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ALLOREACTIVE CYTOTOXIC T CELLS RECOGNIZE MHC CLASS I ANTIGEN WITHOUT PEPTIDE SPECIFICITY

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In this report, experiments are described to differentiate between three potential models of class I MHC allorecognition, namely 1) recognition of peptide-free MHC, 2) peptide-MHC-specific recognition, and 3) peptide-MHC-nonspecific recognition. Using a nucleoprotein peptide (NPP) with a sequence derived from influenza virus nucleoprotein with high affinity for K^d class I MHC molecules, it is shown that target cells rapidly become lysable by K^d-NPP self-restricted cytotoxic T (Tc) cells, and retain sufficient K^d-NPP complexes for at least 72 h. K^d-specific alloreactive Tc cells at the clonal and polyclonal level do not show decreased lysis of K^d-bearing targets in the continuous long term (48 h) presence of NPP. K^d-stimulator cells modified with NPP are able to induce potent K^d-NPP-specific self-restricted Tc cells, however K^d-NPP stimulator cells do not generate K^d-NPP specific alloreactive Tc cells from CBA and B10.A (5R) mouse strains as tested by limiting dilution split clone experiments. Human cells infected with the vaccinia virus recombinant coding for the murine K^d class I MHC Ag can be lysed by murine K^d-specific alloreactive Tc cells. In addition the rate of reemergence of alloreactive and self-restricted Tc cell epitopes on virally infected target cells that had their cell-surface class I MHC Ag removed is identical. These results are consistent with model 3 namely that the majority of Tc precursor and effector cells recognize class I MHC Ag without peptide specificity.

Recognition of target cells by $CD8^+$ T lymphocytes has been well characterized in self MHC restricted responses. The epitope recognized is a complex of a self MHC class I molecule plus a foreign peptide (1). Although the phenomenon of recognition by T cells of foreign MHC (allorecognition) has been investigated for many years, our understanding of what is precisely recognized in such interactions is much more limited. However, a priori, there exist two basic options regarding the form of epitopes that are capable of forming biologically meaningful interactions with the TCR. One, which is in essence the same as that observed for self-restricted TCR/class I interaction, was proposed initially by Matzinger and Bevan (2) and has been extended by Kourilsky and Claverie (3). Thus, individual alloreactive Tc cells recognize allo MHC class I molecules with a specific endogenous peptide. Evidence for the presentation of endogenous peptides is numerous, i.e., Tc [3] cell responses against HY, (4) β_2-Microglobulin (5), and other minor Ag (6). Support for this model has come recently with the finding by Heath et al. (7) which showed that murine alloreactive Tc clones lysed human cells transfected with the relevant murine class I MHC Ag only in the presence of murine peptides.

In contrast, the difficulty in obtaining allorestricted, virus-specific Tc cells (8), the failure to detect minor H-specific alloreactive Tc cells in split clone-limiting dilution assays using targets from congenic mouse strains (9), and the lack of tissue specificity in allograft rejection (10) argue against peptide-specific allorecognition.

Evidence that different interactions are in operation in self-restricted recognition vs allorecognition is indicated by studies on selected mutants of class I Ag involving the β-sheet and α-helices that affect allorecognition differently from self-restricted recognition (11) and experiments showing that a lymphoma mutant, unable to express normal levels of class I MHC Ag because of a defect in endogenous peptide production, can be recognized by alloreactive T cells but is not recognized by minor H Ag-specific, self MHC-restricted T cells (12).

An alternative is that the epitope recognized is determined by the conformation of the polymorphic part of the MHC class I molecule per se, irrespective of the peptide bound. A modified version of this model would be that allorecognition is seeing peptide-free class I MHC, estimated to be in the order of 0.3% of cell-surface expressed class I molecules (13). Evidence that peptide-free class I molecules can be recognized as allogeneic molecules has been shown recently by Elliot and Eisen (14).

Recognition of allo MHC irrespective of peptide would be consistent with the model for restrictive recognition vs allorecognition as proposed by Langman (15). The results presented here suggest that the majority of alloreactive Tc clones recognize allo MHC class I per se, but do not exclude the possibility that a small minority of such clones are specific for endogenous peptides.

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[3] Abbreviations used in this paper: Tc cells, cytotoxic T cells; VV, vaccinia virus; NPP, nucleoprotein peptide; TGM, thioglycollate-induced B/c peritoneal macrophages.

MATERIALS AND METHODS

Animals. Mouse strains CBA/H (K^kD^k) (CBA), B10.A (5R), (K^bD^d) (5R), C3H.H-2° (K^dD^k) (OH), and BALB/c (K^dD^d) (B/c) were obtained from the breeding establishment of the John Curtin School, Canberra, Australia. Only females older than 6 wk were used.

Viruses. A strain influenza viruses A/WSN (H1N1) and A/JAP (H2N2) were grown and titrated as has been described elsewhere (16). The recombinant VV coding for the MHC class I K^d molecule (VV-K^d) and the TK^- control VV or wild-type VV WR (VV-WR) were grown and titrated as described (17).

NPP. The R^- peptide of influenza A virus 147–158 (18) was a generous gift of Peptide Technology, Sydney, Australia. Iodination was performed by the lactoperoxidase method, essentially as follows—10 μg of peptide was labeled using 0.5 mCi of ^{125}I in PBS (pH 7.2) by the addition of lactoperoxidase (2 μg) and hydrogen peroxide (30 ng) for 2 min. Three further additions of hydrogen peroxide were made at 2-min intervals and the reaction was terminated by the addition of BSA (3% w/v in PBS, pH 7.2, and 0.1% (w/v) sodium azide). Free iodine was separated from the peptide by Sephadex G-25 chromatography.

Immunization. Animals were immunized with either 10^7 plaque-forming units of VV-WR or 10^3 hemagglutinating units of A/strain influenza virus A/WSN.

Generation of effector cells. Spleen cells from mice immunized 6 days previously were used for primary VV-immune Tc cells. Cultures for the generation of secondary influenza-immune Tc cells have been described in detail elsewhere (16). For the generation of alloreactive Tc cells, 8×10^7 responder splenocytes were cocultured with 4×10^7 irradiated (2000 r) allogeneic stimulator cells for 5 days in medium with or without NPP as indicated in the text.

Generation of effector cells in microcultures. Alloreactive Tc cells were generated in cocultures of 5×10^3 red cell-depleted responder splenocytes with 2×10^5 irradiated (2000 r) red cell-depleted stimulator splenocytes. The culture medium (F15, GIBCO, Grand Island, NY) was supplemented with 5% FCS plus 10^{-4} M 2-ME (0.2 ml/well) and was conditioned with 10% Con A spleen supernatant (16). In individual cultures, NPP was present at 10^{-4} M concentration. After 5 days in culture, plates were spun at 1500 rpm for 3 min, culture supernatant was discarded, and cells were resuspended in 0.2 ml fresh medium and split into two 0.1-ml aliquots.

Target cells. TGM, P815 cells (H-2^d) L929 cells (H-2^k) methylcholanthrene-induced fibrosarcoma cell lines MC57 (H-2^b), HTG (H-$2K^dD^b$), and OH (H-$2K^dD^k$) and the human cell line 143-B were infected with vaccinia or influenza virus labeled with ^{51}Cr as described in detail elsewhere (16, 17, 19). Target cells were incubated before or after infection for different times with NPP as detailed in results.

Papain treatment of target cells. The method has been described (20).

^{51}Cr-release cytotoxicity assay. The methods used for cell line and macrophage targets have been described in detail elsewhere (16, 19). The duration of the assays were 6 h. The percent specific lysis was calculated using the formula: % specific lysis = ((experimental release − medium release)/(maximum release − medium release)) × 100. Data given for bulk cultures are the means of triplicates. SEM were always <5% and are omitted for clarity. Significance was determined by Student's *t*-test.

RESULTS

Kinetics of uptake and retention of peptide. To assess the role of peptides associated with class I MHC molecules in recognition by alloreactive Tc cells, NPP was used to saturate available K^d on target cells. The kinetics of uptake of I^{125}-labeled, K^d-restricted, synthetic peptide (NPP) with a sequence derived from the nuclear protein of influenza virus, by dividing and nondividing-K^d bearing target cells is shown in Figure 1. Plateau levels were reached at 8 h in both dividing or mitomycin C-treated P815 cells or nondividing B/c TGM, thus indicating the NPP-saturated available K^d (free of endogenous peptide) by 8 h. To investigate the retention of NPP on K^d, nondividing B/c TGM were incubated with 10^{-4} M NPP for 1 h, washed, and left for up to 72 h before being tested for susceptibility to lysis by K^d-restricted, A/WSN influenza-immune Tc cells (Fig. 2). No differences could be observed in target cell lysis for up to 72 h after labeling. This

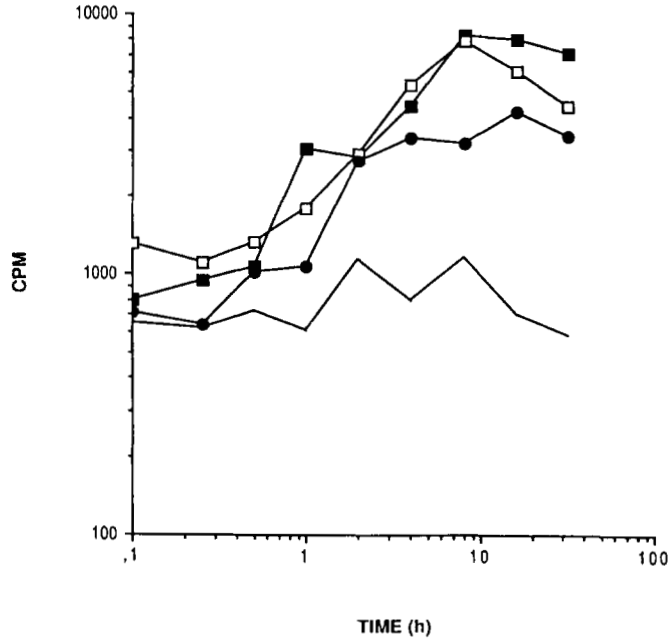

Figure 1. Kinetics of uptake of I^{125} labeled NPP. P815 cells (*squares*) or B/c TGM (*circles*) were either left untreated (*closed symbols*) or treated with mitomycin C (*open symbols*) and incubated with 10^{-4} M ^{125}I-labeled peptide. Cell-associated radioactivity was measured over a 48-h period. Line without symbols gives background radioactivity in the absence of cells.

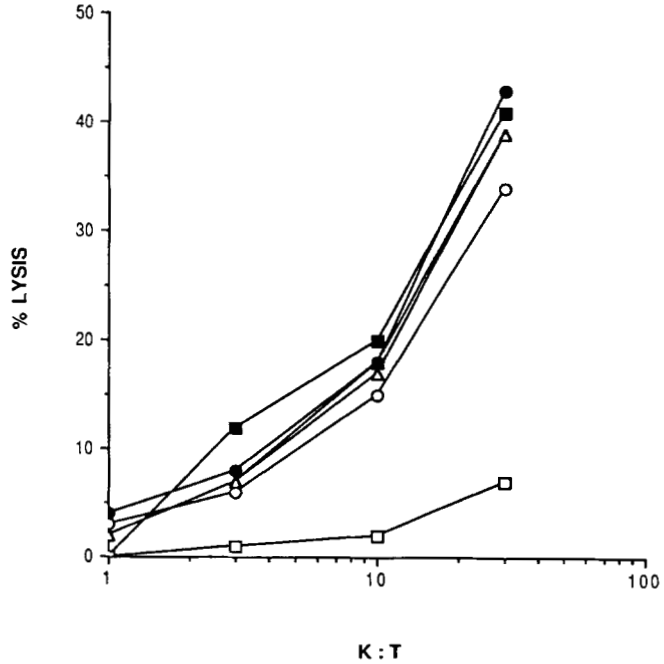

Figure 2. Peptide-modified target cell lysis by influenza-immune Tc cells. Lysis by secondary in vitro A/WSN-immune, K^d-restricted Tc cells of P815 cells modified with 10^{-4} M NPP for 1 h and left for 72 h (■), 48 h (△), 24 h (●), or 1 h (○) before assay, or unmodified (□). Assay duration was 6 h.

suggests that under the conditions employed here, NPP remains associated with K^d in sufficient quantity for optimal Tc cell recognition for at least 72 h.

Continuous presence of NPP does not alter allorecognition. The previous experiments show that K^d can be modified by NPP and that this modification is stable for long periods of time. We therefore investigated the effect of continuous presence of NPP at 10^{-4} M concentration

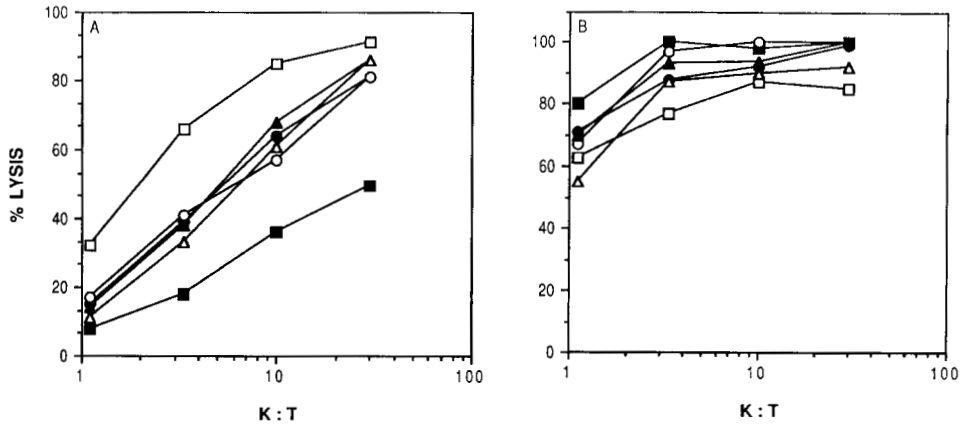

Figure 3. Peptide-modified P815 target cell lysis by Tc cells. Lysis of targets infected with A/WSN (□) left uninfected (■) or cultured in continuous presence of 10^{-4} M NPP over 72 h (△), 48 h (▲), 24 h (○), 1 h (●) by secondary influenza-immune OH effector cells (*A*) or 5R anti-B/c alloreactive Tc cells (*B*). Assay duration was 6 h.

for up to 72 h before the addition of effector cells, on target cell lysis by anti-K^d alloreactive and K^d-restricted, influenza-immune Tc cells (Fig. 3*A*). K^d-restricted, influenza virus-immune Tc cells lysed P815 targets modified with NPP equally whether the NPP was present for 1 or 72 h. A/WSN influenza virus-infected targets were lysed to a greater extent in this experiment, possibly as a result of contributions of other viral peptides recognized by this polyclonal Tc cell population. K^d-specific alloreactive Tc cells from 5R (K^bD^d) anti-B/c (K^dD^d) lysed the panel of targets approximately equally (Fig. 3*B*). Similar observations were obtained with the nondividing B/c TGM targets (Fig. 4). Identical results were obtained with K^d-specific alloreactive Tc cells generated with different haplotype combinations, i.e., CBA (K^kD^k) anti-OH (K^dD^k) (data not shown).

Limiting dilution split clone analyses on NPP saturation modified targets. To test the possibility that long incubation of target cells at high NPP concentration may allow NPP to displace endogenous peptides (bound to K^d) to such an extent as to lose threshold avidity for certain low affinity K^d-reactive clones specific for endogenous peptides, limiting dilution split clone experiments were undertaken. Effector cells were generated from 5R splenic responder cells cocultured with irradiated B/c splenic stimulator cells in microtiter wells. After 5 days' culture the individual cultures were split and tested on normal P815 and P815 incubated for 48 h with 10^{-4} M NPP. NPP was replenished after 24 h and 1 h before assay. The results of two independent representative experiments are shown in Figure 5. In no case did we observe clones that significantly lysed unmodified P815 more than NPP-modified P815. In addition, the same observation was made when K^d-specific alloreactive Tc cells were generated by different haplotype combinations, i.e., CBA (K^kD^k) anti-OH (K^dD^k) (data not shown).

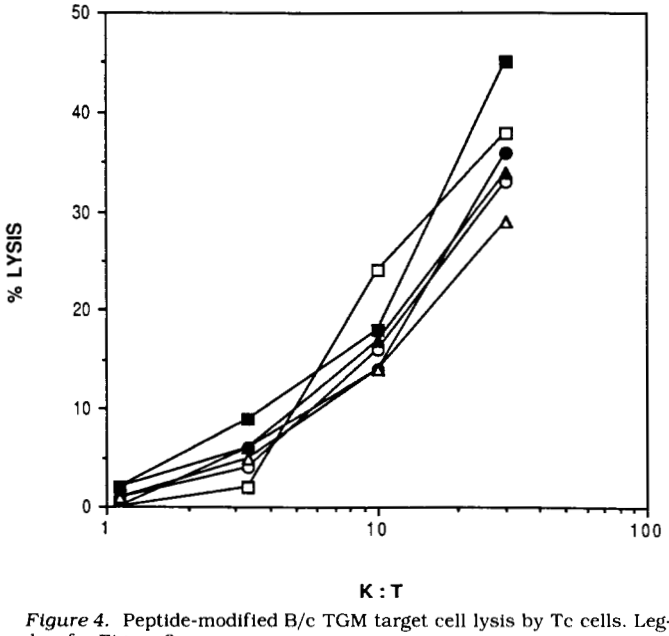

Figure 4. Peptide-modified B/c TGM target cell lysis by Tc cells. Legend as for Figure 3.

K^d modified with NPP can stimulate syngeneic but not allogeneic NPP-specific responses. The possibility that K^d-allospecific Tc cells are cross-reactive (with respect to K^d-bound peptides) at the target cell level, but recognize K^d-peptide specifically at induction was tested in split clone experiments. We tested if B/c-irradiated splenocytes can present K^d-NPP specifically in a syngeneic system. B/c splenocytes were pulsed with 10^{-4} M NPP for 24 h, infected with influenza virus A/WSN, or left uninfected. Splenocytes of A/JAP-primed B/c mice were added for 5 days as a source of NPP-specific, K^d-restricted memory Tc cells. The effector cells were tested for lysis on uninfected, NPP-modified and A/WSN-infected P815 targets (Fig. 6). NPP-modified stimulators induced NPP-specific K^d-restricted effector Tc cells with potency equal to virus-infected stimulators. We have also shown that NPP pulsed stimulator cells are specifically recognized by syngeneic cells in vivo.[4] Thus K^d-NPP can be seen by resting memory and naive, syngeneic, splenic T cells in a specific manner. Using the same stimulator cell conditions we tested for generation of K^d-NPP-specific alloreactive clones in a limiting dilution split clone experiment. A total of 2000 5R anti-B/c-NPP clones (determined from a precursor frequency analysis) were tested on 48-h NPP-modified P815 vs unmodified targets (Fig. 7). No P815-NPP (K^d-NPP) specific clones were found. The same experiment was performed using a different strain combination (CBA anti-OH) with the same results being obtained (data not shown). The failure to detect K^d-NPP-specific clones in two separate strain combinations containing different MHC and background genes reduces the possibility of cross-tolerance or "immunodominance" effects as the sole explanation of the results.

[4] **Müllbacher, A., and R. Tha Hla.** *Manuscript in preparation.*

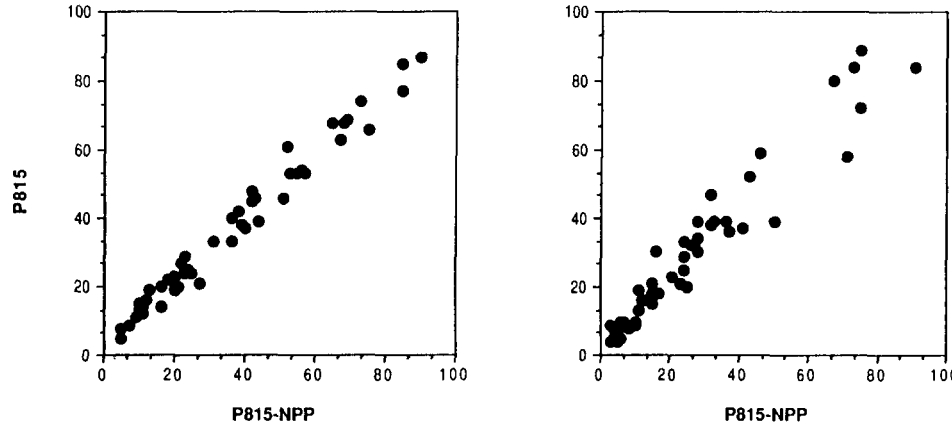

Figure 5. Limiting dilution split clone analysis. K^d-specific alloreactive effector cells were generated in microculture wells (96 wells/panel) by coculture of 5×10^3 5R responders with 2×10^5 B/c-irradiated stimulator cells. Contents of wells were split and tested for lysis of 1×10^4 P815 and 1×10^4 P815 cultured for 48 h in the continuous presence of 10^{-4} M NPP.

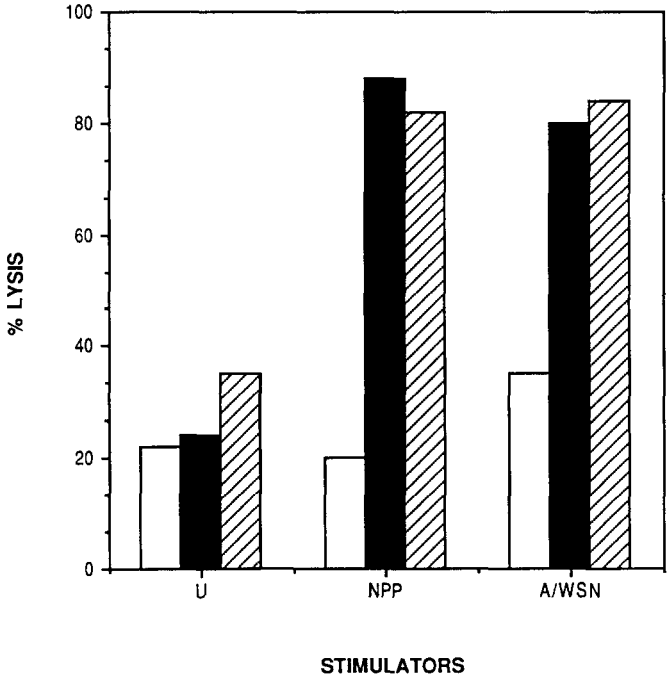

Figure 6. Influenza-specific target cell lysis by in vitro NPP-boosted effector cells. Lysis of P815 cells infected with A/WSN influenza (▨), modified with 10^{-4} M NPP (■), or unmodified (□) by B/c A/WSN-immune Tc cells generated by boosting influenza-primed memory cells in vitro with B/c stimulator splenocytes either left uninfected, modified with 10^{-4} M NPP, or infected with A/WSN. Values taken are from a fourfold titration curve at an E:T ratio of 10:1. Duration of assay was 6 h.

K^d-expressed in a human cell line can be recognized by murine K^d-alloreactive Tc. To further test if endogenous mouse peptide-specific recognition occurs in murine T cell alloreactivity, we infected murine and human target cells with a VV recombinant coding for K^d class I MHC (VV-K^d). The human target cells could not provide mouse-specific endogenous peptides. K^d-specific alloreactive Tc cells (5R anti-B/c) lysed K^d-bearing HTG and OH targets very efficiently (Table I). Significant cross-reactivity on control MC57 targets, uninfected or vaccinia-infected, was observed. However, threefold higher lysis was obtained on targets infected with the K^d-coding recombinant virus. Importantly, however, K^d-specific lysis occurred with the human cell line 143-B infected with VV-K^d. K^d-restricted VV-immune Tc cells (OH anti-VV) lysed MC57 VV-K^d-infected targets threefold more efficiently than 143-B VV-K^d-targets, suggesting that 143-B VV-K^d expresses less K^d than MC57-VV-K^d. Using FACS analysis to investigate K^d expression of these two VV-K^d-infected target cells, a two- to threefold lower expression was found in 143-B target cells than in MC57 (data not shown). These data indicate that mouse-specific endogenous peptides were not essential for recognition by K^d-specific murine alloreactive Tc, but do not exclude a role for endogenous peptides shared by mouse and human.

VV-K^d recombinant virus can induce K^d-alloreactive Tc cell responses of equal magnitude to K^d allogeneic splenocytes. Vaccinia is a cytopathic virus which inhibits its host cell protein synthesis soon after infection. K^d encoded by vaccinia should therefore be exposed predominantly to vaccinia-encoded peptides rather than endogenous mouse peptides. To examine the effects of this scenario on K^d-specific alloreactive Tc induction, 5R responder splenocytes were stimulated in vitro with either B/c or VV-K^d-infected 5R splenocytes. Effector cells were tested for lytic activity on HTG (K^d) targets or L929 (H-2^k) and MC57 (H-2^b) target cells infected with either VV-K^d, control VV, or left uninfected (Table II). 5R anti-B/c effector cells lysed MC57- and L929 VV-K^d-infected targets specifically, although, as shown above, cross-reactivity on VV-infected and uninfected targets was also observed. Lysis of HTG targets was higher than L929-VV-K^d which in turn was higher than MC57-VV-K^d-infected targets, reflecting the K^d Ag concentration as determined by FACS analysis (data not shown). 5R effectors generated in response to VV-K^d-infected 5R cells gave results similar to those stimulated with B/c, thus indicating no effect attributable to vaccinia infection on availability of endogenous mouse cell peptides.

Limiting dilution split clone experiments on murine and human target cells infected with the VV-K^d recombinant virus. The question regarding murine peptide-specific allorecognition was also addressed using a limiting dilution split clone approach. 5R anti-B/c cultures were set up under limiting dilution conditions, and effector clones were split and tested on MC57 VV-K^d vs 143-B VV-K^d targets (Fig. 8).

Murine targets infected with VV-K^d were more susceptible to lysis by anti-K^d alloreactive Tc cell clones than the human 143-B VV-K^d-infected target cells. However, a large number of clones significantly lysed both targets suggesting no role for murine-specific peptides. Quantitative analysis of K^d expression on the target cells revealed that MC57 targets expressed K^d at higher concentrations than 143-B (data not shown), thus indicating a

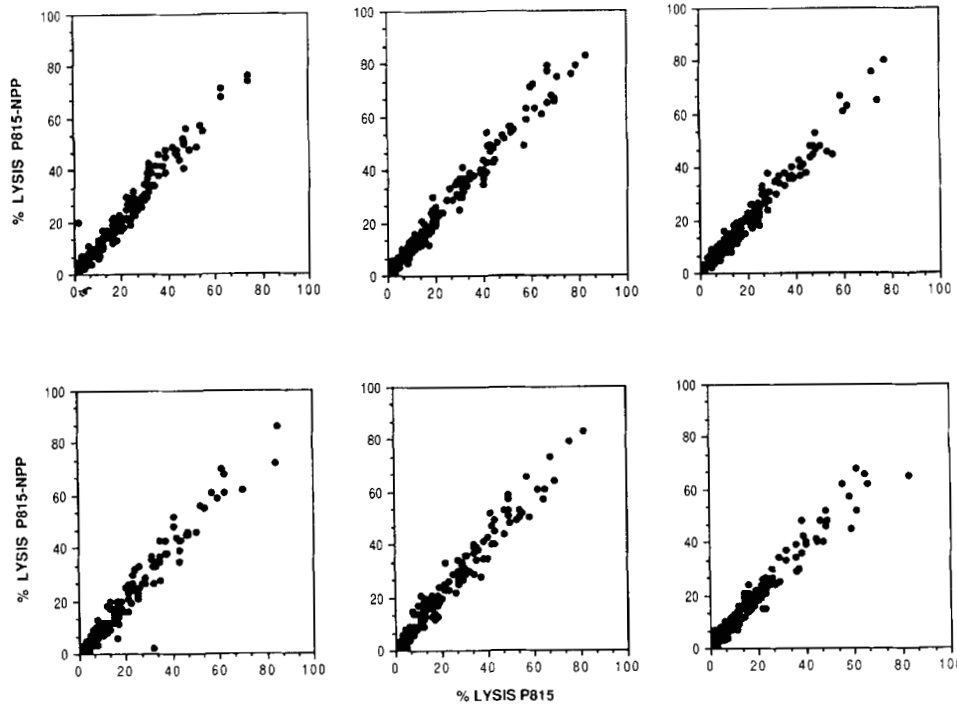

Figure 7. Limiting dilution split clone analysis of NPP-K^d-stimulated alloreactive Tc cells. K^d-NPP-specific alloreactive effector cells were generated in microculture wells by coculture of 5×10^3 5R responder cells with 2×10^5 B/c stimulator cells in the presence of 10^{-4} M NPP. Contents of wells were split and tested for lysis on P815 and P815–48h NPP (10^{-4} M) modified targets. Each panel contains the results of 192 wells. Duration of assay was 6 h.

TABLE I

VV-K^d-infected murine and human target cell lysis by murine alloreactive anti-K^d Tc cells and VV-specific K^d-restricted Tc cells

Effectors	E:T	% Specific ^{51}Cr Release							
		HTG (K^dD^b)	OH (K^dD^k)	MC57 (H-2^b)			143-B (human)		
		U	U	U	VV	VV-K^d	U	VV	VV-K^d
5R anti-B/c^b (anti-K^d)	30	89	87	52	63	82	11	6	36
	10	69	81	24	26	66	5	2	17
	3	39	55	9	9	34	2	1	6
OH anti-VV^c (K^d-restricted)	90	11	40	12	20	83	19	14	61
	30	9	29	8	14	75	12	12	35
	10	5	15	4	8	46	6	5	16

[a] Mean percent ^{51}Cr release from target cells over a 6-h period. Spontaneous release ranged from 10 to 13%. Means of triplicates are given; SE of the mean were never >3%.
[b] In vitro generated K^d-specific alloreactive Tc cells.
[c] Primary in vivo VV-immune Tc cells.

TABLE II

Induction and recognition of allo-K^d expressed by VV-K^d recombinant

Effectors	E:T	% Specific ^{51}Cr Release[a]						
		MC57 (H-2^b)			L929 (H-2^k)			HTG (K^dD^b)
		U	VV	VV-K^d	U	VV	VV-K^d	U
5R anti-B/c^b (anti-K^d)	30	41	37	73	40	47	72	87
	10	17	14	34	19	22	60	79
	3	5	2	15	6	7	45	49
5R anti-VV-K^d[c]	30	52	50	68	48	55	68	83
	10	28	26	42	29	37	61	68
	3	8	7	20	10	14	53	44

[a] As for Table I. Spontaneous release ranged from 9 to 17%.
[b] As for Table I.
[c] Primary in vitro culture of 5R splenocytes stimulated with 5R splenocytes infected with VV-K^d.

quantitative contribution of K^d to differences in lysis of murine and human target cells.

NPP-enhances VV-expressed K^d as recognized by alloreactive Tc cells. Recent results suggest that exogenous peptides that are able to bind to MHC class I Ag facilitate the cell surface expression and or the stability of these molecules (21). Therefore we tested the proposition that K^d-specific alloreactive Tc cells generated in the absence of NPP lyse target cells infected with VV-K^d more efficiently if such cells are pretreated with NPP. Table III shows two such experiments. K^d-alloreactive Tc cells lysed VV-K^d-infected, NPP-treated murine and human targets twofold more efficiently than untreated VV-K^d-infected targets. This result implies that NPP complexed with K^d formed an appropriate target cell epitope for K^d-specific alloreactive Tc cell recognition, even though NPP

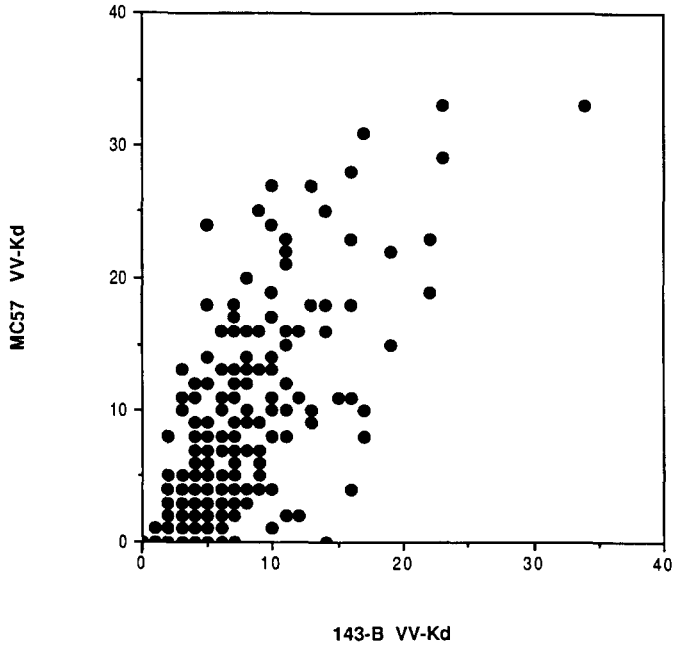

Figure 8. Limiting dilution split clone analysis of murine and human K^d-expressing target cells. 5R anti-B/c alloreactive Tc cells generated in microculture wells were tested by splitting contents of wells for lysis of MC57 and 143-B target cells infected with VV-K^d. Panel represents 284 wells tested. Assay duration 6 h.

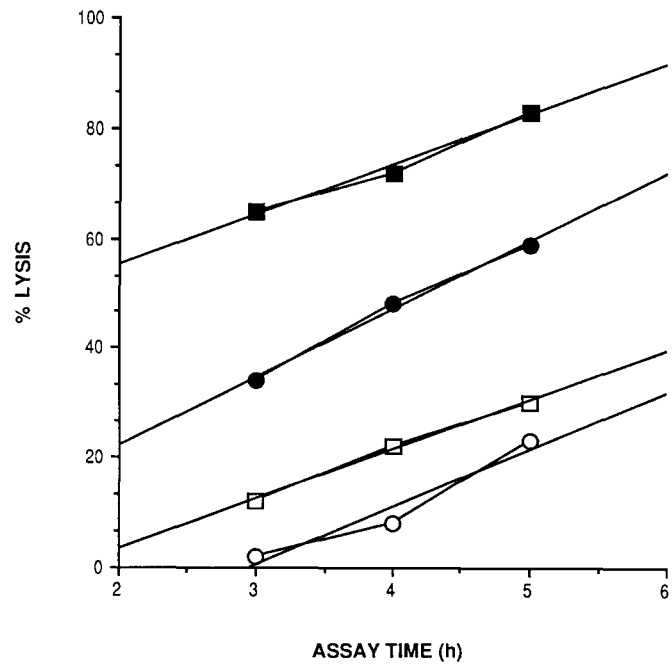

Figure 9. Kinetics of reemergence of T cell epitopes. Lysis of influenza virus-infected targets treated with papain (*open symbols*) or left untreated (*closed symbols*) 3, 4, and 5 h before alloreactive (CBA anti-B/c) (*squares*) or influenza-immune (*circles*) effector were added. All values are from a fourfold titration curve.

TABLE III

Lysis of murine L929- and human 143-B target cells infected with VV-K^d in the presence or absence of NPP by K^d-specific alloreactive Tc cells

Expt. 1 Effectors	E:T	% Specific ^{51}Cr Release from L929 Targets[a]			
		VV	VV-K^d	VV/NPP	VV-K^d/NPP
CBA anti-B/c[b]	30	43	67	53	80
	10	28	48	36	57
	3	2	26	18	32
B/c anti-A/WSN[c]	30	23	26	27	72
	10	14	17	17	50
	3	5	7	7	25
Expt. 2		% Specific ^{51}Cr Release from 143-B Targets[a]			
		U	VV	VV-K^d	VV-K^d/NPP
CBA anti-OH[b]	30	10	11	46	57
	10	4	4	22	38
	3	2	0	9	14

[a] As for Table I. Spontaneous release ranged from 12 to 18%.
[b] As for Table I.
[c] Secondary in vitro culture of B/c A/WSN-immune memory splenocytes stimulated with A/WSN-infected B/c splenocytes.

was not present during the induction of the response, thus suggesting a lack of specificity for peptide in allorecognition.

Kinetics of emergence of K^d-influenza and alloreactive K^d epitopes on papain-treated target cells. Because influenza virus is cytopathic and inhibits host cell protein synthesis soon after infection, influenza-specified peptides should be much more prevalent than endogenous mouse cell peptides in K^d-peptide complexes produced subsequent to infection. Therefore, pre-existing K^d-peptide complexes on P815 target cells were removed by papain treatment 1 h after infection with influenza virus and the treated cells were used as target cells for K^d-alloreactive and K^d-restricted influenza-immune Tc cells (Fig. 9). Lysis of target cells was assayed 3, 4, and 5 h after the addition of effector cells. K^d-alloreactive Tc cells lysed untreated control targets effectively at an E:T ratio of 3:1 giving 65% lysis at 3 h to 84% lysis at 5 h. Papain-treatment of cells reduced the lysis to 11% at 3 h and 30% at 5 h. Influenza virus-immune Tc cells gave a similar picture, although overall lower lysis was obtained. Most importantly, however, the slopes of the lines, a measure of the rate of killing, was the same for both the K^d-alloreactive Tc cells and the influenza-immune Tc cells.

DISCUSSION

The experiments described in this paper are an attempt to differentiate between three possible models of alloreactive Tc cell recognition of class I MHC. Namely, a) only peptide-free MHC is recognized, b) recognition is specific for an epitope formed by a complex of an allo class I MHC molecule plus an endogenous peptide, analogous to MHC-restricted responses, or c) allogeneic class I MHC molecules complexed with endogenous peptides are recognized by Tc cells regardless of which particular peptide is present.

We could test the first possibility by using a synthetic peptide (NPP) resembling the sequence of the nucleoprotein of influenza virus, which has a very high affinity for K^d and which forms with K^d a dominant epitope recognized by K^d-restricted influenza-immune Tc cells (18). The initial experiments showed that target cells expressing K^d reached plateau levels of NPP binding by 8 h and retained NPP for at least 72 h. Thus the continuous presence of NPP over a 48-h period should saturate all available K^d (free of endogenous peptides) estimated to be less than 0.3% of all cell-surface K^d (13). However, polyclonal K^d-alloreactive Tc cell populations did not show decreased lysis of peptide-modified targets compared with unmodified controls. Clonal analysis using the split-clone limiting dilution technique also did not reveal a

single K^d-reactive clone that was unable to lyse NPP-modified K^d targets, although it is known that individual alloreactive Tc cell clones are heterogeneous with respect to their ability to lyse target cells with low levels of class I MHC cell-surface expression (22). Thus these data strongly suggest that peptide-free class I molecules are not the principle ligand for alloreactive Tc cells.

We then asked if the K^d-NPP epitope can be recognized during the induction of an alloreactive Tc cell response. The immunogenicity of K^d-NPP was clearly established, as self K^d-restricted influenza-primed splenocytes could be stimulated in vitro with NPP-armed splenocytes to differentiate into K^d-NPP-specific Tc cells. Employing the same conditions with NPP-armed splenocytes we tested in limiting dilution a total of 2000 alloreactive 5R (K^b,D^d) anti-K^d Tc cell precursors. The results were unambiguous; no K^d-NPP specific clones could be detected. The same experiment was performed using CBA splenocytes as responders, to test the possibility that thymic positive selection or cross-tolerance to MHC or background Ag in 5R skewed the T cell repertoire in such a way that K^d-NPP recognition is reduced. Again it was found that K^d-NPP specific alloreactive Tc cells were not induced implying that recognition of K^d by alloreactive Tc cells was not specific for peptide.

Three further results provide additional evidence compatible with peptide-nonspecific allorecognition. First, K^d expressed in human 143B cells by infection with recombinant VV encoding K^d (VV-K^d) can render these cells susceptible to lysis by murine alloreactive Tc cells from both polyclonal and limiting dilution monoclonal cultures, although the level of lysis was about threefold lower than on VV-K^d-infected MC57 murine target cells. This difference could be explained by a combination of the observed cross-reactivity of 5R anti-B/c (anti-K^d) effectors on MC57 (K^bD^b) targets (Tables I and II), a two- to threefold concentration difference of cell-surface K^d molecules evident by FACS analysis and possibly reduced efficiency of lymphocyte function-associated Ag-intercellular adhesion molecule interactions across the species barrier (23). The same targets showed a threefold difference in lysis by VV-immune K^d-restricted effectors that recognize vaccinia-derived peptides. These results suggested that rather than a lack of endogenous mouse-specific peptides, the concentration of cell-surface K^d expression was a limiting factor (24, 25) in the avidity of Tc cell binding required for effector function of Tc cells against VV-K^d-infected 143B target cells. Such quantitative differences could also have contributed to the findings by Heath et al. (7) which were interpreted qualitatively as evidence for peptide-specific recognition by alloreactive Tc cells. In this latter case, addition of murine peptides to target cells may have increased the quantity of murine class I MHC expressed by human target cells transfected with a murine class I gene (21) (see Table III discussed further below).

Using the same VV-K^d recombinant virus to infect 5R (K^bD^d) stimulator cells we were able to induce potent alloreactive K^d-specific 5R Tc cells that lysed uninfected HTG (K^d,D^b) targets. Because VV infection inhibits host cell protein synthesis, which would reduce availability of endogenous murine peptides, the VV-encoded K^d produced in infected 5R stimulator cells may have been predominantly exposed to VV peptides as indicated by VV-specific K^d-restricted recognition. This again points to the possibility of K^d alloreactive Tc cells without peptide specificity.

Second, the results presented in Table III are in agreement with recent reports (21) indicating that exogenously applied peptides facilitate MHC class I cell-surface expression and stability. L929 and human 143-B cells infected with VV-K^d and pretreated with NPP were more susceptible to lysis by CBA anti-K^d-alloreactive Tc cells than VV-K^d-infected targets. Under these conditions it is reasonable to propose that the increased lysis was caused by increased expression of K^d complexed with NPP, a T cell epitope not present during induction of the Tc cell response.

Finally, influenza virus-infected target cells that had their class I MHC cell-surface molecules removed by papain cleavage displayed an identical rate of reemergence of allo and self-restricted T cell epitopes. Because influenza infection inhibits host cell protein synthesis, it would be expected that class I MHC reexpressed on papain-treated influenza-infected cells would be complexed predominantly with influenza-specified peptides. Thus these results also point to the possibility that MHC class I-influenza peptide complexes are recognized by alloreactive peptide-nonspecific Tc cells.

There are two possible explanations as to why allorecognition differs in its molecular model from self-restricted recognition. First, it is now established that self MHC must be bound by TCR during thymic development of T cells (positive selection), but the specificity of this event with respect to peptides is unknown (26). Self tolerance mechanisms then delete or control T cells reactive to normal self MHC epitopes (negative selection) (26), but permit the survival of T cells with affinity for allo MHC. There is no a priori reason to believe that peptide specificity is *essential* with respect to allo MHC recognition. Furthermore, two recent studies indicate that it is possible for alloreactive Tc cells to recognize peptide-free allo class I MHC molecules (12, 14). The present results suggest that peptide-free MHC is not essential for allorecognition and that peptide specificity is often irrelevant to allo MHC binding by the TCR. In contrast, because of self tolerance, self MHC-restricted Tc can bind to self class I molecules with sufficient affinity to trigger activation, *only* when a foreign peptide occupies the cleft in the MHC molecule.

Second, the results are also in agreement with the mechanism proposed by Langman and Cohn for alloreactivity by the one receptor dual recognition model of TCR (15). In this model, one chain of the TCR-$\alpha\beta$ is selected in the thymus for restricted recognition of self MHC (e.g., α-chain) and variants of the other chain (e.g., β-chain) randomly associate with the first chain to give a family of heterodimers, which constitute the self-MHC-restricted repertoire. The rules of self tolerance apply as outlined above. This model permits allo MHC recognition by the random repertoire of the second chain (e.g., β) regardless of peptide specificity.

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