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INDUCTION OF CYTOTOXIC T LYMPHOCYTES BY PRIMARY IN VITRO STIMULATION WITH PEPTIDES

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The detection of foreign viral or minor histocompatibility antigen by cytotoxic T lymphocytes (CTL) requires the corecognition of cell surface glycoproteins encoded by the class I loci of the MHC (1–4). Recently it was demonstrated (5, 6) that the expression of truncated versions of viral genes in transfected cell lines can render them susceptible to CTL lysis. It was subsequently shown (7–9) that synthetic peptides could effectively substitute for foreign antigens during MHC-restricted CTL-mediated lysis. For example, Townsend et al. (7) showed that a synthetic peptide from the influenza nucleoprotein sequence was effective in sensitizing uninfected target cells for influenza-specific CTL recognition.

As a consequence of these observations, it has been suggested that endogenously expressed viral antigens may in fact be fragmented by some cellular degradation mechanism before expression on the surface of the cell in conjunction with MHC ready for T cell recognition (6, 10, 11). Virtually all cellular proteins, in addition to viral antigens, could therefore generate relevant peptide fragments that serve as potential MHC class I ligands. While a direct peptide–class I MHC protein interaction has yet to be demonstrated for either foreign or self antigens, recent crystallographic data on a human class I glycoprotein are consistent with this idea (12, 13). Given this view, it is clear that any particular MHC/peptide complex would represent only a small proportion of the total MHC-encoded molecules found on the cell surface. This type of argument has been presented previously to explain the high frequency of T cells responding to stimulator cells that express allogeneic MHC products (alloreactive T cells). On the one hand, it has been proposed that the high frequency of T cells responsive to stimulators expressing allogeneic MHC products results from the enormous multiplicity of new peptide/MHC complexes on the surface (14). Alternatively, it has been proposed that alloreactivity may be due to the fact that some determinants on the allo-MHC molecule will be expressed at a much higher density than any one self-restricted peptide/MHC determinant (15, 16).

We reasoned that the association of exogenous, soluble peptides with MHC class I products during in vitro culture may be more efficient than would be the case for the comparable endogenously produced determinant. This efficient association would

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result in a disproportionately high density of MHC class I protein/peptide complex on the cell surface. Soluble peptide could then potentially generate a stronger T cell response than is usually observed for class I-restricted antigens. We show here that peptide fragments can effectively generate a strong primary in vitro CTL response. This response is both peptide specific and MHC restricted but clearly differs from the secondary CTL response seen after conventional in vivo immunization with live virus.

Materials and Methods

Mice. C57BL/6, B10.A(4R), and B10.A(5R) mice were obtained from the Scripps Clinic and Research Foundation vivarium.

Monoclonal Antibodies. mAbs used in this study were 13.4 (anti-Thy-1.2, reference 17), RL.172.4 (anti-CD4 (L3T4), reference 18), and 3.168 (anti-CD8 (Lyt-2), reference 19). These were used with rabbit complement to deplete the appropriate effector cell populations immediately before addition to cytotoxicity assays.

Antigens. OVA (Grade VI; Sigma Chemical Co., St. Louis, MO) was used in native form or after cleavage with trypsin (T OVA)[1] or cyanogen bromide (CN OVA). For T OVA, 100–250 mg of OVA was reduced in 0.2 M 2-ME/8 M urea overnight and then alkylated with 0.25 M iodoacetic acid for 2 h. The alkylated protein was exhaustively dialyzed and then cleaved with 1% trypsin in 50 mM NH_4CO_4, pH 8.0, overnight. For CN OVA, 250 mg of OVA was reacted overnight with 500 mg of CN dissolved in 70% formic acid. The solvent was evaporated under a stream of nitrogen and the residue was suspended in water and lyophilized. No intact OVA remained in the CN OVA and T OVA preparations, as assessed by SDS gel electrophoresis. The synthetic peptide antigens $NP_{365-380}$ (IASNENMET-MESSTLE) and $OVA_{111-122}$ (YPILPEYLQCVK) were synthesized on an automated peptide synthesizer (Applied Biosystems, Inc., Foster City, CA) and their purity was assessed by HPLC and amino acid analysis.

Influenza Virus Strains. Influenza virus stocks of A/PR/8 and A/HK/68 were obtained from A. Vitiello, Scripps Clinic and Research Foundation. Virus was grown in the allantoic sacs of 11-d-old embryonated chicken eggs and stored as infectious allantoic fluid at −70°C.

Target Cells for Cytotoxicity Assays. The tumor cell lines used were the Ia^- lines, EL4 (C57BL/6, H-2^b thymoma), P815 (DBA/2, H-2^d mastocytoma), CBA-D1 (CBA, H-2^k lymphoma), and R1.E (TL^-) (C58/J, H-2^k thymoma). The R1.E(TL^-) line also fails to express class I MHC gene products on the surface due to a defect in β_2-microglobulin expression (20). EL4 was transfected with OVA cDNA in a plasmid construct with the human β-actin promoter to derive the OVA-producing cell line E.G7-OVA, which will be described in detail elsewhere (Moore, M. W., M. J. Bevan, and F. R. Carbone, manuscript in preparation). Peritoneal exudate cells for use as targets were induced by injecting 1.5 ml of 3% wt/vol thioglycolate (Difco Laboratories Inc., Detroit, MI) intraperitoneally 4 d before harvesting.

Primary Peptide-specific Cytotoxic Effector Populations. Unless otherwise stated, 60 × 10^6 spleen cells from unprimed 6–8-wk-old C57BL/6 mice were incubated for 5 d in 10 ml of RP10 (RPMI-1640 with 10% FCS and 50 μM 2-ME) in upright 25-cm^2 flasks at 37°C in 7% CO_2/air. Concentrations of peptides added to the cultures were 100 μg/ml for CN OVA and T OVA or 5 μg/ml for $NP_{365-380}$.

Long-Term CTL Lines. Primary cytotoxic effector populations were harvested after 7 d, and 5 × 10^6 recovered cells were restimulated with 20 × 10^6 irradiated (3,000 rad) syngeneic spleen cells in 10 ml of RP10 at the same peptide concentrations as the primary cultures. Subsequent weekly restimulations were carried out with 2–4 × 10^6 responder cells and 20 × 10^6 irradiated syngeneic spleen cells in 10 ml of RP10 with 5% supernatant from Con A-stimulated rat spleen cells and 50 mM α-methyl mannoside in upright 25-cm^2 flasks. Peptides were used at the same concentrations as in primary cultures.

[1] *Abbreviations used in this paper:* CN OVA, cyanogen bromide-cleaved ovalbumin; T OVA, trypsinized ovalbumin.

CN OVA-specific CTL Clones. Responder cells recovered from long-term CN OVA-specific populations were cloned at 10, 3, 1, and 0.3 cells/well in 96-well plates (No. 3596; Costar, Cambridge, MA). Irradiated syngeneic spleen cells (5×10^5 cells/well) and CN OVA (100 μg/ml) in medium containing 5% supernatant from Con A-stimulated rat spleen cells were used for cloning. Cells from wells with positive signs of growth (from 3 and 1 cell/well plates) were expanded and tested for CN OVA-specific lysis. Clones were maintained by weekly restimulation of $1-2 \times 10^5$ cells with 5×10^6 irradiated syngeneic cells in 2 ml of CN OVA containing medium in 24-well plates (No. 3484; Costar).

Generation of Anti-influenza A/PR/8 CTL. In vitro secondary anti-A/PR/8 effectors were derived using responder spleen cells from C57BL/6 mice immunized 4 wk earlier with 100 HAU of A/PR/8 influenza virus intravenously. Stimulator cells were prepared by infecting 10^8 irradiated (3,000 rad) C57BL/6 cells with 0.5 ml infectious allantoic fluid in 4 ml of RPMI-1640 for 90 min. Responder cells (25×10^6 cells) and stimulator cells (25×10^6 cells) were incubated in 20 ml of RP10 medium for 5 d in upright 25-cm^2 flasks. Influenza A/PR/8-reactive cells were selectively expanded from anti-$NP_{365-380}$ CTL populations after the third or fourth in vitro stimulation. In this case, $4-6 \times 10^6$ responder cells and 20×10^6 A/PR/8-infected irradiated syngeneic spleen cells were incubated for 5 d in 20 ml medium in upright 25-cm^2 flasks.

Cytotoxicity Assay. For peptide-specific lysis, 10^6 target cells in 600 μl of RP10 were labeled with 300 μCi sodium [^{51}Cr]chromate for 45 min. For influenza-specific lysis, 10^6 target cells in 600 μl RPMI-1640 were labeled with 300 μCi of ^{51}Cr and simultaneously infected with 100 μl of infectious allantoic fluid for 90 min. After washing, 10^4 labeled targets and serial dilutions of effector cells were incubated in 200 μl of RP10 with appropriate peptides. Peptide concentrations used in the assays were 100 μg/ml CN OVA and T OVA, or 10 μg/ml of $NP_{365-380}$ and $OVA_{111-122}$. After a 4-h incubation at 37°C, 100 μl of supernatant was collected and specific lysis was determined as: Percent specific lysis = 100 × [(release by CTL − spontaneous release)/(maximal release − spontaneous release)]. Spontaneous release in the absence of CTL was <25% of maximal release by detergent in all experiments.

Results

Induction of Cytotoxic Effectors by Primary In Vitro Stimulation with Peptides. Both T OVA and CN OVA were used as a source of peptides of varying lengths to assess their ability to generate a primary in vitro CTL response. A 5-d incubation of spleen cells from unimmunized C57BL/6 mice with 100 μg/ml CN OVA generated effectors that efficiently lysed the syngeneic tumor target EL4 in the presence of CN OVA (Fig. 1 *a*). These effector cells caused only a low level of specific target cell lysis in the presence of T OVA, and no significant enhancement of killing was seen in the presence of native OVA when compared with EL4 targets alone (Fig. 1 *a*). Fig. 1 *b* shows that effectors of reciprocal specificity resulted when T OVA was used as the in vitro immunogen. A 5-d incubation of spleen cells with native OVA (Fig. 1 *c*) failed to generate effectors capable of lysing targets in the presence of T OVA, CN OVA, or native OVA.

Synthetic peptides can substitute for endogenously synthesized viral proteins during CTL recognition (7–9). We attempted to raise CTL by primary in vitro stimulation using one such peptide, $NP_{365-380}$, which corresponds to the fragment between residues 365 and 380 from the influenza virus A/PR/8 nucleoprotein (21). Fig. 1 *d* shows that incubating spleen cells from unprimed C57BL/6 mice with $NP_{365-380}$ generated effectors capable of lysing EL4 targets only in the presence of this synthetic peptide and eliminated the possibility that the primary in vitro antipeptide activity was due to an artifact peculiar to the OVA protein.

Effect of Cell Density on the Primary Response. During the course of our studies we

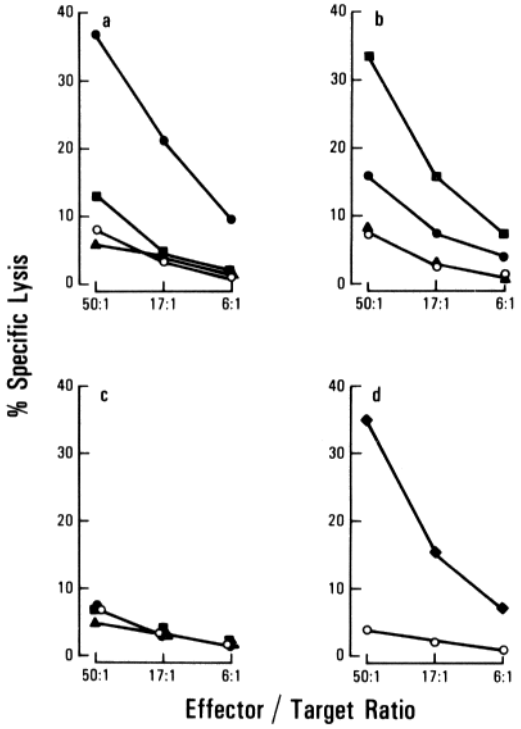

FIGURE 1. The ability of peptides to elicit specific CTL after primary in vitro stimulation. Spleen cells from unprimed C57BL/6 mice (60×10^6 cells per flask) were incubated for 5 d in the presence of 100 μg/ml (*a*) CN OVA, (*b*) T OVA, (*c*) native OVA, or (*d*) 5 μg/ml $NP_{365-380}$. The resulting effectors were then tested for lysis of ^{51}Cr-labeled EL4 target cells in the presence of (●)100 μg/ml CN OVA, (■)100 μg/ml T OVA, (▲)100 μg/ml native OVA, (◆)10 μg/ml $NP_{365-380}$, or (○) medium alone.

noticed that the most critical parameter affecting the primary in vitro response was the starting cell density, as demonstrated for CN OVA in Fig. 2. In this experiment, starting cell numbers in the range of 40–80 $\times$ 10^6 cells/flask resulted in the stimulation of a strong CN OVA-specific activity, with 20 $\times$ 10^6 cells/flask giving much weaker activity. Such weak stimulation was generally observed for most attempts at priming using < 40 $\times$ 10^6 cells/flask. Cell numbers of ~60 $\times$ 10^6 were routinely used for our experiments. All three peptide preparations were capable of priming at this density, with CN OVA giving the best response, followed by T OVA and then $NP_{365-380}$.

Antipeptide Cytotoxic Effector Cells are Thy-1$^+$ and CD8$^+$ Cells. Peptide-specific lines were established by repeated antigen stimulation of effectors generated from the primary in vitro cultures. To determine whether peptide-specific recognition was indeed due to T cell activity, these lines were treated with anti-Thy-1, anti-CD8 or anti-CD4 antibodies, and/or complement immediately before their addition to assay cultures. Fig. 3 shows that depletion of CD8$^+$ or Thy-1$^+$ cells from both anti-CN OVA and anti-$NP_{365-380}$ lines abrogated all specific lytic activity. Neither complement alone nor anti-CD4 had any effect. Similarly, all the T OVA activity is sensitive to anti-Thy-1 and anti-CD8 antibody and complement treatment (data not shown). Effectors were therefore classical CD8$^+$ T cells.

$OVA_{111-122}$ Is a Target Peptide for Anti-T OVA CTL. Based on the preliminary analysis of a chromatographic separation of an OVA tryptic digest (not shown), a limited

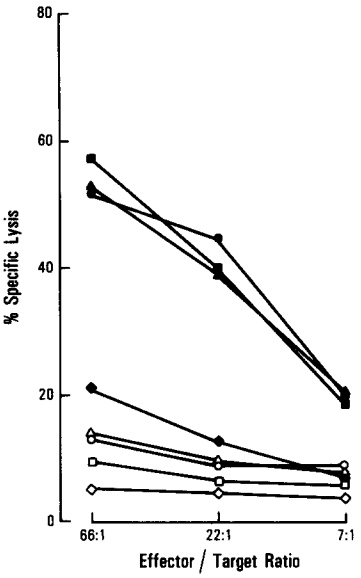

FIGURE 2. The effect of cell density on primary in vitro peptide stimulation. Spleen cells from unprimed C57BL/6 mice (●, ○: 80 × 10^6; ■, □: 60 × 10^6; ▲, △: 40 × 10^6; ◆, ◇: 20 × 10^6 cells) were incubated for 5 d in the presence of 100 μg/ml CN OVA. The resulting effectors were then tested for lysis of ^{51}Cr-labeled EL4 target cells in the absence (*open symbols*) or presence (*closed symbols*) of 100 μg/ml CN OVA.

number of peptides were synthesized in an attempt to identify the fragments recognized by the OVA digest-specific CTL. Anti-T OVA CTL lines showed strong lysis of the H-2-compatible target EL4 in the presence of one such peptide, synthetic tryptic fragment $OVA_{111-122}$ (Table I). This peptide did not sensitize targets to the same extent as the whole T OVA digest, suggesting that $OVA_{111-122}$ represented a major but not exclusive determinant for the bulk of anti-T OVA CTL. In other experiments we estimated that the activity against this peptide accounted for 10–100% of the total CTL activity.

Peptide-specific Lysis Is Class I Restricted. Thioglycolate-induced peritoneal exudate cells from C57L/6 (K^b, D^b), B10.A(4R) (K^k, D^b), and B10.A(5R) (K^b, D^d) mice served as convenient targets for mapping the restriction element used by the three different effector populations. Table II shows that anti-$NP_{365-380}$ CTL lysed

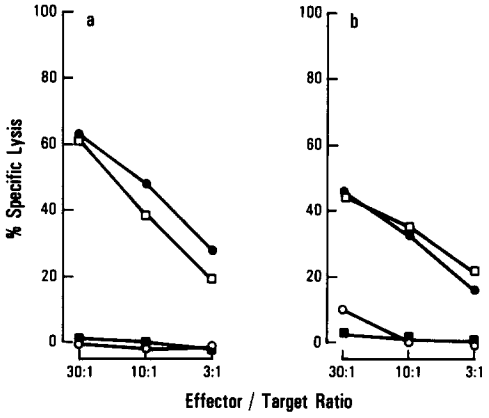

FIGURE 3. Peptide-specific effectors are $CD8^+$ T cells. Long-term (*a*) anti-CN OVA and (*b*) anti-$NP_{365-380}$-specific effector populations were treated with complement in the presence of (■) anti-Thy-1, (●) anti-CD4, (○) anti-CD8, or (□) no antibody before addition to ^{51}Cr-labeled EL4 in the presence of (*a*) 100 μg/ml CN OVA or (*b*) 10μg/ml $NP_{365-380}$.

TABLE I

Peptide Specificity of Anti-T OVA CTL

E/T	Specific Lysis in the presence of*		
	Medium control	T OVA‡	$OVA_{111-122}$§
		%	
30:1	9	67	48
15:1	4	47	23
8:1	1	22	10

* ^{51}Cr-labeled EL4 cells were used as targets for lysis by anti-T OVA CTL population derived from primary in vitro stimulation.
‡ 100 μg/ml T OVA digest.
§ 10 μg/ml $OVA_{111-122}$.

C57BL/6 and B10.A(4R) but not B10.A(5R) cells in the presence of $NP_{365-380}$. Recognition of the $NP_{365-380}$ peptide was clearly restricted to the D^b locus. This coincides with the fact that $NP_{365-380}$ CTL derived from virus-infected C57BL/6 mice are also D^b restricted (7, 22). The anti-T OVA CTL response was also predominantly D^b restricted since strong peptide-specific killing was observed for both C57BL/6 and B10.A(4R) targets (Table II). In this case, weak lysis was apparent for B10.A(5R) targets in the presence of the T OVA digest, suggesting a minor K^b-restricted activity.

Anti-CN OVA CTL efficiently recognized all three peritoneal exudate targets in the presence of CN OVA (Table II). In addition, this line also lysed the class II-negative tumor targets P815 (H-2^d) and CBA-D1(H-2^k) in a peptide-specific manner (Fig. 4 *a*). The C58/J (H-2^k)-derived line R1.E (TL^-) is β_2-microglobulin-negative, and accordingly, does not express class I MHC gene products on its surface. The failure of anti-CN OVA CTL to lyse R1.E (TL^-) (Fig. 4 *a*) suggests that anti-CN OVA CTL are indeed class I restricted despite an unusual crossreaction on at least three

TABLE II

MHC Restriction of Peptide-specific CTL Recognition

CTL effector	Added peptides	Specific lysis of targets from:					
		C57BL/6*		B10.A(4R)		B10.A(5R)	
		10:1	3:1‡	10:1	3:1	10:1	3:1
		%		%		%	
anti-$NP_{365-380}$	$NP_{365-380}$§	50	29	47	31	−4	−1
	None	0	−1	−3	−3	2	0
anti-T OVA	T-OVA¶	35	32	30	20	12	7
	None	−3	0	8	7	4	5
anti-CN OVA	CN-OVA¶	64	62	62	52	57	42
	None	5	7	8	7	4	0

* ^{51}Cr-labeled thioglycolate-induced peritoneal exudate cells from C57BL/6, B10.A(4R), and B10.A(5R) mice were used as targets for lysis by CTL populations derived from primary in vitro peptide stimulation.
‡ E/T Ratio.
§ 10 μg/ml $NP_{365-380}$.
¶ 100 μg/ml OVA digest.

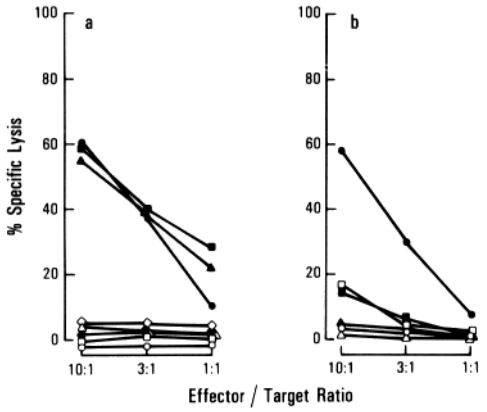

FIGURE 4. Peptide-specific lysis of various tumor targets by antipeptide CTL. ^{51}Cr-release assay for specific CTL lysis of tumor targets (●, ○) EL4, (▲, △) P815, (■, □) CBA-D1, and (◆, ◇) R1.E(TL$^-$) in the presence (*closed symbols*) or absence (*open symbols*) of peptides (*a*) 100 μg/ml CN OVA for anti-CN OVA effectors and (*b*) 100 μg/ml T OVA for anti-T OVA effectors.

different H-2 haplotypes. No peptide-specific lysis of tumor targets other than the class II-negative EL4 was observed for anti-T OVA CTL (Fig. 4 *b*) and anti-$NP_{365-380}$ (data not shown), confirming that these activities show classical class I MHC restriction.

Individual CN OVA-specific Clones Recognize Peptide in the Context of H-2^b, H-2^d, and H-2^k. The broad MHC haplotype crossreactivity shown by the anti-CN OVA population may have been a consequence of a more limited recognition by individual T cell clones of different specificities within the whole population. For example, there may be some clones that recognize CN OVA in association with H-2^b and H-2^k but not H-2^d, while others are restricted to H-2^b and H-2^d. This possibility was ruled out by examing the anti-CN OVA response at the clonal level. Five CN OVA-specific clones were established by limiting dilution from the bulk population used in the preceding section. All five clones exhibited CN OVA-specific lysis on each of the three tumor targets (Table III). Consequently, individual clones could recognize the

TABLE III

Lysis of a Range of Target Cells by CN OVA-specific CTL Clones

Target	Added peptide	Specific lysis of targets by CTL clones:* C3 10:1	C3 3:1‡	C7 3:1	C7 1:1	C11 10:1	C11 3:1	C12 10:1	C12 3:1	C13 3:1	C13 1:1
		%		%		%		%		%	
EL4	CN OVA§	37	24	40	22	47	34	50	41	52	40
	None	4	3	3	2	3	0	8	6	15	10
P815	CN OVA	42	30	59	36	68	52	55	57	66	50
	None	4	1	6	0	4	0	17	11	28	8
CBA-D1	CN OVA	46	38	78	56	66	57	58	57	62	55
	None	2	−1	14	3	5	4	9	7	10	3

* ^{51}Cr-labeled EL4, P815, and CBA-D1 tumor cells were used as targets for lysis by CN OVA-specific CTL clones.

‡ E/T ratio.

§ 100 μg/ml CN OVA digest.

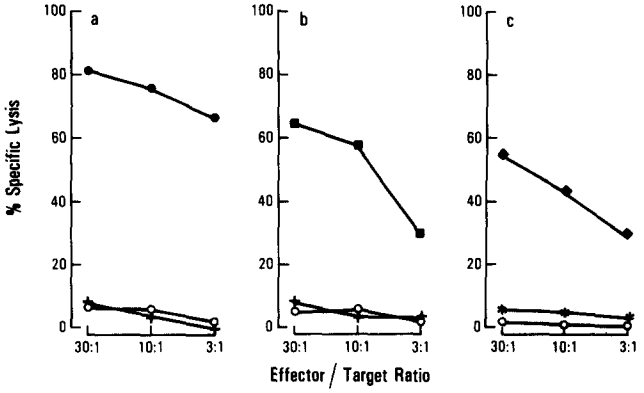

FIGURE 5. Antipeptide CTL fail to lyse targets that endogenously express the foreign antigens. Long-term (*a*) anti-CN OVA, (*b*) anti-T OVA, and (*c*) anti-$NP_{365-380}$ CTL lines were tested for killing on ^{51}Cr target cells: (●)EL4 + 100 μg/ml CN OVA, (■)EL4 + 100 μg/ml T OVA, (◆)EL4 + 10 μg/ml $NP_{365-380}$, + E.G7-OVA, (*)A/PR/8-infected EL4, or (O)EL4 alone.

CN OVA digest on restricting elements encoded by the H-2^b, H-2^d, and H-2^k haplotypes. We have not determined whether the peptide is the same for all five clones nor have we identified the peptide(s) responsible for target sensitization.

The Majority of In Vitro-induced Antipeptide CTL Fail to Lyse Cells that Endogenously Express the Foreign Antigen. The CTL lines generated from primary in vitro stimulation were assessed for their ability to lyse targets expressing endogenous forms of the appropriate antigens. Neither the anti-CN OVA CTL (Fig. 5 *a*) nor the anti-T OVA CTL (Fig. 5 *b*) could lyse the OVA-expressing transfectant E.G7-OVA. This lack of recognition was not due to defective antigen presentation, since E.G7-OVA is effectively lysed by OVA-specific H-2^b-restricted CTL derived from mice previously immunized with this transfectant (Moore, M. W., M. J. Bevan, and F. R. Carbone, manuscript in preparation). Similarly, the anti-$NP_{365-380}$ line failed to show significant lysis of influenza A/PR/8-infected EL4 cells even at high E/T ratios (Fig. 5 *c*).

The anti-$NP_{365-380}$ CTL line used for the experiment shown in Fig. 5 *c* was split and stimulated for a further 5 d in the presence of either $NP_{365-380}$ or A/PR/8-infected spleen cells. The A/PR/8-selected line showed a dramatic increase in lysis of A/PR/8-infected EL4 target cells (Fig. 6 *b*) when compared with the peptide-stimulated line (Fig. 6 *a*). The virus-selected anti-$NP_{365-380}$ line did not recognize A/HK/68-infected targets. In contrast, A/HK/68-infected EL4 cells were efficiently recognized by secondary CTL derived from A/PR/8-infected C57BL/6 mice (Fig. 6 *c*). This virus crossreactivity has been attributed to a nucleoprotein-specific subpopulation within the secondary A/PR/8 response (23, 24). Spleen cells from the same batch of unimmunized C57BL/6 mice used to set up the initial anti-$NP_{365-380}$ line failed to mount any primary in vitro response to A/PR/8-infected syngeneic spleen cells (Fig. 6 *d*). This inability to elicit a primary antiviral response in conjunction with the expansion of only substrain-specific CTL strongly suggested that the A/PR/8 activity shown in Fig. 6 *b* was derived as a consequence of peptide priming and not from inadvertent influenza infection of the mice used in this study. The E.G7-OVA-transfected line could neither elicit a primary response nor could it selectively expand any transfectant-specific effector from the CTL populations derived by primary OVA-peptide stimulation (data not shown). Furthermore, we were not

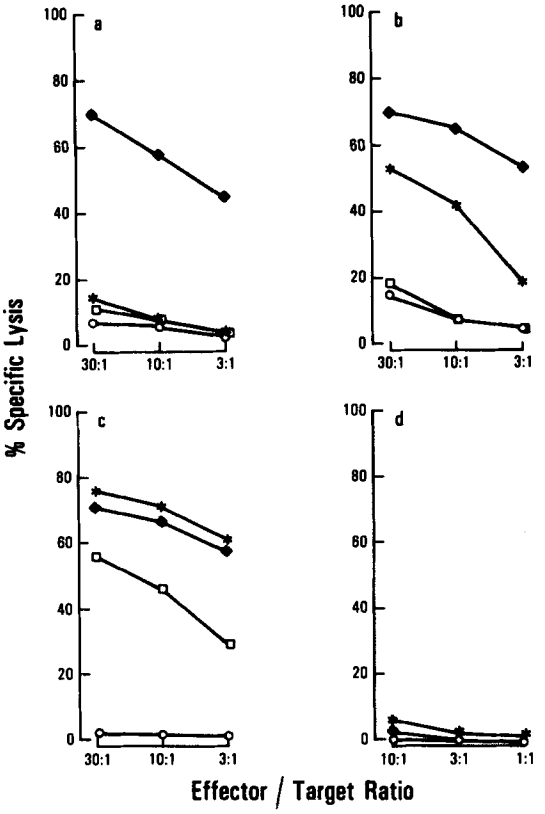

FIGURE 6. Selection for A/PR/8-specific CTL. CTL used in the assay shown in Fig. 5 *c* were stimulated for a further 5 d in the presence of (*a*) $NP_{365-380}$ or (*b*) A/PR/8-infected spleen cells. Spleen cells from (*c*) A/PR/8-infected or (*d*) uninfected C57BL/6 mice were stimulated for 5 d in the presence of A/PR/8-infected spleen cells. Specific lysis was measured on the following targets: (◆)EL4 + 10μg/ml $NP_{365-380}$, (*)A/PR/8-infected EL4, (□) A/HK/68-infected EL4, or (○)EL4 alone.

able to select a population of CTL capable of lysing A/PR/8-infected EL4 cells from the OVA digest-specific CTL lines.

Discussion

The major finding of our work is that peptides can elicit strong, MHC-restricted CTL responses measurable after 5 d of culture with spleen cells from unprimed mice. Conventional presentation of MHC-restricted antigens, such as by virus-infected or minor histocompatibility-disparate cells, does not result in measurable primary in vitro responses. This was evident by the failure of influenza A/PR/8-infected cells to induce a primary response (Fig. 6 *d*). Previously, only MHC-different stimulators (25–27) or hapten-modified syngeneic cells (28, 29) have been reported to give vigorous primary responses. The antipeptide responses reported here are much weaker than alloreactive responses and are very dependent on a high cell density in the cultures. However, it is clear that while influenza virus-infected cells cannot stimulate the response, one antigeneic peptide from the NP component of the virus can. Similarly, we would predict that while it is not possible to observe a primary in vitro CTL response of H-2^b female spleen cells to the male-specific H-Y antigen by stimulation with male cells, if we knew the sequence of one of the H-Y antigenic peptides we would be able to use such a peptide to generate a response.

To explain the success of exogenous peptide immunization and the failure of endogenous peptide presentation in vitro, we invoke the determinant density argument. It has been argued previously that the strong response to MHC allogeneic cells is due to the high density of MHC determinants (15, 16). It is possible that an analogous situation pertains as a consequence of the exogenous addition of peptides at high concentrations. Under these circumstances it may be that a large fraction of MHC molecules are occupied by the added peptide, while in the case of endogenous protein synthesis the analogous peptide would occupy a much smaller fraction of the MHC molecules. With a higher surface density of a particular peptide/MHC complex we would expect T cells with lower receptor affinity to be able to respond. Thus, the bulk of antipeptide CTL may be unable to lyse cells expressing endogenous determinants. This is exactly what was observed for anti-$NP_{365-380}$ CTL; the majority of effectors failed to lyse A/PR/8-infected targets (Fig. 5 *c*). Within this population there existed a small proportion of CTL with high receptor affinity, and these were selectively expanded by stimulating with the A/PR/8-infected spleen cells (Fig. 6 *b*).

In contrast to the anti-$NP_{365-380}$ CTL, no corresponding E.G7-OVA-reactive CTL were detected within the anti-CN OVA or anti-T OVA populations. We attribute this to differences between the OVA peptides and the $NP_{365-380}$ peptide related to antigen processing. The nucleoprotein peptide mimics the endogenous CTL determinant derived by the cellular degradation of the A/PR/8 nucleoprotein. It is possible that the OVA peptides used for in vitro priming, such as $OVA_{111-122}$, are not produced as a consequence of cellular processing of the OVA gene product. Consistent with this suggestion, CTL derived by direct stimulation with $OVA_{111-122}$-containing digest would then fail to lyse E.G7-OVA since it does not express the appropriate determinant (Fig. 5 *b*). Furthermore, we have observed that in vivo immunization with E.G7-OVA elicits OVA-specific CTL that fail to lyse targets in the presence of $OVA_{111-122}$ (Moore, M. W., M. J. Bevan, and F. R. Carbone, manuscript in preparation).

It is likely that our success in eliciting a class I-restricted CTL response is due in part to the direct association of degraded antigen with the MHC products. This may have obviated the need for any form of cellular processing. Antigen processing combined with intracellular trafficking are thought to be the essential mechanisms that discriminate between class I- and class II-restricted antigen presentation (10, 11). The two processing pathways are thought to be distinct. Native, exogenous proteins are endocytosed and degraded within the endosomal compartment before reexpression on the cell surface in association with class II MHC molecules (30, 31). In contrast, endogenous class I-restricted antigens are thought to be degraded by an alternative mechanism (32, 33). Thus, native OVA, which must be degraded for antigen presentation, failed to elicit primary effectors in vitro and was incapable of sensitizing targets for lysis by peptide-specific CTL. Recently, Staerz et al. (34) have disputed the suggestion that class I and class II antigen presentation represent separate pathways. They have claimed that native protein can elicit peptide-specific, class I-restricted CTL by in vivo priming. We suggest that many of their results may be explained on the basis of direct in vitro peptide stimulation rather than successful in vivo immunization with native protein.

There may be other features that distinguish endogenous and exogenous peptide

presentation in addition to the differences in processing requirements and cell surface densities discussed above. For example, the broad MHC haplotype crossreactivity noted for the in vitro-induced anti-CN OVA CTL (Fig. 4 *a*) may represent a unique association of peptide with class I protein at sites that are not accessible to endogenous peptide determinants. Such sites may exist at monomorphic regions of the K-, D-, or L-encoded proteins or on the proteins encoded by the relatively conserved Qa/Tla genes. However, despite all these differences, it is clear that exogenous peptides can effectively substitute for conventional CTL determinants, such as viral antigens, both for target sensitization (7, 9) and for CTL priming, as demonstrated here for $NP_{365-380}$.

The ability to elicit CTL by peptide stimulation affords us a relatively simple technique with which to study class I-restricted T cell recognition. Since T cells are specific for denatured rather than native forms of foreign antigens, peptides clearly represent a potential means of effective vaccination. In the particular case of influenza, specific CTL populations (35) and clones (36, 37) have proven effective in protecting naive individuals against lethal influenza infection. It may be of interest to ascertain whether the virus-reactive CTL derived from in vitro peptide priming can also provide the same protection in vivo. If that is the case, then primary in vitro CTL stimulation could provide us with information that will prove useful in the design of efficient antiviral vaccines.

Summary

Antigen-specific cytotoxic T cells can be generated by primary in vitro stimulation of spleen cells from C57BL/6 mice with appropriate peptide fragments. This response can be elicited without prior in vivo immunization. Chicken OVA fragmented with either cyanogen bromide (CN OVA) or trypsin (T OVA) was used as a source of mixed peptides. A synthetic peptide, $NP_{365-380}$, representing the sequence 365–380 from influenza virus A/PR/8 nucleoprotein, was also used, since this contains the main determinants recognized by CTL generated from H-2^b mice infected with A/PR/8 virus. The primary in vitro cytotoxic T cell response was peptide specific, since targets were lysed only in the presence of appropriate peptide antigens. Native OVA could not elicit primary effectors in vitro nor could it sensitize targets for lysis by OVA digest-specific CTL. A synthetic peptide corresponding to residues 111–122 within the OVA sequence could sensitize targets for lysis by effectors induced against T OVA.

Effectors generated by in vitro stimulation were $CD8^+$, $CD4^-$, and H-$2D^b$-restricted for $NP_{365-380}$ and T OVA recognition. CN OVA-specific effectors were also $CD8^+$, $CD4^-$, but surprisingly, were able to lyse a range of H-2-different targets in an antigen-specific manner. These effectors failed to lyse a tumor line that does not express class I MHC molecules. This broad MHC restriction pattern was also apparent at the clonal level.

In all cases, the antipeptide CTL generated by primary in vitro stimulation were inefficient in lysing target cells expressing engogenous forms of antigens, such as influenza virus-infected cells or cells transfected with the OVA cDNA. However, cytotoxic T cell lines generated in vitro against the $NP_{365-380}$ peptide did contain a minor population of virus-reactive cells that could be selectively expanded by stimulation with A/PR/8-infected spleen cells. These results are discussed in terms of

class I-restricted T cell stimulation in the absence of antigen processing by high surface densities of peptide/MHC complexes.

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