A Subpopulation of Mouse Cytotoxic T Lymphocytes Recognizes Allogeneic H-2 Class I Antigens in the Context of Other H-2 Class I Molecules

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Summary

Recently, independent lines of evidence strongly suggested that peptides derived from one foreign major histocompatibility complex (MHC) molecule bound to another MHC molecule can give rise to multiple composite MHC complexes that are able to stimulate allo-(xeno)-reactive T cells. In this study, we describe that in vivo immunization of mice with cells mismatched with the recipient for a single class I antigen results in the induction of $CD8^+$ cytotoxic T lymphocytes (CTL) specific for allogeneic class I locus products (D^d, K^d, D^q) in the context of other class I molecules (K^s, D^d, K^k) present on stimulator cells. Evidently, the target antigen for these class I–restricted alloreactive CTL is not the native class I molecule but peptides derived from endogenous processing of allogeneic class I products presented by class I molecules. Using a combination of limiting dilution and split-well analyses, we estimated for K^k-restricted D^q-specific alloreactive CTL a precursor frequency (CTLpf) that was ~10 times lower than the CTLpf for "classical" nonrestricted D^q-specific alloreactive CTL. These data suggest that H-2 class I peptides presented by intact H-2 class I molecules are allostimulatory, supporting the concept that the capacity for presentation of MHC peptides by MHC molecules constitutes a part of the allogeneic immune response.

Virus-specific T cells recognize peptides derived from viral proteins produced in the infected cell. These peptides are presented by self-MHC class I or II molecules, as indicated by functional (1, 2) and crystallographic data (3, 4). Consequently, T cells recognize nominal antigens only on cells expressing MHC molecules identical to those of the responder. This is in contrast to the apparently nonrestricted recognition of alloantigens as alloreactive T cells recognize MHC antigens on whichever cell.

Based on the information that T cell recognition of nominal antigens involves trimolecular interactions between receptors on T cells, antigenic peptides, and class I or II MHC molecules, several current models have been suggested to account for allorecognition (reviewed in references 5–7). From most of the models, it appears that MHC alloreactivity probably involves a tripartite structure consisting of an allogeneic MHC molecule, an endogenous peptide, and a TCR. However, this does not rule out that some alloreactive CTL may recognize MHC molecules in the absence of peptide adducts, i.e., recognizing "naked" class I MHC molecules (8). Recent studies on alloreactivity also suggest that allogeneic responses include the recognition of MHC peptides derived from one MHC molecule and being presented to T cells by a second MHC molecule (9–15). Additional examples of the presentation of MHC peptides by MHC molecules emerge from studies on the xenogeneic response of murine T cells to human MHC products (16–19). In some of these studies, CTL clones were isolated for which the recognition of xenogeneic MHC peptides was restricted by self-MHC, like the recognition of nominal antigens (17). In other studies, the preferential binding of xeno-MHC peptides by their native MHC molecules has been suggested (20).

Thus, at least a part of the MHC molecules on stimulator and target cells might be occupied by derivatives of other self-MHC molecules. An intriguing question emerging from this concept is whether such composite MHC molecules play a role in alloimmune response in vivo by the induction of CTL specific for peptides derived from MHC alloantigens and presented by other MHC molecules expressed either on responder or stimulator cells. We report here that a subset of CTL indeed recognized allogeneic class I MHC locus products in the context of other class I molecules. This population of class I–restricted alloreactive CTL was detected only after in vivo priming at a frequency that was ~10 times lower than the classical nonrestricted alloreactive CTL population. These experiments suggest a further example of the capacity

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of MHC molecules to present MHC peptides, and show that allorecognition is a complex phenomenon in which multiple T cell populations seem to play a role. The results might have consequences for allogeneic transplantations in sensitized recipients.

Materials and Methods

Mice. All mice were obtained from the Animal House of our institute and bred under specific pathogen-free conditions. Mice were primed by one intraperitoneal of 10^7 allogeneic spleen cells in PBS 3–6 wk before use.

CTL Analysis. Limiting dilution (LD)[1] and split-well assays have been described in detail elsewhere (21). Briefly, for split-well analysis, 2,500 responder lymph node cells of naive or primed mice were cocultured with 50,000 irradiated (25 Gy) allogeneic spleen cells per well in the presence of 20 U/ml rat IL-2. For each split-well experiment, 96 wells were seeded. After 7 d, microcultures were divided into three (60-μl) or four (45-μl) aliquots and transferred into wells containing 140 or 155 μl of medium and 5,000 ^{51}Cr-labeled target cells. Culture medium was IMDM supplemented with 10% (wt/vol) FCS and 100 IU/ml penicilline, 100 μg/ml streptomycine, and 5×10^{-5} M 2-ME. After 4 h of incubation at 37°C, the supernatants were automatically harvested by Titertek systems and counted in a gamma counter. Responding cultures were defined as those in which the ^{51}Cr release values exceeded in the mean spontaneous ^{51}Cr release plus three times the SD. LD cultures were set up starting from 64,000 to 500 responder lymph node cells/well in 24 replicates. Stimulation, culture, and ^{51}Cr release conditions, as well as the splitting of LD cultures into four aliquots, were the same as for split-well analysis.

Target Cells. Spleen cells cultured for 3 d in the presence of LPS (30 μl/ml) were used as target cells. Before labeling, target cells were centrifuged on Ficoll. For blocking studies with H-2-reactive antibodies, target cells were incubated with 100 μl of hybridoma culture supernatant for 30 min at 37°C followed by the same period on ice. Cells were washed twice with culture medium before the addition of CTL. For blocking studies with anti-CD8, target cells and CTL were incubated in the presence of 20 μl of anti-CD8 antibodies (1:1,000). Antibodies used in this study were: anti-D^q-(28.14.8S [22]), anti-K^k-(36.7.5S [23]), and anti-Ly2.2-specific mAb (New England Nuclear, Boston, MA).

Calculation of CTL Precursor Frequencies (CTLpf). CTLpf were calculated by scoring wells with responding and nonresponding cultures. Minimal estimates of CTLpf were calculated using the jackknife version of the maximum likelihood procedure with a 95% confidence interval determined according to the method described by Strijbosch et al. (24). The correlation coefficient of the experiments given was ≥ 0.90. Computer programs for the above-described CTLpf calculations are available from the Computer Applications Group (Tilburg University, Tilburg, The Netherlands).

Results and Discussion

To analyze whether a fraction of alloreactive CTL recognize MHC alloantigens in the context of other MHC antigens, we compared primary and secondary alloreactive CTL responses of mice induced by a single H-2 class I difference. Because CTL bulk cultures are in general not restricted by MHC, we generated CTL cultures at the level of precursor frequency. For this propose, lymph node cells from naive and primed mice were stimulated in microcultures with allogeneic spleen cells. Each microculture was split into three or four aliquots and tested for cytotoxicity against selected target cells.

Table 1 shows the split-well analysis of three independent experiments. Before priming, all CTL microcultures were reactive with target cells expressing the stimulating class I H-2 antigen. Target cell lysis was not dependent on non-MHC genes, i.e., background differences (Exp. 1) or coexpression of other class I H-2 molecules on responder, stimulator, or target cells (Exps. 1–3). These observations are general and reported for a broad field of classical alloreactive CTL research.

After priming, the majority of responding T cells exhibited the same reactivity pattern. However, another population of CTL lysed exclusively those target cells expressing in addition to the stimulating class I molecules, class I molecules shared by both responder and stimulator cells. It appears that after priming in vivo and restimulation in vitro, for a small population of CTL, the recognition of the alloantigen is restricted by syngeneic class I H-2 molecules. Obviously, recognition of alloantigens is restricted here by H-2 private determinants. The percentage of H-2 class I–restricted alloreactive CTL seems to be low, but it may well be that part of the restricted CTL were overgrown by the classical non-MHC-restricted alloreactive CTL. For that reason, we made an effort to block target cell lysis by H-2-specific antibodies. To ensure the involvement of $CD8^+$ T cells, only those microcultures were taken into account that were blocked by anti-CD8 antibodies. Table 2 shows that 49 microcultures contained $CD8^+$ T cells reactive with the stimulator cells. Of these 49 cultures, 28 were blocked by anti-D^q antibodies, suggesting a direct recognition of allogeneic D^q epitopes. 11 CTL microcultures were not blocked by anti-D^q but by K^k-reactive antibodies. Here, the K^k molecule seems to be involved in recognizing allogeneic D^q antigens. The peptide model valid for nominal antigen recognition provides the best model for explaining the specificity of the latter alloreactive CTL. The recognized alloantigen seems to be formed by K^k molecules associated with a class I peptide derived from D^q molecules. The K^k molecules on the target cells (LPS blasts) apparently contained sufficient amounts of D^q peptides to be lysed by the CTL. Evidently, D^q molecules are processed in the target cells and presented to K^k molecules by the endogenous pathway of antigen presentation. Interactions on the target cell membrane between T cell receptors and K^k molecules associated with D^q peptides will be blocked by K^k-reactive mAbs rather than by anti-D^q antibodies. Because B10.BR and B10.AKM mice differ only in class I D^q or K^k gene products, our findings suggest that a low percentage of the syngeneic class I H-2 molecules is occupied with peptides of other class I H-2 molecules. This has recently also been shown in the human system for class I HLA molecules of which the processed peptides were bound by HLA class II expressed on the same cell surface (13, 15). Hence, endogenous processing and presentation of class I peptides normally occurs in vitro and in vivo. However, our results in the mouse

[1] *Abbreviations used in this paper:* CTLpf, cytotoxic T lymphocyte precursor frequencies; LD, limiting dilution.

Table 1. *Split-well Analysis of Primary and Secondary Alloreactive CTL*

Exp.	Mouse strain	H-2 K	H-2 D	Split-well reaction pattern of CTL microcultures: Before priming		After priming	
1							
Responder	A.SW	s	s				
Stimulator	A.TH	s	d				
Target: 1	R			−	−	−	−
2	S			+	+	+	+
3	B10.D2	d	d	+	−	+	−
4	B10.HTT	s	d	+	+	+	+
				43*	0	49	7
2							
Responder	B10.BR	k	k				
Stimulator	B10.OH	d	k				
Target: 1	S			+	+	+	+
2	B10.D2	d	d	+	−	+	−
3	B10.GD	d	b	+	−	+	−
				37	0	50	5
3							
Responder	B10.BR	k	k				
Stimulator	B10.AKM	k	q				
Target: 1	S			+	+	+	+
2	B10.Q	q	q	+	−	+	−
3	B10.MBR	b	q	+	−	+	−
				27	0	38	5

Responder (R) lymph node cells of naive or primed mice were seeded into 96 wells (2,500/well) and cocultured with irradiated stimulator (S) spleen cells (50,000/well). Microcultures were split into three or four aliquots and tested on ^{51}Cr-labeled target cells in a final volume of 200 μl. +, target cell lysis; −, no target cell lysis.

* Expressed is the number out of 96 CTL microcultures exhibiting the indicated split-well pattern of target cell lysis.

model suggest that for class I molecules, peptide presentation is more efficient after sensibilization in vivo. Probably, expression of the minimal number of MHC/peptide complexes required for T cell activation is stimulated by priming and differs from the number of complexes necessary for target cell lysis (25, 26).

Several other publications demonstrated that MHC class I peptides can be recognized when presented by allogeneic (9, 10) or xenogeneic (16, 17) MHC molecules or that such peptides might inhibit allo- (11, 27) or xenoreactivity (28). In this context, it is of interest to know at which frequency CTL recognize such composite MHC antigens. Using a combination of LD and split-well analysis, we estimated the frequency of CTL precursors in lymph node cell populations before and after sensitization to a single class I difference. Table 3 shows that the frequency of class I–restricted alloreactive CTL is ~10 times lower than of the nonrestricted classical alloreactive CTLp and that the majority of restricted CTL is contributed by previously primed cells. Thus, in vivo immunization with allogeneic class I molecules stimulates CTL specific for composite determinants consisting of allogeneic class I peptides presented by other class I molecules. Together with the information that CTL are also able to recognize class I peptides presented by class II molecules (13, 15), it can be speculated that both class I and class II MHC molecules present either class I– or class II–derived peptides. Recently, it has been postulated that such complexes might play a role in the determination of T cell repertoires (15) or in T cell suppressor networks (29). Possibly, peptides derived from one MHC gene product will bind to the intact products of the same gene (20) and may in this way participate in elimination or inactivation of T cells with self-MHC specificities.

Taken together, our results demonstrate that the recognition of allogeneic class I antigens is for a limited number of $CD8^+$ T cells restricted by class I MHC molecules. The low CTLpf suggests that these restricted alloreactive CTL are only a minor part of the total T cell population and are therefore

Table 2. *Split-well Analysis of Secondary Allo-(D^q)-reactive CTL in the Presence of CD8- or H-2-specific mAbs*

		H-2 K	H-2 D	Split-well reaction pattern of CTL microcultures	
Responder	B10.BR	k	k		
Stimulator	B10.AKM	k	q		
Inhibition by:					
	1. –			0	0
	2. Anti-CD8			+	+
	3. Anti-D^q			+	0
	4. Anti-K^k			0	+
				38*	11

96 microcultures of alloreactive CTL were seeded as indicated in the legend to Table 1. Lymph node cells of primed mice were used as responder cells. +, blocking of target cell lysis; 0, no blocking of target cell lysis.
* Expressed is the number out of 96 CTL microcultures with the indicated split-well pattern of target cell lysis. Targets were: stimulator cells (*1*), stimulator cells in the presence of anti-CD8 mAbs (*2*), and stimulator cells previously incubated with D^q- (*3*) or K^k- (*4*) specific mAbs.

not easily detectable in CTL bulk cultures. Nevertheless, especially in those individuals who became sensitized to MHC alloantigens, class I–restricted alloreactivity may be involved in the rejection of allogeneic grafts as allogeneic MHC class I peptides are presented by class I molecules expressed on either donor or recipient cells.

Table 3. *Precursor Frequencies of Primary and Secondary Allo-(D^q)-reactive CTL*

Exp.	H-2K	H-2D	Frequency*	95% CI‡
Responder B10.BR	k	k		
Stimulator B10.AKM	k	q		
1§ (before priming)				
Targets: R			125,000	100,000–166,667‖
S			3,788	2,759–5,528
S + anti-D^q			89,909	63,407–95,956‖
S + anti-K^k			4,409	3,667–8,657
2 (after priming)				
Targets: R			99,997	66,667–166,667‖
S			2,392	1,896–3,333
S + anti-D^q			25,900	24,641–33,741
S + anti-K^k			3,185	2,463–4,685
3 (after priming)				
Targets: R			86,878	66,667–166,667‖
S			3,257	2,475–4,364
S + anti-D^q			32,171	24,011–40,376
S + anti-K^k			3,744	2,851–4,977

Wells were tested on responder (R), stimulator (S), and stimulator cells previously incubated with H-2-specific mAbs. Experiments are representative and undependent examples.
* Reciprocal CTL precursor frequency.
‡ 95% Confidence interval.
§ CTLpf were estimated in individual mice by a combination of LD and split-well analysis.
‖ CTLpf and 95% CI are calculated using extrapolation to responder cell numbers not used in the LD assay.

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