

## H-2 RESTRICTION AS A CONSEQUENCE OF INTENTIONAL PRIMING

Frequency Analysis of Alloantigen-restricted,  
Trinitrophenyl-specific Cytotoxic T Lymphocyte  
Precursors within Thymocytes of Normal Mice\*

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Our understanding of the influence of the thymus on the repertoire of maturing T cells is still limited. Experimental results obtained with lymphocytes from chimeric mice have indicated that self major histocompatibility complex (MHC)<sup>1</sup>-restriction of peripheral T cells does not represent antigen-driven selection of precommitted lymphocytes, but is imposed during their intrathymic maturation phase independent of the presence of antigen (1-6). The mechanism operating is viewed as a selection process (2, 4, 7-9), whereafter the T cells that are finally exported into the periphery are selected according to the MHC type of the thymic epithelial cells (10) or macrophages (11). Because MHC-linked Ir gene responsiveness of T cells were shown not to parallel their own MHC genotype, but that of the thymus (3, 4), it was implied that intrathymically operating positive selection of MHC-restricted T cells also determines the Ir gene-controlled immunoresponsiveness.

Several recent experimental results, however, can not easily be incorporated within this conceptional framework. Not only has it been shown that *in vitro* immunocompetent T lymphocytes from fully allogeneic chimeric mice were restricted either to their own or to the allogeneic thymic MHC-type (12-15), but also cytotoxic T lymphocytes (CTL) as generated from lymphocytes that were derived from athymic (nu/nu) mice already exhibited self-MHC restriction (16, 17). Moreover, after acute depletion of alloreactive CTL precursors, the negatively selected splenic T cells from normal mice contained antigen-specific T cell clones restricted to the allo-MHC type in question (18-21). This phenomenon, which initially had been termed "aberrant recognition," implied that the thymus exports alloantigen-restricted CTL precursors and that MHC restriction of CTL is, at least in part, a consequence of priming.

This report deals with the existence of allo-MHC-restricted CTL precursors within the thymus of normal mice. Furthermore, a frequency analysis was performed to quantitate their actual numbers.

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<sup>1</sup> Abbreviations used in this paper: Con A, concanavalin A; CTL, cytotoxic T lymphocyte; CTL-P, CTL precursor; IL-2, Interleukin 2; MHC, major histocompatibility complex; TNP, trinitrophenyl.

### Materials and Methods

*Mice.* CBA (H-2<sup>k</sup>), C3H (H-2<sup>k</sup>), BALB/c (H-2<sup>d</sup>), and C57BL/6 (H-2<sup>b</sup>) mice were obtained from G. L. Bomholtgaard, Ry, Denmark.

*Cell Culture.* CTL were induced in mixed lymphocyte cultures as described (22). In short, thymocytes of the various mouse strains were used as source of responding cells, and irradiated spleen cells as stimulator cells. To bypass the requirement of T helper cells, optimal concentrations of T cell-derived helper factor (Interleukin 2) were added to the cultures as described (23). Before culture, stimulator cells were irradiated with a dose of 2,000 rad (RT 200; Philips Electronic Instruments, Inc., Mahwah, N. J.) at a dose rate of 620 rad/min.

*Induction of Trinitrophenyl (TNP)-specific CTL.* The method described by Shearer et al. (22) was used to induce TNP-specific CTL.

*Target Cells.* Lymphoblasts stimulated by the mitogen concanavalin A (Con A) (48 h; 2.5  $\mu\text{g/ml}$ ) were used as target cells. TNP conjugation of target cells was performed as described previously (24).

*Absorption of Alloreactive Cytotoxic CTL Precursors (CTL-P).* Basically, the protocol described by Schnagl and Boyle (25) was used to absorb CTL-P. In short, monolayers were constructed from spleen cells or thymocytes that had been preincubated in culture medium overnight at 37°C (viability >90%). About  $3 \times 10^7$  preincubated cells were attached to surface-treated petri dishes (35-mm diameter; 10 mm high; 8160; Greiner, Nürtingen, Federal Republic of Germany) previously coated with poly-L-lysine (70,000 mol wt, Sigma Chemical Co., Munich, Federal Republic of Germany). The monolayers were washed three times in phosphate-buffered saline, overlaid with 1 ml culture medium, and irradiated with 2,000 rad before use as absorbing monolayer. Responder cells ( $1 \times 10^7$ ) in 1 ml of culture medium were added to each monolayer. After 1 h, the nonadherent responder cells were recovered by gentle agitation and the procedure was repeated by attaching to a second monolayer. After 1 h, the nonadherent cells were recovered, washed once, and used as responder cells acutely depleted for the appropriate alloantigen. The cell recovery under those conditions was between 70 and 90% of the starting cell number.

#### *Limiting-Dilution Technique*

*CULTURE MEDIUM.* A mixture of Click's and RPMI-1640 tissue culture media (50:50) (Seromed, München, Federal Republic of Germany) was supplemented with 10% fetal calf serum, 2-mercaptoethanol ( $5 \times 10^{-5}$  M), 10 mM Hepes buffer, and an optimal concentration of Interleukin 2 (IL-2) (23). IL-2 is used to bypass the T helper cell requirement in the induction of CTL.

*T CELL-DERIVED GROWTH FACTOR (IL-2).* Mouse-derived splenic lymphocytes were cultured in serum-free medium that contained 1  $\mu\text{g/ml}$  Con A for 24 h. The culture supernate was concentrated 20-fold, eluted from a Sephadex G-100 column, and the fractions that demonstrated IL-2 activity were pooled and concentrated 20-fold (23).

*CULTURE SYSTEM.* 16–24 replicates of limiting numbers of responder lymphocytes were cultured together with  $5 \times 10^6$  irradiated (2,000 rad) stimulator cells in V-shaped microtiter plates (Greiner). The cultures were maintained for 6 d at 37°C in a 5% CO<sub>2</sub> atmosphere.

*CYTOTOXIC ASSAY AND CTL-P FREQUENCY.* Cultures were centrifuged, and the culture medium removed. To each cell pellet, 1,000 <sup>51</sup>Cr-labeled targets (~2–3 cpm/cell) were added in a total vol of 150  $\mu\text{l}$ . The plates were centrifuged for 1 min at 100 g, and incubated for 4 h at 37°C. The radioactivity released within individual wells was determined by counting 100  $\mu\text{l}$  of supernate in a gamma counter. A well was scored positive if the <sup>51</sup>Cr release exceeded the mean spontaneous release by at least 3 SD. This had been determined by incubating target cells with stimulator cells only. The percent nonresponding cultures were calculated for each responder lymphocyte concentration set up. The frequencies of CTL precursors were based on a slope determined by the linear regression analysis and fit to the Poisson equation:  $\ln y = -fx + \ln a$ , where  $y$  = the percentage of nonresponding cultures,  $f$  = CTL-P frequency equal to the negative slope, and  $a$  =  $y$  axis intercept (26).

### Results

*Demonstration of MHC-restricted CTL-P within Thymocytes.* Previous work of this (23, 27, 28) and other (26) laboratories have described high CTL reactivity within

thymocytes, provided the thymic responder cells were sensitized in the presence of activated T-helper cells (28–31) or a T-helper cell product, IL-2 (26, 28, 32–35). This refers to both alloreactive as well as H-2-restricted, TNP-specific CTL responses (26, 27). Table I depicts frequency data of alloreactive and H-2 restricted TNP-specific CTL-P in the thymus and spleen of different inbred mice.

As a first step, we wished to establish whether the acute in vitro depletion protocol described previously (21) would allow selective removal of alloreactive CTL-P within thymocytes. The results given in Fig. 1 show evidence that after the plating of BALB/c thymocytes on H-2<sup>k</sup> monolayers, the nonadherent thymocyte population had lost the capacity to mount anti-H-2<sup>k</sup> CTL responses, whereas responses to third-

TABLE I  
Frequencies of Alloreactive and H-2-restricted, TNP-specific CTL-P

Responder cells	Stimulator cells	Number of determination	Frequency	Range
C57BL/6 thymocytes	BALB/c (H-2 <sup>d</sup> )	10	1/10,580	1/5,000–1/20,600
CBA/J thymocytes	C57BL/6 (H-2 <sup>b</sup> )	6	1/24,800	1/18,000–1/31,000
BALB/c thymocytes	CBA/J (H-2 <sup>k</sup> )	3	1/47,300	1/45,000–1/54,000
C57BL/6 thymocytes	C57BL/6 (TNP)	6	1/11,000	1/5,300–1/20,000
CBA/J thymocytes	CBA/J (TNP)	7	1/13,600	1/8,500–1/20,000
BALB/c thymocytes	BALB/c (TNP)	4	1/28,700	1/20,000–1/37,000
C57BL/6 spleen cells	BALB/c (H-2 <sup>d</sup> )	10	1/582	1/300–1/1,000
C57BL/6 spleen cells	C57BL/6 (TNP)	5	1/9,090	1/1,500–1/15,000

CTL-P frequencies within thymocytes of various strain combinations were determined by linear-regression analysis (26). For each responder cell concentration, 16 wells were set up and cultured for 6 d. Thereafter, individual microcultures were tested for specific <sup>51</sup>Cr release in a 4-h assay. As target cells, P815 (H-2<sup>d</sup>), LS (H-2<sup>k</sup>), or EL<sub>4</sub> (H-2<sup>b</sup>) tumor cells were used. The spontaneous <sup>51</sup>Cr release of the target cells never exceeded 15%.

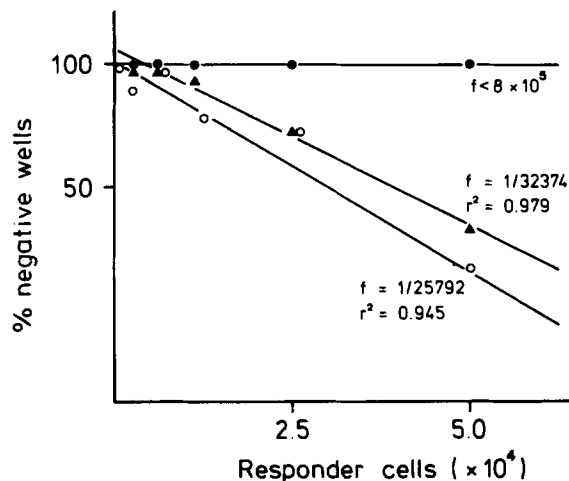


FIG. 1. Thymocytes of BALB/c (H-2<sup>d</sup>) mice were first negatively selected for anti-H-2<sup>k</sup> alloreactivity, and subsequently cultivated under limiting-dilution conditions either with H-2<sup>k</sup> (●) or H-2<sup>b</sup> (▲) stimulator cells. In addition, unselected BALB/c thymocytes were sensitized toward H-2<sup>b</sup> (○) stimulator cells. All responder cell concentrations were set up 16-fold and tested after 6 d of culture on <sup>51</sup>Cr-labeled LS (H-2<sup>k</sup>) or EL<sub>4</sub> (H-2<sup>b</sup>) tumor target cells. Cultures that gave a <sup>51</sup>Cr release exceeding the mean spontaneous release by 3 SD were considered positive.

party alloantigens remained intact. Because similar results were obtained with several haplotype combinations (data not shown) we concluded that, similar to the method for selective removal of splenic cells (21), the monolayer adherence technique allowed selective removal of thymic alloreactive CTL-P.

After the experimental conditions allowing selective removal of alloantigen-specific CTL-P within thymocytes were established, we next tested negatively selected T cells for the presence of allo-MHC-restricted, TNP-specific CTL-P. To accomplish this, negatively selected cells were sensitized toward three stimulator cells: (a) TNP-conjugated syngeneic cells, (b) allogeneic cells used for depletion, and (c) the very same allogeneic cells after conjugation with TNP (Table II). Clearly, after negative selection, the responder cells were depleted for alloreactivity, although they still exhibited CTL responsiveness to TNP-conjugated syngeneic stimulator cells. The critical observation, however, was that the negatively selected cells, shown to be unresponsive toward alloantigen, could effectively be sensitized to become CTL, provided the allogeneic stimulator cells were conjugated with TNP. More importantly, the CTL induced were able to lyse efficiently TNP-conjugated allogeneic targets, but could lyse unconjugated allogeneic targets or TNP-conjugated syngeneic targets only to a marginal degree. We therefore concluded that thymocytes contain TNP-specific allo-MHC-restricted CTL precursors.

*Frequency Analysis of Allo-MHC-restricted Thymic CTL-P.* Using the very same negative selection protocol, we next investigated within thymocytes the frequency of TNP-specific, self MHC-restricted CTL-P. The results depicted in Fig. 2 described two haplotype combinations tested. Obviously, the negative selection protocol did not affect the frequency of self-MHC-restricted CTL-P when compared with unselected thymocytes. After negative selection, the existence of TNP-specific, allo-MHC-restricted CTL-P could be detected with frequencies ranging between 1/27,000 and 1/142,000. It should be noted that when compared with self-MHC-restricted CTL-P, allo-MHC-restricted CTL were detected with a three- to ninefold lower frequency. Information in regard to the specificity of the CTL tested in Fig. 2 is given in Table II.

The frequencies of CTL detected toward TNP-conjugated allogeneic targets by far exceeded those toward allogeneic unmodified, or syngeneic TNP-conjugated target

TABLE II  
*Specificity of Allo-H-2<sup>b</sup>-restricted CTL-P Within CBA Thymocytes Acutely Depleted for Anti-H-2<sup>b</sup> Alloreactivity*

Responder cells	Stimulator cells	Percent specific lysis of target cells*				
		H-2 <sup>b</sup> (TNP)	H-2 <sup>b</sup>	H-2 <sup>k</sup> (TNP)	H-2 <sup>k</sup>	H-2 <sup>d</sup>
CBA (H-2 <sup>k</sup> ) thymocytes (acutely depleted for anti-H-2 <sup>b</sup> alloreactivity)	C57BL/6	ND <sup>‡</sup>	<5	ND	ND	ND
	C57BL/6 (TNP)	45	<5	<5	ND	<5
	CBA (TNP)	<5	<5	38	9	<5

Thymocytes of CBA mice were first acutely depleted for anti-H-2<sup>b</sup> alloreactivity and subsequently sensitized toward H-2<sup>b</sup> stimulator cells, TNP-conjugated H-2<sup>b</sup> stimulator cells, and TNP-conjugated H-2<sup>k</sup> stimulator cells. After 6 d of culture, effector cells were tested on Con A-induced, <sup>51</sup>Cr-labeled blast cells in a 3-h <sup>51</sup>Cr-release assay. The spontaneous <sup>51</sup>Cr release of the target cells never exceeded 13%.

\* Effector:target cell ratio, 25:1.

<sup>‡</sup> Not done.

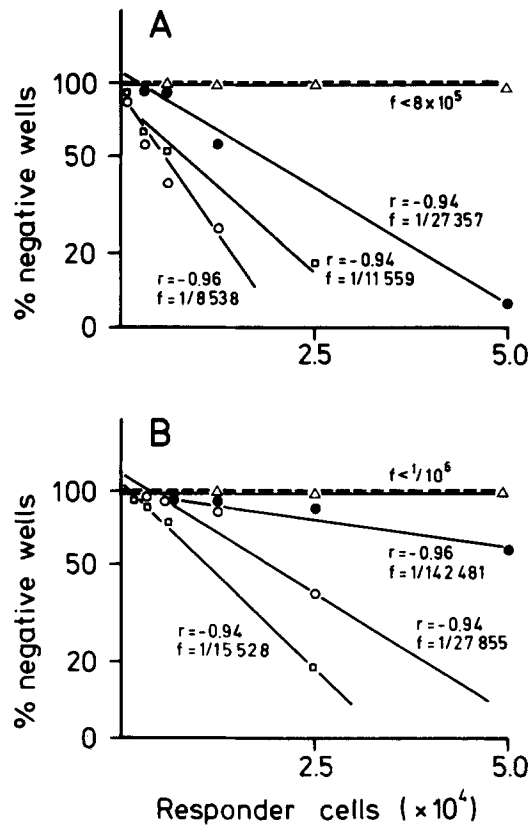


FIG. 2. The frequency analysis of allo-MHC-restricted, TNP-specific CTL-P within thymocytes of normal mice. (A) Thymocytes of CBA mice were acutely depleted for anti-H-2<sup>d</sup> alloreactivity ( $\Delta$ ) and subsequently sensitized toward TNP-conjugated H-2<sup>d</sup> ( $\bullet$ ) or TNP-conjugated H-2<sup>k</sup> ( $\circ$ ) stimulator cells. (B) Thymocytes of BALB/c mice were negatively selected for anti-H-2<sup>k</sup> alloreactivity ( $\Delta$ ) and subsequently cocultivated with TNP-conjugated H-2<sup>k</sup> ( $\bullet$ ) or TNP-conjugated H-2<sup>d</sup> ( $\circ$ ) stimulator cells. To compare the frequency of self-MHC-restricted CTL-P between unselected and negatively selected thymocytes, data from unselected thymocytes cocultivated with the relevant TNP-conjugated syngeneic stimulator cells ( $\square$ ) are incorporated. The spontaneous <sup>51</sup>Cr-release of the blast cells never exceeded 20%.

cells. However these data still were insufficient to exclude that allo-MHC-restricted CTL in fact represent cross-reactive self-MHC-restricted CTL. Therefore, a segregation analysis of the clonal distribution of allo- versus self-MHC-restricted CTL was performed.

*Specificity Analysis of Self- versus Allo-MHC-restricted CTL-P.* Limiting numbers of negatively selected BALB/c responder cells were sensitized toward TNP-conjugated allogeneic CBA stimulator cells. 96 microcultures established simultaneously were assayed for specificity by dividing each individual microculture and testing them against distinct target cells. In addition, limiting numbers of the very same negatively selected responder cells were sensitized toward TNP-conjugated syngeneic stimulator cells. The 96 microcultures established were also assayed individually for specificity of the CTL generated. Independently, i.e., whether sensitization was directed toward TNP-conjugated syngeneic or TNP-conjugated allogeneic stimulator cells, ~40% of

the microcultures were found to be positive ( $^{51}\text{Cr}$  release exceeded the mean of spontaneous release by  $>3$  SD). As can be seen in Fig. 3, only 5% of the positive wells exhibited cross-reactive target cell lysis. We therefore concluded, that the specificity of allo-MHC-restricted CTL is comparable to that of self-MHC-restricted CTL.

### Discussion

We have investigated the existence, the frequency, and the specificity of allo-MHC-restricted, TNP-specific CTL-P within murine thymocytes. After acute in vitro depletion of alloreactive CTL-P, we observed allo-MHC restricted TNP-specific CTL-P with a frequency of 1/27,000–1/142,000 in the negatively selected cells; these values were about three- to ninefold below those obtained for self-MHC-restricted CTL-P. The specificity of allo-MHC restricted, thymocyte-derived CTL generated under limiting-dilution conditions and tested individually against distinct target cells, was comparable to that of self-MHC-restricted, TNP-specific CTL. These results are consistent with our previous work (21) demonstrating the existence of high numbers of antigen-specific allo-MHC-restricted CTL-P in the spleen of normal mice. On the basis of our data presented here and elsewhere (18, 19, 21) we draw two conclusions: First, independent of its own MHC-type, the thymus generates and exports high numbers of allo-MHC-restricted, antigen-specific CTL-P. Second, the existence of allo-MHC-restricted CTL-P implies that the phenotypical manifestation of the MHC restriction is a result of antigen-driven selection of precommitted T cells.

The in vitro acute-depletion protocol used in this study has advantages and limitations. Compared to the in vivo depletion protocol (18, 19), one advantage is that, in vitro, a 70–95% recovery of negatively selected cells can be achieved. The recovery data stress the selectivity of the in vitro absorption method used. Compared with the use of chimeric mice (1–6, 13–15) a limitation of the acute in vitro depletion

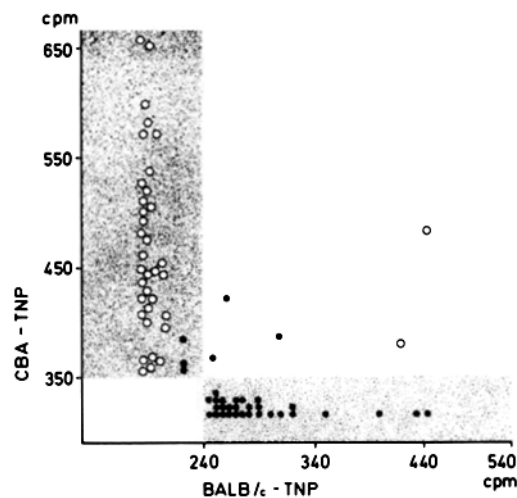


FIG. 3. Specificity of clonally distributed allo-MHC-restricted CTL-P. Thymocytes of BALB/c mice were negatively selected for anti-H-2<sup>k</sup> alloreactivity and subsequently sensitized toward TNP-conjugated allogeneic (H-2<sup>k</sup>) (○) or syngeneic (H-2<sup>d</sup>) (●) stimulator cells under limiting-dilution conditions. After 6 d of culture, each microwell was split and tested either on TNP-conjugated allogeneic or syngeneic Con A-induced blast cells as described in Materials and Methods. The spontaneous  $^{51}\text{Cr}$  release of the target cells never exceeded 25%.

protocol is that it does not give information on possibly intrathymic operating mechanism resulting in the generation of MHC-restricted CTL-P, but only furnishes data on the immune responsiveness of a given lymphocyte population at a given time. Nevertheless, simple removal of alloreactive CTL-P has allowed us to detect and to quantitate allo-MHC-restricted thymic CTL-P.

After twofold absorption on an allogeneic monolayer, the frequency of alloreactive thymic CTL-P could be reduced from  $\sim 1/20,000$  to  $< 1/800,000$  (Fig. 1). Upon sensitization of the negatively selected cells toward TNP-modified allogeneic stimulator cells, allo-MHC-restricted CTL-P with a frequency of  $1/20,000$ – $1/142,000$  could be detected within thymocytes (Fig. 2). A specificity analysis of the CTL obtained under bulk culture conditions established that only those target cells that displayed the TNP-conjugated alloantigens used for sensitization (Table 2) were effectively lysed. Moreover, by sensitizing negatively selected thymic CTL-P under limiting-dilution conditions, the CTL generated in individual microcultures were positive, in 95% of the cases, only toward targets that displayed the TNP-modified alloantigens (Fig. 3). The reverse was found with CTL sensitized toward TNP-conjugated syngeneic stimulator cells. These data established the exclusive specificity of allo-MHC-restricted CTL. The clear specificity pattern obtained with thymocytes toward Con A-induced target cells obviously rules out the possibility that the lytic effect seen is a result of *in vitro* activated natural killer cells.

Several different techniques were applied to analyze the question whether T cells of a given MHC-type can respond to foreign antigen presented in the context of an allogeneic MHC type (1–15, 36–38). Although no unifying results have yet emerged from these approaches, it has been assumed that tolerance to the allogeneic MHC-type is a prerequisite for responsiveness to be generated (1–6, 13–15, 36–38). Because after acute depletion *in vitro*, allo-MHC-restricted T cells can be detected in all lymphocyte populations so far tested ([21]; and Table II), it follows that in unselected lymphocytes allo-MHC-restricted CTL-P must also be present. Our data therefore imply that tolerance to alloantigens is not a prerequisite for the generation of allo-MHC-restricted CTL-P.

The positive demonstration of allo-MHC-restricted CTL-P in lymphocytes of normal mice brings up the question again why T cells of semi- and fully allogeneic chimeric mice fail to respond *in vivo* to foreign antigens presented in the context of the allo-MHC type (1, 2, 10), whereas T lymphocytes of similar chimeras are responsive when tested *in vitro* (12, 14, 21). That *in vivo* suppressor T cells may cause unresponsiveness has been suggested by Miller et al. (39), whereas the work of Zinkernagel et al. (40) appears to exclude this possibility. Because the thymus of a normal mouse contains allo-MHC-restricted CTL-P, we intend to reexamine the *in vitro* T cell responsiveness of lymphocytes from (A  $\times$  B) $\rightarrow$ A chimeric mice under limiting-dilution conditions.

The results of this study were obtained in the TNP system in which the work of Shearer et al. (22) has indicated the critical role of the epitope density for eliciting optimal CTL responses. Furthermore it is not yet clear how much of an anti-TNP response is classically restricted and how much of it may be an alloresponse. The results described here were obtained at a TNP-epitope density described by Shearer et al. to be optimal. Furthermore the H-2 restriction specificity of anti TNP-reactive CTL (Fig. 3) excluded the possibility that we were dealing with alloreactive CTL.

Therefore we feel confident that our conclusion of the existence of high numbers of allo-MHC-restricted CTL-P within thymocytes is justified. The results also agree with those obtained by Doherty and Bennink (19) in an in vivo negative selection protocol. The existence of allo-MHC-restricted, hapten-specific CTL in the thymus of normal mice calls into question the necessity to postulate intrathymic operating mechanisms as a consequence of which only a self-MHC-restricted T cell receptor repertoire is generated (4). That self-MHC-restricted CTL-P can be generated even without intrathymic processing has recently been indicated in the nu/nu mouse system. Lymphocytes derived from nu/nu mice already express a repertoire enabling them to mount both alloreactive (16, 41, 42) and self-MHC-restricted CTL responses (16, 17). The observation that the frequency of allo-MHC-restricted antigen-specific T cells is about three- to ninefold lower compared with self-MHC-restricted T cells suggests the existence of mechanisms on the basis of which preferentially self-MHC-restricted T cells are generated. A frequency analysis of CTL-P in fully allogeneic chimeric mice indicated that the self-MHC preference is not controlled by the thymus (43). Studies at present conducted in this laboratory aim at an analysis of the H-2 restriction phenotypes of CTL-P from athymic (nu/nu) mice.

In conclusion, we provide evidence that independent of its own MHC type, the thymus allows the generation and export in both self-MHC-restricted as well as of allo-MHC-restricted T lymphocytes. Upon sensitization, the H-2 restriction phenotype will depend on antigen-driven selection of precommitted T cells. Because the results described here were obtained in the TNP system, we now wish to extend the findings to virus-specific CTL responses in H-2-congenic strains of mice.

### Summary

An in vitro acute-depletion protocol was used to detect trinitrophenyl (TNP)-specific, allo-major histocompatibility complex (MHC)-restricted cytotoxic T lymphocytes (CTL) within thymocytes of inbred mice. After removal of alloreactivity, the negatively selected cells could be sensitized to become TNP-specific, allo-MHC-restricted cytotoxic T cells. A precursor frequency analysis revealed a three- to ninefold lower frequency of allo-MHC-restricted CTL precursors (CTL-P) as compared to self-MHC-restricted CTL-P. The specificity analysis of clonally distributed allo-MHC-restricted CTL-P excluded cross-reactivity as an explanation of allo-MHC restriction. These results provide direct evidence that thymic T cells are composed of a mixture of self-MHC- and allo-MHC-restricted immunocompetent T cells and that antigen-driven selection of precommitted T cells dictates the H-2-restriction phenotype, i.e., H-2 restriction is a consequence of priming.

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