text{-}P_{III}$ is influenced by the presence of antibody H100-27/55. An intermediate inhibition is observed at cell doses correlating with $CTL\text{-}P_{II}$.

Fig. 3 c and d represent target inhibition of limiting dilution experiments in which only two CTL subsets could be demonstrated (see Fig. 2 c) and which, according to their frequency, correspond to subpopulations CTL_{II} and CTL_{III}. The target inhibition pattern confirmed this assumption. The mAb H142-45/2 displayed a strong inhibitory capacity of clones generated at high cell input, but only medium inhibition at inputs of 625 and 2,500 cells/well, both of which, in this experiment, represent population II (see Fig. 3 c). On the other hand, H100-27/55 showed only marginal inhibition at high cell doses and medium inhibition at lower cell doses (see Fig. 3 d). Thus, both frequency and fine specificity analysis suggest that in these experiments the subpopulation CTL_I is missing. Target inhibition of limiting dilution experiments of the biphasic type depicted in Fig. 2 b clearly demonstrated that these two populations represent CTL_I and CTL_{II} (data not shown). For the sake of brevity only some typical blocking experiments are shown.

Quantification of the Inhibitory Effect of Different mAb on CTL Clones. Experimental data of target inhibition studies as given in Fig. 3 indicate that the killer populations generated in the limiting dilution system are composed of clones that differ with respect to their fine specificity. For dissection of the receptor repertoire of each subset, we determined precursor frequencies of those CTL directed against particular epitopes of the K^k molecule, the killing ability of which is blocked directly or by steric inhibition by a corresponding mc anti-H-2. Thus, in a Poisson analysis the number of responder cells per well is plotted semilogarithmically against the fraction of those cultures lacking the relevant precursor cell. In this case the fraction of nonresponding wells is defined as those wells that do not contain CTL, plus those wells that are cytolytic but not inhibited by antibody. The precursor estimations obtained by the inhibition analysis can be compared with the frequencies of total CTL-P directed against the $H\text{-}2K^k$ molecule, which are determined from the corresponding part of the split microcultures in the same experiment.

Representative results for mAb H100-5/28 and H142-23/3, which belong to the two different clusters on the K^k molecule, are depicted in Figs. 4 and 5. Both experiments are of the type shown in Fig. 2 c, in which the CTL_I population is missing. The upper panels (4 a and 5 a) show the frequency of the sum of all CTL clones in populations CTL_{II} and CTL_{III} (no target inhibition), and the lower panels (4 b and 5 b) describe the Poisson distribution of only those CTL directed against H-2 regions or epitopes covered by antibody. In both cases (Figs. 4 b and 5 b), the

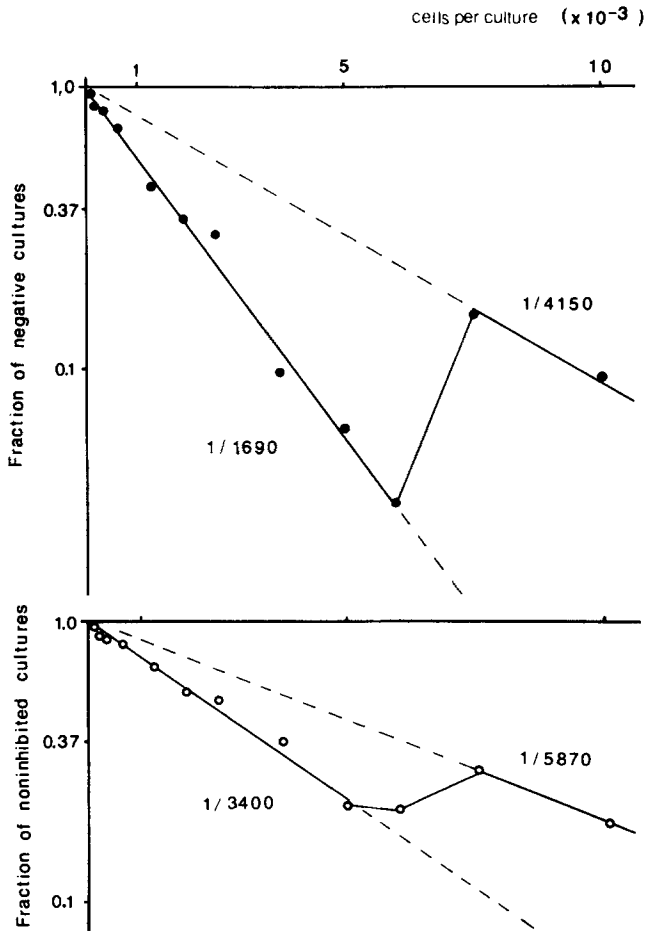


FIG. 4. Determination of frequency of CTL precursors directed against that epitope or part of the H-2K^k molecule covered by mc anti-H-2 H142-23/3 (cluster B). Limiting dilution wells were split and cytolytic activity determined in the absence and presence of mAb. Fig. 4a shows a biphasic Poisson distribution curve of the type 2c (see Fig. 2c) with frequencies of CTL-P against the sum of all H-2K^k determinants (no target inhibition by antibody), whereas Fig. 4b shows frequencies of CTL-P against antibody-covered sites. Calculations were performed as described in Materials and Methods.

semilogarithmic plots of the fraction of noninhibited wells vs. the responder cell input describe two sets of points that fit to two straight lines crossing the ordinate at ~1.0. We conclude that the Poisson distribution is correctly used to analyze the phenomenon of inhibition, the limiting event being the precursor cell of killers inhibited by an mAb. Both antibodies (H142-23/3 and H100-5/28) block ~50% of the CTL clones belonging to CTL$_{II}$. In contrast, 70% of CTL$_{III}$ clones are inhibited by mAb 142-23/3, whereas only 30% are blocked by mAb H100-5/28. Analogous target inhibition studies with all six mAb show that these distinctive reactivity patterns are characteristic of the two alloantigenic clusters A and B.

Averaged data of all inhibition experiments are summarized in Table III. It is obvious from Fig. 3 and Table III that there is a shift in the inhibitory effect of mAb

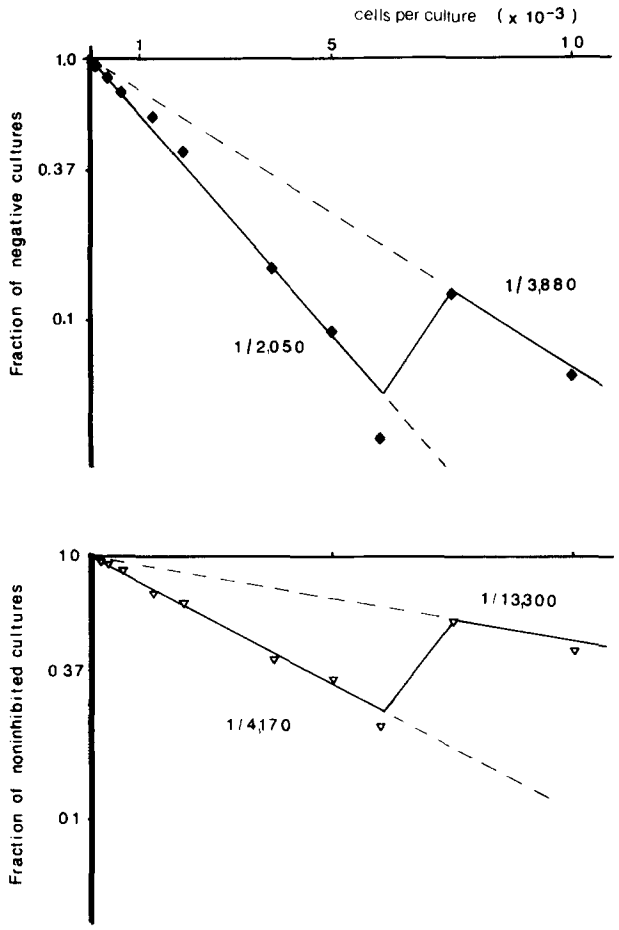


FIG. 5. Frequency of CTL precursors directed against the sum of all H-2K^k epitopes (a) and against sites covered by antibody H100-5/28 (cluster A) (b). The experiment shows a biphasic curve of the type 2c (Fig. 2c) in which only CTL$_{II}$ and CTL$_{III}$ are observed. See also legend to Fig. 4.

with regard to different CTL subpopulations. Thus, antibodies belonging to cluster A display a strong inhibition of CTL$_I$, an intermediate inhibition of CTL$_{II}$, and a rather weak inhibition of CTL$_{III}$. The opposite pattern was found for antibodies describing cluster B. The effect on CTL$_I$ was hardly detectable, while most of CTL$_{III}$ clones are blocked. In addition, Table III includes a summary of inhibition experiments with CTL generated in a bulk culture that shows, similarly to the CTL$_{III}$ population, a dominance of CTL with specificity for cluster B.

It is also noted in Table III that a mixture of all mAb as well as an anti-H-2K^k alloantiserum is highly effective in target cell blockade for all different CTL populations, confirming that the vast majority of CTL is indeed reactive against H-2K^k epitopes.

Discussion

A surprisingly large fraction of T cells are CTL-P cells that respond against foreign H-2 antigens (10–14). Information about the quantitative contribution of different

TABLE III

Summary of Target Inhibitions With MC Anti-H-2K^k of H-2K^k Specific CTL Clones and CTL Generated in Bulk Culture

Inhibitor	Cluster	Percent of clones inhibited by CTL subpopulations in limiting dilution*			Inhibition of CTL from bulk culture LU/10^6 cells‡
		CTL_I	CTL_{II}	CTL_{III}	
-	-	-	-	-	14.1
H100-5/28 (m3)	A	74	53	29	1.03
H100-27/55 (m4)	A	52	41	24	0.84
H116-22/7 (m1)	A	48	10	8	1.33
H100-30/23 (m5)	B	0	55	65	0.35
H142-23/3 (m9)	B	0	52	68	0.35
H142-45/2 (m10)	B	0	59	72	0.12
Mixture of all mAb	A + B §	85	90	95	0.02
BALB/c ā-A/J serum	A + B	76	85	80	NT‖

* The percentage of inhibited CTL clones was calculated by comparing the frequencies of clones directed against the sum of all H-2K^k determinants with frequencies of clones directed only against antibody-covered sites (for example compare Fig. 4a with 4b or 5a with 5b).

‡ H-2K^k specific CTL were generated for 5 d in bulk culture (10×10^6 DBA/2 responders plus 12×10^6 A/J stimulators) and lytic units (LU) have been determined on the basis of CTL effector titrations in the absence and presence of mc anti-H-2K^k. One LU corresponds to the number of effectors required to lyse 50% of target cells. Data adapted from Weyand et al. (5).

§ It is assumed but has not been determined that the hyperimmune BALB/c antii-A/J alloantiserum contains antibodies to determinants of both clusters.

‖ Not tested.

clonotypes with specificity for different H-2 epitopes is still scarce, although recent work with H-2 mutants has provided new insights (15). In addition, it is not clear whether CTL recognize the same alloantigenic determinants or domains on H-2 molecules defined by alloantibodies. In the present study the receptor repertoire of H-2K^k-specific CTL was dissected on a clonal level in a limiting dilution system.

H-2K^k CTL clones were generated in a limiting dilution system and their fine specificity assessed by target inhibition with a panel of monoclonal anti-H-2K^k antibodies. The Poisson distribution analysis resulted in three straight lines indicating the existence of three precursor populations that differ in their precursor frequency (Fig. 2a). Average frequency data are 1/400 for CTL_I, 1/2,000 for CTL_{II} and 1/4,000 for CTL_{III}. The limiting dilution curves suggest that the more frequent CTL populations are subject to a suppressive mechanism. Only in this context can the less frequent populations be demonstrated in the limiting dilution system. It should be emphasized that very often we observe only biphasic curves representing in some experiments CTL populations I and II and in others CTL_{II} and CTL_{III}. The reasons for these variations are not clear but the quality of TCGF, the immune status of mice, etc., may contribute to the generation of particular subpopulations. Frequency determinations of CTL-P by other authors also show considerable variations and lie in a range of 1/170–1/3,000 (10–14, 16). It is possible that in these experiments

different subpopulations of CTL have also been generated depending on culture conditions, etc.

At first glance one is tempted either to ignore the multiphasic Poisson distribution curves as laboratory artifacts or to smooth the curves by a single best-fitting line. However, we consistently observe these multiphasic curves with comparable precursor frequency for the different CTL subsets. In addition, biphasic limiting dilution curves have also been reported by Goronzy et al. (16) for CTL precursors activated polyclonally by Con A. It is not clear why such curves have not been described by more investigators. Goronzy et al. stated that they did not find multiphasic curves when responders were stimulated by a whole H-2 difference, whereas in our experiments CTL are only activated against the K end. H-2 haplotypes and strain combinations may also play a role. Allotype does not seem to be important because limiting dilution analysis of B10.D2 anti-B10.A CTL resulted in multiphasic curves as well (data not shown).

The most compelling evidence for the existence of different CTL subpopulations is our observation that the CTL subsets differ in their receptor repertoire, which does not seem to be randomly distributed. This conclusion was derived from target inhibition studies with a panel of mc anti-H-2K^k antibodies that have previously been shown to define two separate clusters of antigenic determinants on the K^k molecule (4). Thus mc anti-H-2K^k, which define cluster A, are highly effective in blocking the target determinants for clones from CTL_I, whereas they inhibit much less the low frequent preparation III. In a reciprocal relationship, target inhibitions obtained with mAb belonging to cluster B, which do not affect the high frequent population CTL_I, strongly block CTL_{III}. Subpopulation CTL_{II} is blocked almost equally well by both groups of antibodies. The differential inhibition pattern of mAb indicates that the inhibitory effect can indeed be related to the target specificity of both antibodies and CTL. The results could not be explained by other properties of the antibodies such as affinity. In fact all mAb display very similar affinities (17 and H. Lemke, personal communication). Antibody H142-45 is clearly the strongest inhibitor, possibly because it belongs to the IgM class while all other antibodies are IgG2a or IgG2b, respectively.

It should be mentioned that detailed immunochemical analysis (2-D gel electrophoresis, etc.) demonstrated that all mAb react with the same H-2K^k molecule (S. Koch, unpublished observations).

Application of a single antibody blocks only a fraction of CTL clones, whereas CTL target interaction was totally prevented when numerous epitopes were covered by a mixture of mAb or by an alloantiserum (Table III). In addition each antibody exhibited a characteristic inhibition pattern. Hence, each CTL subpopulation appears to consist of various different clonotypes with specificity for different target epitopes. The multitude of antigenic determinants present on alloantigens could be a possible explanation for the high frequency of alloreactive T cells. Similar conclusions have been drawn by Sherman (15), who investigated the clonal repertoire of CTL against H-2 mutant antigens.

Inhibition by mc anti-H-2 antibodies of heterogeneous CTL derived from bulk cultures has been described by several authors (18, 19). Neither these studies nor the present one indicate conclusively whether CTL recognize exactly the same H-2 determinants as antibodies; that is, whether T and B cells display the same receptor repertoire. The fact that for a number of CTL clones only partial inhibition can be

achieved by mAb suggests that in at least some cases sterical hindrance of antibodies bound to spatially related determinants plays a role. An alternative but less likely explanation is that antibodies block killing activity by inducing conformational changes in the H-2 molecule. But even such an assumption would not change the conclusion that the different CTL subpopulations revealed by limiting dilution analysis display distinct fine specificity for H-2 epitopes. Altogether the results provide strong evidence for the notion that alloreactive CTL recognize in general antigenic determinants within the same polymorphic domains on H-2 molecules characterized by antibodies. These domains may possibly correlate to polymorphic regions defined by amino acid sequence analysis (20).

The generation of CTL subpopulations with specificity for particular domains appears to be controlled by suppressive mechanisms and it is startling to see that a very high frequent population (CTL_I) appears to be "wasted" by suppression. In fact, the A-specific CTL population is hardly seen in a H-2^d anti-K^k CTL bulk culture dominated by CTL specific to cluster B determinants (5). Thus a bulk culture resembles CTL_{III} of the limiting dilution system. Altogether these data allow the conclusion that clones expressed in a bulk culture do not necessarily reflect the true T cell repertoire due to regulatory events.

It is not clear whether cluster A-specific CTL are also suppressed in vivo, which may provide different microenvironments. There are several reports on the generation of suppressor cells during in vitro primary alloreactive cytotoxic responses that inhibit the activation of CTL-P (21–25). The nature of the suppressive mechanism observed in our limiting dilution system is at this stage only a subject for speculation. It is possible that clones within a CTL population (e.g., CTL_I) are idiotypically similar, rendering them susceptible to a restricted spectrum of anti-idiotypic T suppressor cells. Alternatively, suppressive cells specific for epitopes of cluster A or for characteristic surface determinants on particular CTL precursor populations could exist. In view of the different specificity of the CTL subpopulations it is unlikely that they represent different stages of differentiation, e.g., unprimed and memory cells that may display distinct sensitivity to suppressor cells as suggested by Goronzy et al. (16). In any case, if indeed suppressor cells are responsible for this finding, then they must occur in a very high frequency, because suppression is already observed at doses between 500 and 1,000 cells/well.

The suppression-induced shift of the expressed repertoire observed in our limiting dilution system indicates that complicated regulatory phenomena determine those T cell clones that are eventually allowed to proliferate. Similar conclusions have been made by F. Bach (personal communication), who also found, in another limiting dilution system, that populations with a particular specificity are subject to suppression. We would like to add that in H-2-restricted CTL systems we also observed that suppressive influences determine the occurrence of CTL populations that recognize foreign antigen in association with either cluster A or cluster B epitopes (manuscript in preparation).

Summary

In the present communication, the repertoire of alloreactive cytotoxic T lymphocytes (CTL) clones was quantitatively investigated by limiting dilution analysis and by target inhibition with a panel of monoclonal antibodies (mAb). These mAb have

previously been shown to define two distinct alloantigenic domains, A and B, on the H-2K^k molecule. The Poisson distribution analysis of H-2K^k-specific CTL clones generated in a limiting dilution system revealed three CTL populations with different precursor frequencies. The high frequent population is suppressed by an unknown suppressive mechanism that allows less frequent CTL populations to become visible. Target inhibition studies with a panel of K^k-specific mAb showed that these CTL populations differ not only in their precursor frequency but also in their specificity for different H-2 epitopes on the K^k molecule. Thus clones of the high frequency population are almost exclusively specific for determinants within domain A. In contrast, the low frequency population displays predominant specificity for determinants of domain B, while the population with medium frequency is blocked equally well by mAb against either domains A or B.

Each mAb blocked only a fraction of clones indicating that each CTL subpopulation may consist of a large number of clonotypes with specificity for different H-2 epitopes. The data suggest that CTL recognize basically the same polymorphic domains on the H-2K^k molecule defined by antibodies, and they show that regulatory mechanisms determine the expressed repertoire in CTL populations.

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