Identification of Naturally Processed Viral Nonapeptides Allows Their Quantification in Infected Cells and Suggests an Allele-specific T Cell Epitope Forecast

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Summary

Virus-specific cytotoxic T lymphocytes (CTL) recognize virus-derived peptides presented by major histocompatibility complex (MHC) class I molecules on virus-infected cells. Such peptides have been isolated from infected cells and were compared to synthetic peptides. We found previously the K^d- or D^b-restricted natural influenza nucleoprotein peptides to coelute on reversed phase high performance liquid chromatography columns with certain peptidic by-products present in synthetic peptide preparations. Here we show by extensive biochemical and immunological comparison that the natural peptides in all respects behave as the surmised synthetic nonapeptides, and thus, must be identical to them. The absolute amounts of these natural peptides contained in infected cells could be determined to be between 220 and 540 copies by comparing with defined amounts of pure synthetic nonapeptides. The comparison of the natural K^d-restricted peptide with published synthetic peptides known to contain other K^d-restricted CTL epitopes suggested a new MHC allele-specific T cell epitope forecast method, based on the defined length of nine amino acid residues and on critical amino acid residues at the second and the last position.

The function of MHC class I molecules is to present peptides derived from cellular proteins to $CD8^+$, MHC class I–restricted T cells (1–7). A considerable fraction of alloreactive T cells also recognizes peptides derived from cellular proteins (K. Falk, unpublished data). Such peptides can, in principle, be derived from protein occurring in different cellular compartments (8). It is not known, however, where and how the peptides are made from the protein, and how they come into contact with MHC class I molecules. Based on the blocking of MHC class I–restricted antigen presentation with Brefeldin A (9, 10) and other experiments (11), it was speculated that MHC molecules bind peptides during or shortly after assuming their final structure in the endoplasmic reticulum. It is not clear, however, whether nascent MHC molecules bind preformed peptides, or rather larger molecules, or even complete protein, which then might be trimmed to those peptides found later on bound to mature MHC molecules (12).

To better understand these processes, the exact knowledge of the identity of MHC-presented peptides should be useful. We have recently shown that cellular peptides can be isolated from complete cells, or from purified MHC molecules, and that the peptides isolated from complete cells, without prior enrichment for MHC molecules, are still heavily dependent on MHC expression in the respective cells (6, 12, 13; K. Falk, unpublished data).

For the minor histocompatibility and viral antigens tested, the respective MHC-dependent peptide as seen by a given CTL always elutes a single, sharp activity peak from reversed phase HPLC columns, indicating that the cell makes exactly one well-defined peptide to be seen by a given CTL. The peptide presented by influenza-infected $H\text{-}2^d$ cells to K^d-restricted, influenza nucleoprotein-specific CTL coelutes on a reversed phase HPLC column together with a minor byproduct present in a synthetic peptide preparation made according to a peptide previously reported to contain a K^d-restricted influenza nucleoprotein epitope, TYQRTRALVRTG (6, 14). The minor byproduct was determined by ion spray mass spectrometry to be a nonapeptide contained in the above preparation. Similar observations were made for a D^b-restricted CTL epitope formerly described to be contained in a 16–amino acid residue peptide also from influenza nucleoprotein, IASNENMETMESSTLE (2, 6).

To test whether such synthetic nonapeptides really are identical with the naturally processed peptides, we did extensive biochemical and immunological comparisons. Based on the

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data obtained, we quantified the number of nucleoprotein peptide copies present in influenza-infected cells. In addition, we introduce a new hypothesis on the prediction of T cell epitopes in an MHC allele-specific way, which is based on the knowledge of the identity of naturally processed viral peptides.

Materials and Methods

Virus and Cells. Influenza A/PR/8/34 virus was grown in the allantoic sacs of 11-d-old embryonated chicken eggs and stored at −70°C. Infectivity was tested by determining the titer of hemagglutinating units (HAU;[1] 15). EL4 (H-2^b), P815 (H-2^d), and P1.HTR (H-2^d) tumor cells were grown in RPMI 1640 supplemented with 5% FCS and antibiotics. P1.HTR is a subline of P815 selected for high transfection efficiency (16) and is easier infectable with influenza virus than is P815 in our hands (H. Schild, unpublished data). This line was erroneously called P815.TR in a previous publication (6). The CTL line HASI is specific for influenza nucleoprotein peptide T-V (for abbreviations of peptides, see Tables 1 and 2) and is K^d restricted (6). The line 28B90 was derived by stimulating spleen cells of a C57BL/6 mouse (preimmunized with 50 HAU of virus), with crude IASNENMET-MESSTLE peptide (1 μg/ml) in vitro. This line has essentially the same specificity as has the line LS9 (6), i.e., is specific for A-M and is D^b restricted. Both CTL lines were kept by weekly restimulation with irradiated (33 Gy) syngeneic spleen cells and the respective peptide in α medium supplemented with 10% FCS and antibiotics and Con A–induced rat spleen cell supernatant as a source of IL-2.

Virus Infections. EL4 cells were grown in roller bottles to a density of 1–2 × 10^6 cells/ml. Cells were infected by incubation with 15 × 10^4 HAU of virus for 10^9 cells in PBS (without FCS) for 12 h at 37°C; P1.HTR cells were grown in plastic petri dishes (14.5-cm diameter) to near confluent density (~10^8 cells/dish). Medium was removed and replaced by PBS (without FCS) containing infectious virus (15 × 10^4 HAU/10^9 cells). Incubation at 37°C was for 12 h. Success of infections was controlled for by testing a sample of infected cells for recognition by virus-specific CTL.

Extraction of Viral Peptides from Infected Cells. 10^9 virus-infected cells were pelleted and suspended with 15 ml of 0.1% (vol/vol) TFA. Cells were disrupted by douncing (10 strokes) followed by ultrasonication (20 pulses of 1 s; Sonifier BIS; Branson, Heusenstamm, Germany). Suspensions were then stirred for 30 min at 4°C; pH was kept at 2.0 with 1% TFA. The supernatant taken after centrifugation (30 min; 180,000 *g*; 4°C) was lyophilized, resolved in 0.1% TFA, and subjected to a G25 Sephadex (Pharmacia Fine Chemicals, Piscataway, NJ) column (bed volume, 50 ml; sample volume, 3 ml; eluent, 0.1% TFA; flow rate, 0.5 ml/min). Material of M_r <5,000 (excluded from this column) was collected, lyophilized, and resolved in 1 ml of 0.1% TFA for HPLC separation.

Synthetic Peptides. Peptides (Tables 1 and 2) with COOH-terminal amino acid residue L, M, or E, respectively, were synthesized by solid phase techniques on a peptide synthesizer (model 430 A; Applied Biosystems, Foster City, CA) using our own Fmoc program and commercially available Fmoc-amino acid *p*-benzyloxybenzylalcohol-resins and Fmoc-protected amino acids (Novabiochem, Sandhausen, Germany). Fmoc-amino acids were side chain protected as follows: Gln(Trt), Thr(tBu), Ser(tBu), Glu(tBu), Asn(Trt), Arg(Mtr), Tyr(tBu). The coupling of amino acids was carried out in 10-fold excess within 1 h. Fmoc-amino acids (1.5 mmol) were activated in situ with 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU) (481 mg; 1.5 mmol), VHOBt-DMF (1.1 M; 1.5 ml), and VDIEA-DMF (1 M; 2.3 ml) in 1.5 ml DMF (Merck, Darmstadt, Germany), and transferred to resin. All other peptides were synthesized simultaneously on a fully automated multiple peptide synthesizer (model 350; Zinsser, Frankfurt/M., Germany). The couplings were performed using the 10-fold excess of Fmoc-amino acids and DCC/HOBt within 40 min. Peptides were deprotected and simultaneously removed from the resin by treatment with anhydrous TFA in the presence of 3% (vol/vol) thioanisol and *p*-thiocresol (Fluka, Neu-Ulm, Germany) (3 h at the temperatures indicated below). Nucleoprotein peptides of the K^d-restricted series starting with Thr were deprotected and removed from the resin before cleaving off the NH_2-terminal Fmoc-protecting group with piperidine/DMF, 1:1 (vol/vol). Peptides containing Arg(Mtr) were completely deprotected by treatment with TFA plus scavenger for 1 h at 25°C and for additional 2 h at 50°C. All other peptides were kept for only 1 h at 50°C. The crude peptides were separated from the coupling reagents by extraction with ether and were lyophilized subsequently, whereby each peptide was obtained as a colorless powder. Ion spray mass spectrometry (Sciex, Toronto, Ontario) indicated complete deprotection of peptides (correct molecular ion peak) and the appearance of the respective multiple charged species; identity of peptides was confirmed by amino acid analysis (ABI).

HPLC Separation of Natural and Synthetic Peptides. All separations were done on a reversed phase Superpac Pep-S column (C2/C18, 5-μm particles, 4.0 × 250 mm; Pharmacia LKB) using Pharmacia LKB equipment. Samples were injected in volumes of 1,000 μl in starting solution. Four different elution procedures were used. (*a*) TFA/acetonitrile, standard resolution: solution A, 0.1% TFA in H_2O; solution B, 0.1% TFA in acetonitrile. 0–5 min, 100% A; 5–40 min, linear increase to 60% B; 40–45 min, 60% B; 45–50 min, linear decrease to 0% B. Flow rate, 1 ml/min; fraction size, 1 ml (used in Fig. 2). (*b*) Same solutions; 0.5% increase of B per min; fraction size, 0.5 ml (used in Fig. 1, *a* and *b*). (*c*) Phosphate buffer/acetonitrile; solution A, 0.1 M sodium phosphate (pH 3.3 in Fig. 1 *c*; pH 6.5 in Fig. 1 *d*); solution B, acetonitrile. Gradient as indicated in Fig. 1, *c* and *d*; flow rate 1 ml/min; fraction size 0.5 ml. (*d*) HFB/acetonitrile; solution A, 0.1% heptafluorobutyric acid (HFB) in H_2O (vol/vol); solution B, 0.1% HFB in acetonitrile. Gradient as indicated in Fig. 1, *g* and *h*. Flow rate, 1 ml/min; fraction size, 0.5 ml. Elution was monitored by measuring ultraviolet light absorption at 220 nm in a continuous flow detector. Individual samples were dried by vacuum centrifugation and stored at −70°C, if not immediately used for CTL assays.

CTL Assays. EL4 (H-2^b) or P815 (H-2^d) target cells were labeled with ^{51}Cr according to standard methods. Synthetic or natural peptides were titrated into 96-well round-bottomed microtiter plates (50 μl/well). Target cells (10^4/well in 100 μl of medium) were added, followed by incubation at 37°C for 90 min. CTL suspensions (50 μl) were added to give a CTL/target ratio of 2:1 to 10:1. Assay plates were incubated for 6 h at 37°C. 100-μl supernatant from each well was harvested and the radioactivity detected in a gamma counter. Specific lysis was determined using the formula: 100 × (cpm released by CTL − cpm released with medium alone)/(cpm released by detergent − cpm released with medium alone). For assays, α medium containing 10% FCS was used. Detergent used to determine maximum release of target cells was 10% Zaponin (Coulter Instruments, Hialeah, FL). Spontaneous ^{51}Cr release of target cells was between 9 and 28%.

[1] *Abbreviations used in this paper:* HAU, hemagglutinating units; VSV, vesicular stomatitis virus.

Results

The Naturally Processed K^d-restricted Peptide from Influenza Nucleoprotein. P1.HTR tumor cells (H-2^d) were infected with A/PR/8/34 influenza virus. Peptides were extracted by acid elution, separated by reversed phase HPLC (TFA/acetonitrile), and tested for recognition by K^d-restricted, influenza nucleoprotein-specific HASI CTL as described (6; Fig. 1 *a*). We found that the natural peptide coeluted with a synthetic byproduct of M_r 1,107 contained in a preparation of TYQRTRALVRTG (abbreviation, T-G; M_r 1,421; 14). Therefore, peptides according to TYQRTRALV (T-V; M_r 1,107) and several other peptides contained in or overlapping with T-G with M_r ~1,000 (see Table 1) were synthesized, and chromatographed under the same conditions as the natural peptide. The elution behavior of these peptides is indicated in Fig. 1 *a* by marks. Three of the synthetic peptides eluting at similar positions as the natural peptide were chromatographed under different conditions (phosphate buffer/acetonitril; Fig. 1 *c*) and compared to the elution behavior of the natural peptide under the same new conditions (Fig. 1 *e*). The TYQRTRALV peak coeluted exactly with the natural peptide, whereas the other two synthetic peptides eluted with considerably different retention times compared to the natural one. Both TYQRTRALV and the natural peptide were chromatographed using yet another parameter (heptafluorobutyric acid [HFB]/acetonitrile; Fig. 1 *g*); again, they both behaved identically. Thus, the naturally processed K^d-restricted peptide from influenza nucleoprotein must be TYQRTRALV. Indeed, synthetic T-V is recognized very well by K^d-restricted CTL HASI (down to a concentration of 60 pg/ml [54 pM]), whereas some of the other peptides in Table 1 are recognized with much inferior efficiency.

The Natural D^b-restricted Peptide from Influenza Nucleoprotein. Acid extracts from influenza-infected EL4 tumor cells (H-2^b) were separated and analyzed as for the H-2^d tumor cells, except that D^b-restricted CTL 28B90 were used for detection (Fig. 1 *b*). The elution behavior of synthetic peptide ASNENMETM, which had been suspected to be the one coeluting with the natural D^b-restricted peptide (6), as well as some other peptides of similar molecular mass (Table 2), contained in the IASNENMETMESSTLE (2) sequence is indicated in Fig. 1 *b* by marks. Three peptides eluting similarly as the natural peptide, peptides A-M, S-M, and A-E, as well as the natural peptide, were chromatographed using a phosphate buffer/acetonitrile gradient (Fig. 1, *d* and *f*). Only ASNENMETM coeluted with the natural peptide; note the shoulder in the activity peak of the natural peptide, which parallels the shoulder in the activity peak of A-M (Fig. 1 *f*). Coelution of both peptides was also observed with the HFB/acetonitrile gradient (Fig. 1 *h*). Thus, the natural D^b-restricted influenza nucleoprotein peptide must be ASNENMETM. In line with this is the highly efficient recognition (<1 pg/ml [1 pM]) of A-M by D^b-restricted CTL as compared to the other peptides (Table 2). Thus, both K^d- and D^b-restricted

Table 1. *Recognition of Synthetic Influenza Nucleoprotein Peptides by K^d-restricted CTL*

NP amino acid residues	Sequence*	M_r	Abbreviation	Recognition by CTL‡
146–154	ATYQRTRAL	1079	A-L	93/92/84/ 5/ 0/ 0/ 1/ 0
146–155	ATYQRTRALV	1178	A-V	92/90/89/97/85/25/ 2/ 0
147–154	TYQRTRAL	1009	T-L	89/91/87/23/ 0/ 0/ 1/ 0
147–155	TYQRTRALV	1107	T-V	92/83/89/88/97/56/ 1/ – 2
147–158	TYQRTRALVRTG	1422	T-G	82/73/86/70/ 7/ 2/ 0/ – 1
148–154	YQRTRAL	907	Y-L	78/ 8/ 3/
148–155	YQRTRALV	1006	Y-V	90/98/87/68/ 5/ 0/ 1/ – 2
149–154	QRTRAL	744	Q-L	1/ 2/ 2
149–155	QRTRALV	843	Q-V	5/ 3/ – 1
150–157	RTRALVRT	972	R-T	3/ 4/ 4
	TYQRTRALV . TG§	1265		78/90/83/98/76/ 7/ 1/ 0
	TYQ . TRALV . TG	1109		84/30/ 0
	TYQ . T . ALVRTG	1109		62/ 4/ 1
	TYQRT . ALV . TG	1109		83/65/ 6

NP, nucleoprotein.
* Single letter amino acid code.
‡ P815 cells (H-2^d) were incubated with titrated concentrations of the respective peptides (crude) and assayed for killing with HASI CTL. The numbers indicate specific lysis at 1 μg/100 ng/10 ng/1 ng/100 pg/10 pg/1 pg/100 fg peptide per well (150 μl).
§ (.) Deletion of a residue.

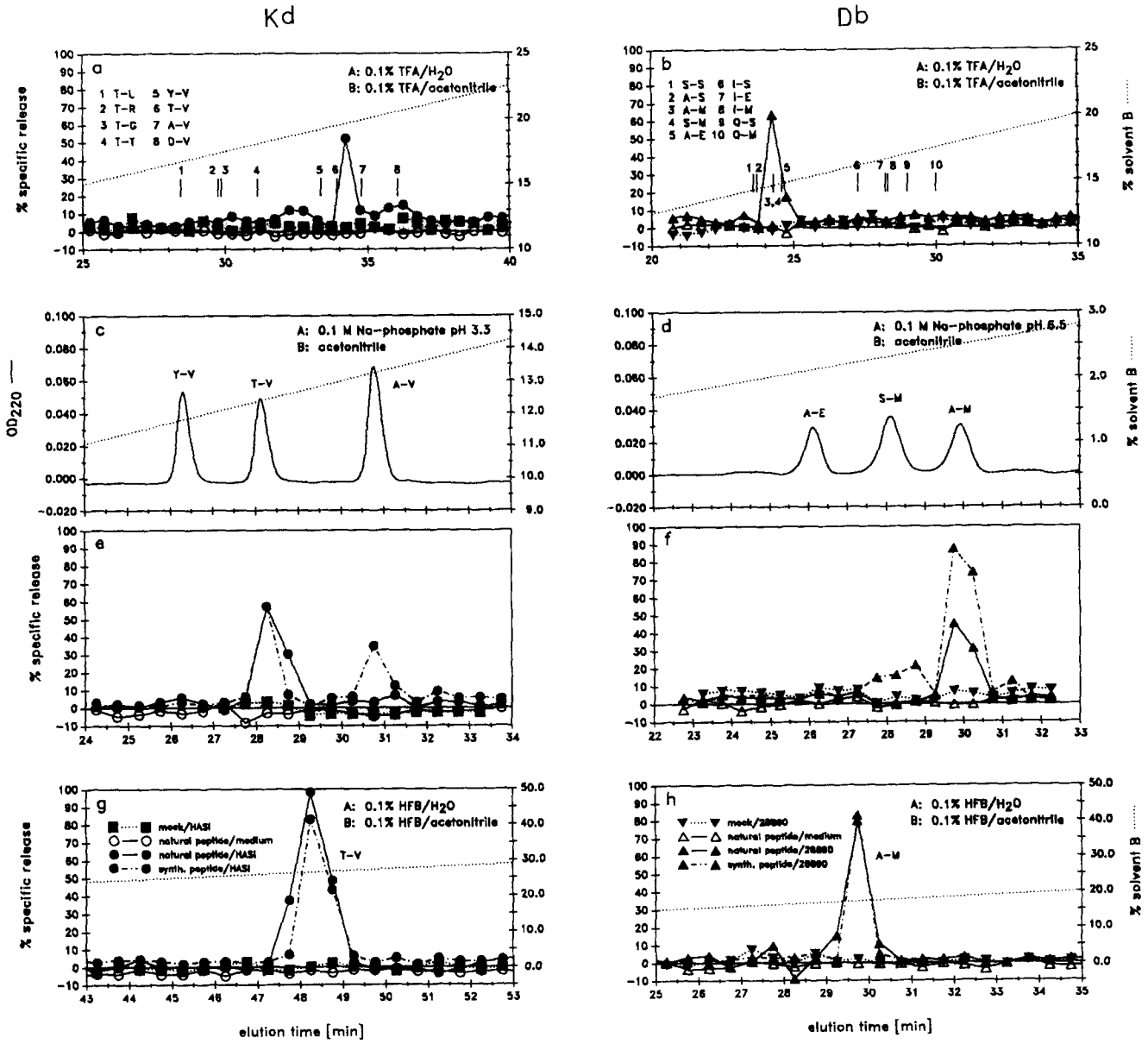

Figure 1. Identification of naturally processed viral peptides by biochemical comparison to synthetic peptides. Naturally processed influenza virus peptides were extracted from infected P1.HTR cells and HPLC separated as described (6). The fraction recognized by the influenza nucleoprotein–specific, K^d-restricted CTL line HASI was further separated using different HPLC parameters (*left panels*). Similarly, the naturally processed influenza peptide recognized by D^b-restricted CTL 28B90 was extracted from EL4 cells, separated by HPLC, and subjected to further HPLC separation (*right panels*). Recognition of individual fractions by CTL was monitored by incubating the respective fractions with P815 target cells and measuring their lysis using HASI CTL (*squares* and *circles*), or by incubating fractions with EL4 target cells and measuring their lysis with 28B90 CTL (*upright* and *inverted triangles*). (■·····■; ▼·····▼) Fractions from control gradients performed without samples. This is an important control, since minor peptide contaminations left over from previous runs might interfere with the extremely sensitive CTL assay. These control gradients were always run before peptide separations. (○———○, △———△) Natural peptide, no CTL (control for toxicity of fractions). (●) Natural K^d-restricted peptide, HASI CTL. (▲) Natural D^b-restricted peptide, 28B90 CTL. (●—·—·—●) Synthetic peptides Y-V, T-V, or A-V, HASI CTL. (▲—·—·—▲) Synthetic peptides A-E, S-M, or A-M, 28B90 CTL. (*a* and *b*) TFA/acetonitrile gradient. (●———●, ▲———▲) The activity peaks of the natural peptides. The numbered marks indicate the elution behavior of synthetic peptides. See Tables 1 and 2 for identity of these peptides. Samples of 10 ng were loaded onto the HPLC column. Loading less peptide (10 ng) resulted in a shift of elution time of ~20 s for T-V, which would move the T-V mark right under the activity peak in *a*. (*c* and *d*) Phosphate buffer/acetonitrile gradient. The three synthetic peptides Y-V, T-V, and A-V (*c*) or A-E, S-M, and A-M (*d*) were mixed (10 ng of each) and applied to the HPLC column. Elution profiles are indicated. Peaks were identified by comparison with single-peptide HPLC profiles. (*e* and *f*) CTL recognition of natural and synthetic peptides eluted from the gradient in *c* and *d*. (*g* and *h*) HFB/acetonitrile gradient. Activity profile of the natural peptides and T-V (*g*) or A-M (*h*) is shown.

Table 2. *Recognition of Synthetic Influenza Nucleoprotein Peptides by D^b-restricted CTL*

NP amino acid residues	Sequence	M_r	Abbreviation	Recognition by CTL*
364–373	QIASNENMET	1136	Q-T	88/87/ 17/ 4
364–374	QIASNENMETM	1267	Q-M	81/93/ 96/90/97/70/13/ 4
364–376	QIASNENMETMES	1484	Q-S	100/99/100/94/10/ 8/ 5
365–373	IASNENMET	1008	I-T	88/88/ 60/ 9/ 6
365–374	IASNENMETM	1139	I-M	54/70/ 80/77/83/30/ 2
365–375	IASNENMETME	1268	I-E	64/67/ 19/ 1
365–376	IASNENMETMES	1355	I-S	90/97/100/97/99/33/10/ 3
365–380	IASNENMETMESSTLE	1786		55/68/ 77/57/13/ 4/ 5
366–373	ASNENMET	895	A-T	93/91/100/50/ 10/ 3
366–374	ASNENMETM	1026	A-M	93/91/100/93/94/99/90/28
366–375	ASNENMETME	1155	A-E	64/84/ 80/ 3/ 5/ 1
366–376	ASNENMETMES	1242	A-S	83/89/100/99/97/93/19/13
367–374	SNENMETM	955	S-M	54/65/ 73/49/15/ 5/ 0
367–375	SNENMETME	1084	S-E	35/ 2/ −2
367–376	SNENMETMES	1171	S-S	93/95/100/99/26/ 8/ 9/11
367–374	NENMETM	868	N-M	23/ 7/ 6/ 6
368–375	NENMETME	997	N-E	−3/ 7/ 0

* EL4 target cells (H-2^b) were incubated with titrated amounts of the respective peptide (crude) and assayed for killing with 28B90 CTL. The numbers indicate specific lysis at 1 μg/100 ng/10 ng/1 ng/100 pg/10 pg/1 pg/100 fg peptide per well (150 μl).

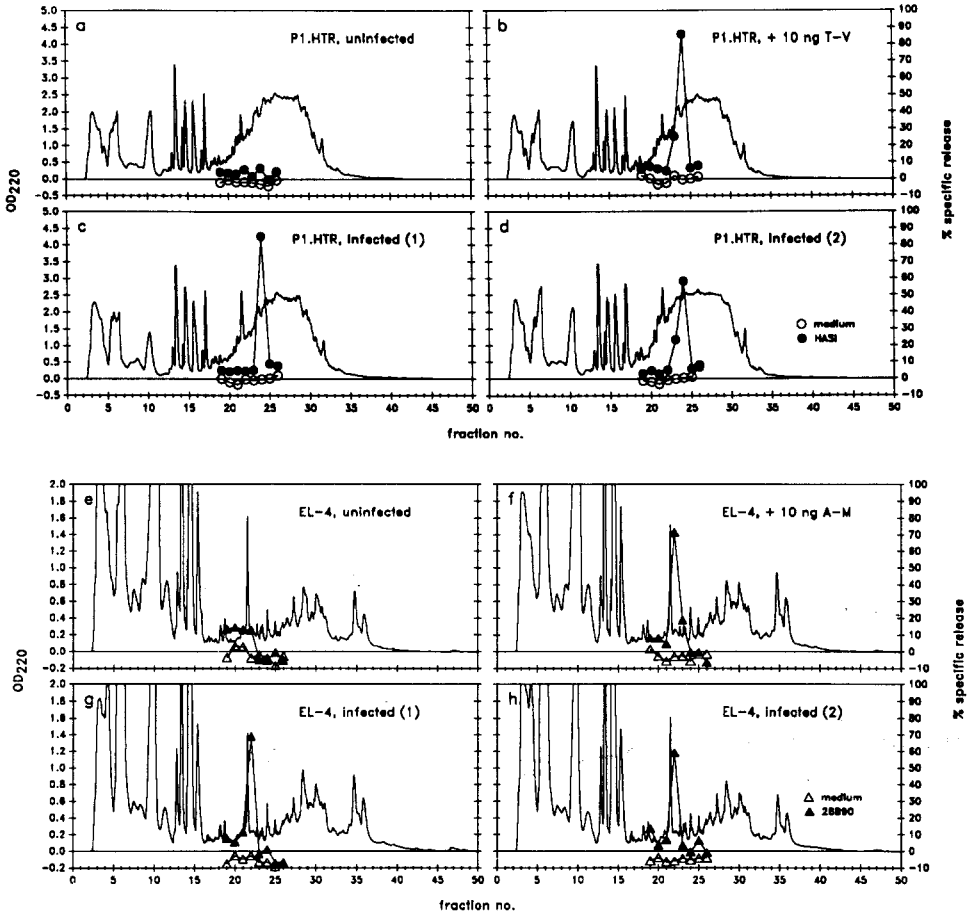

Figure 2. Comparative extraction of naturally processed peptides and of defined amounts of synthetic peptides admixed to cells. (*a–d*) Aliquots of 10^9 P1.HTR (H-2^d) cells were left uninfected (*a* and *b*), were mixed with 10 ng T-V synthetic peptide (*b*) or were infected with influenza virus (*c* and *d*). (*e–h*) Aliquots of 10^9 EL4 cells were left uninfected (*e* and *f*), were mixed with 10 ng A-M synthetic peptide (*f*), or were infected with influenza virus (*g* and *h*). Each aliquot was subjected to acid extraction. The resulting material was separated by reversed phase HPLC and monitored by measuring ultraviolet light absorption at 220 nm (_____). Individual fractions were tested for recognition by HASI CTL (K^d-restricted) on P815 target cells (●) or by 28B90 CTL (D^b-restricted) on EL4 target cells (▲), or were incubated with P815 (○) or EL4 (△) target cells without CTL to control for fraction toxicity.

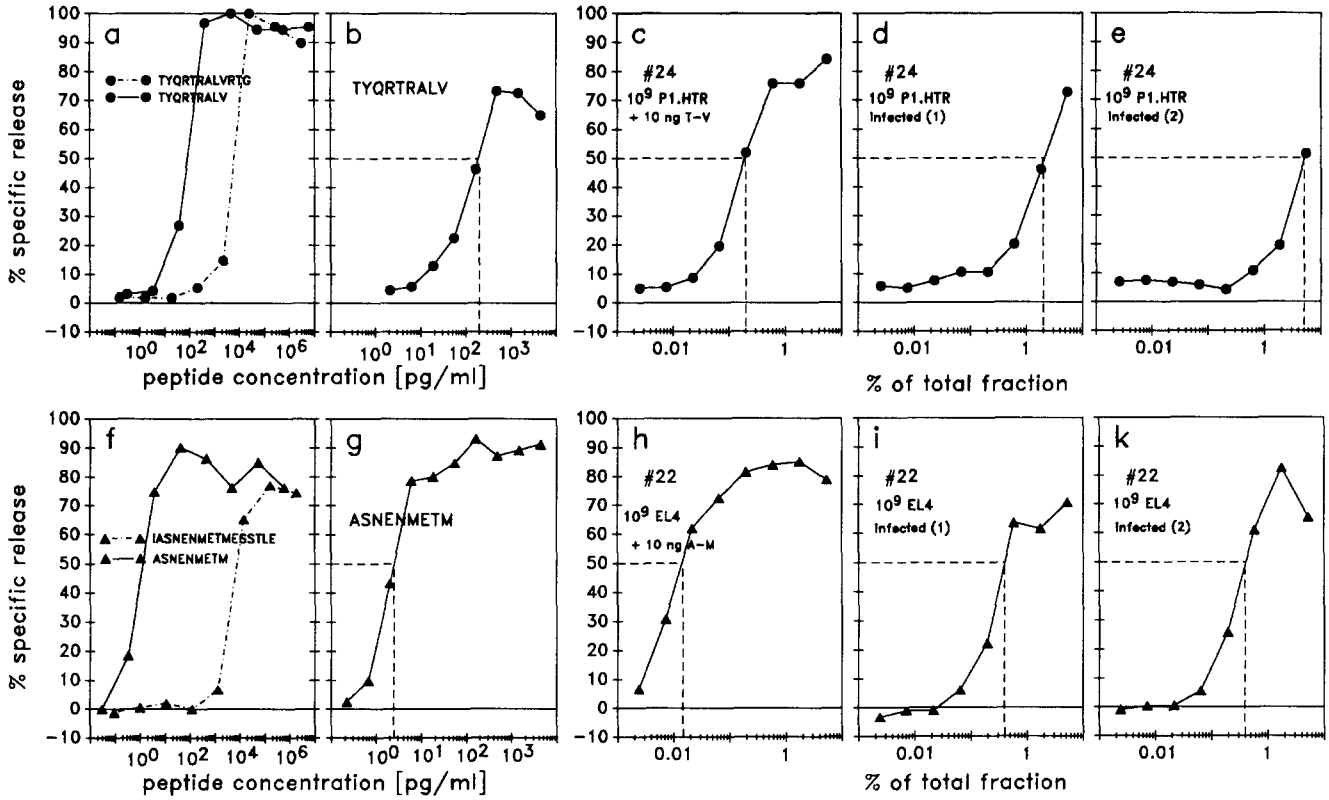

Figure 3. Comparative titrations of synthetic peptides and of naturally processed peptides. (*a–e*) Synthetic and natural peptides were serially diluted, incubated with P815 target cells, and tested for lysis by HASI CTL. (*a* and *b*) synthetic T-G (●—·—·—●) and T-V (●—·—·—●). The titration in *b* was done simultaneously with those in the next three panels. (*c–e*) titrations of fractions 24 each of Fig. 2 *b* (admixed T-V peptide subjected to acid extraction) (*c*); of Fig. 2 *c* (natural peptide, first batch) (*d*), and of Fig. 2 *d* (natural peptide, second batch) (*e*). The data in *c–e* (and also in *h–k*) are plotted as to represent percent of the total amount of fraction 24 (22 in *h–k*) present in the assay microculture. Comparison of *b* and *c* indicates the 50% level of lysis in this experiment to correspond with a peptide concentration of 200 pg/ml (corresponding to 30 pg per microculture [volume, 150 μl] and with 0.2% of the admixed 10 ng of T-V; thus, the recovery of admixed peptide is roughly quantitative. Comparison of *c* with *d* and *e* indicates that in *d*, 10-fold less (natural) T-V than in *c* are detected, and in *e*, 25-fold less than in *c* is detected. Thus, the two batches of 10^9 virus-infected cells contained 1 ng, respectively, 0.4 ng of natural T-V. (*f–k*) synthetic and natural peptides were serially diluted, incubated with EL4 target cells, and tested for lysis by 28B90 CTL. (*f* and *g*) Synthetic IASNENMETMESSTLE (▲—·—·—▲) and A-M (▲_____▲). (*h–k*) Titration of fraction 22 each of Fig. 2 (admixed A-M) (*h*), of Fig. 2 *g* (natural peptide, batch 1) (*i*), and of Fig. 2 *h* (natural peptide, batch 2) (*k*). Comparison of *g* and *h* indicates a recovery of 25% of the A-M peptide admixed to cells. Comparison of *h* with *i* and *k* indicates that both batches of infected cells contained 27 fold less (natural) A-M than detected in *h*. Thus, the two batches of 10^9 virus-infected EL4 cells each contained 375 pg of natural A-M.

CTL epitopes of influenza nucleoprotein are recognized 100- (T-V) or 10,000-fold (A-M) better than the peptides originally described to contain the respective CTL epitope (see also below).

Quantitation of Peptides in Infected Cells. Aliquots of 10^9 P1.HTR or EL4 cells were either mixed with 10 ng T-V or A-M, respectively, or were infected with influenza virus. Extracted peptides from each aliquot were separated and analyzed as described earlier (6). The admixed synthetic peptides as well as the natural peptides eluted at the expected positions (Fig. 2). Fractions containing active peptides were retested in titrated concentrations (Fig. 3). By comparison of the titration curves, it was determined that the recovery of admixed synthetic T-V from P1.HTR cells was approximately quantitative (Fig. 3, *b* and *c*), and that from the first batch of infected H-2^d cells 1 ng of natural T-V was eluted (Fig. 3 *d*). From the second batch, 0.4 ng of natural T-V was eluted (Fig. 3 *e*). Thus, from 10^9 infected H-2^d cells, 1 ng T-V was recovered; this corresponds to 540 T-V molecules per cell. The 0.4 ng T-V from the second batch correspond to 220 copies per cell.

The recovery of admixed synthetic A-M from EL4 cells was 25% (Fig. 3, *g* and *h*); assuming the same recovery rate, both batches of infected EL4 cells contained 0.4 ng of natural A-M, or 220 A-M molecules per cell.

Comparison of Natural Peptides to Other Peptides Known to Contain CTL Epitopes. Table 3 shows a comparison of the naturally processed K^d-restricted influenza nucleoprotein peptide T-V with other peptides known to contain K^d-restricted epitopes. It is evident that all have a tyrosine residue; since T-V has its Tyr at position 2, we have lined up all other peptides accordingly. If we assume that K^d-restricted peptides consist of nine amino acid residues, and that Tyr is always at position 2, the naturally processed K^d-restricted peptides would be predicted to be those printed in bold letters. On comparing these lined-up epitopes, some features are evi-

Table 3. *Comparison of Natural K^d-restricted Peptides to Other Peptides Known to Contain K^d-restricted CTL Epitopes*

Organism	Protein	Amino acid residues	Sequence*	Restriction	Reference
Influenza A/PR/8/34	Nucleoprotein	147–155	**TYQRTRALV**	K^d	6
Influenza A/JAP/305	Hemagglutinin	523–545	**VYQILAIYA**TVAGSLSLAIMMAG	K^d	23
Influenza A/JAP/305	Hemagglutinin	523–545	VYQILA **IYATVAGSL**SLAIMMAG	K^d	23
Influenza A/PR/8	Hemagglutinin	518–528	**IYSTVASSL**VL	K^d	24
Influenza A/JAP/305	Hemagglutinin	202–221	RT**LYQNVGTYV**SVGTSTLNK	K^d	23
Influenza A/JAP/305	Hemagglutinin	202–221	RT**LYQNVGTYVSVGTST**LNK	K^d	23
Human	HLA-A24	170–182	**RYLENGKET**LQRA	K^d	25
Human	HLA-Cw3	171–186	**RYLKNGKET**LQRA	K^d	25
Mouse tumor (P815)	Unknown		**LYQAVTTTL**EE	K^d	26
Plasmodium berghei	CS protein‡	249–260	NDD**SYIPSAEKI**	K^d	27
Plasmodium yoelii	CS protein‡	276–280	NED**SYVPSAEQI**LEFVKQI	K^d	27
Plasmodium yoelii	CS protein‡	281–296	**SYVPSAEQI**LEFVKQI	K^d	28
Influenza A/PR/8/34	Nucleoprotein	366–374	**ASNENMETM**	D^b	6
VSV	Nucleoprotein	52– 59	**RGYVYQGL**	K^b	7

* Natural peptides (underlined) or predicted natural peptides are printed in bold letters. In some cases, two different alignments for the same peptide are shown.
‡ Circumsporozoite protein.

dent: apart from the uniform Tyr at position 2, the last position is always occupied by an amino acid residue with a side chain methyl group (Val, Ile, Thr, Ala, or Leu). The third position is frequently Glu, Leu, or Val; in the fifth position Val, Asn, and Ser; and in the sixth Glu and Ala are found repeatedly. On the other hand, the fourth position is in all cases occupied by a different amino acid, with the exception of the homologous peptides. Similarly, positions 1, 7, and 8 are highly variable. A comparison of the natural D^b-restricted influenza nucleoprotein peptide with other peptides known to contain D^b-restricted epitopes was not informative, because the available database is smaller, and since a sentinel residue such as Tyr for K^d-restricted peptides was not evident. It is clear, however, that the D^b-restricted peptide does not fit into the pattern observed for K^d-restricted peptides; neither does the naturally K^b-restricted peptide of vesicular stomatitis virus (7).

Discussion

The naturally processed influenza nucleoprotein peptide presented by K^d molecules to CTL was determined to be the nonapeptide TYQRTRALV. The corresponding D^b-restricted peptide is ASNENMETM. Knowing exactly the peptides presented by virus-infected cells, it was possible to quantitate the number of peptide copies present in a virus-infected cell by comparing in a CTL assay the titration curves of eluted natural peptides with those of defined concentrations of the corresponding synthetic peptides. From infected P1.HTR tumor cells (H-2^d), between 220 and 540 copies of TYQRTRALV were extracted, and from infected EL4 tumor cells (H-2^b), 220 copies of ASNENMETM.

An unknown parameter may have biased these figures, namely the efficiency of peptide extraction from cells. The efficiency of peptide recovery during HPLC has been controlled for. Most of the naturally processed peptides are likely to be eluted from MHC molecules located in the cytoplasm or on the plasma membrane. Although the condition used for extraction (0.1% TFA) may suggest quantitative dissociation of MHC molecules and peptides, we cannot measure this parameter. On the other hand, we do not know how many of the peptide copies isolated come from the cytoplasm and how many from the plasma membrane. There is no easy way to allocate the origin of eluted peptide to either of the two compartments. Under the assumption that half of the peptides eluted from whole cells come from MHC molecules inserted into the plasma membrane, the number of peptides presented on the cell surface would be ~100 for infected EL4 cells, and ~100–300 for infected P1.HTR cells. These numbers are consistent with the minimal number of peptide/MHC complexes for stimulating MHC class II–restricted T cells (17, 18). If the tumor cells used in our study express 300,000 K^d or D^b molecules per cell, respectively, these should be able to present 1,000–3,000 different peptides simultaneously in sufficient densities to allow T cell recognition. In normal cells, all of these are self peptides; indeed, normal cells can be recognized by quite a lot of different peptide-specific CTL (12, 13; K. Falk, unpublished data). The body cells of individual human beings typically express four different MHC class I molecules capable of presenting peptides (HLA-A and -B in two allelic forms each); normal outbred mice have four or six such molecules. This implicates that one cell (with high MHC expression) is able to present 12,000 different pep-

tides simultaneously. This appears a high number at first glance; the number, however, is too low to guarantee a full covering of any mutation occurring in the genes for the 10,000 proteins or so expressed in a cell, since each average sized protein could give rise to 100 or more different nonapeptides. The number of simultaneously presented peptides is probably even lower than estimated above, since the self peptide mixtures eluted from MHC class I molecules contain 7–10 dominant peptides likely to occupy much >300 MHC molecules per cell (7; O. Rötzschke, unpublished data). Thus, if CTL are involved in the surveillance of normal cells against somatic mutation, as has been speculated, this surveillance has to be incomplete, even if our calculation should grossly underestimate the actual number of different peptides simultaneously presented by a cell.

In the case of cells infected with virus or larger parasites, however, the situation is not so hopeless for a successful surveillance by CTL. Each of the parasites needs at least several of its proteins expressed intracellularly; if, on average, the cell can afford to present one peptide per protein to CTL, the chance for hitting a peptide derived from a foreign protein expressed in addition to the 10,000 or so self proteins is quite high in infected cells. It should be emphasized in this context that a cell has no possibility to distinguish between self or nonself origin of its proteins.

The data base of naturally processed peptides presented by MHC class I molecules consists of only three peptides up to now; in addition to the K^d- and D^b-restricted influenza epitopes dealt with here, a K^b-restricted vesicular stomatitis virus (VSV) epitope has been reported to be an octapeptide (7). This limited information does not allow yet to generalize that MHC class I–restricted T cell epitopes are always nona- or octapeptides, although this is not unlikely. Before a larger data base on naturally processed epitopes is available, however, the comparison of natural peptides to other peptides known to contain CTL epitopes can be informative, as discussed below.

It has been observed that a Tyr residue is common to peptides containing K^d-restricted epitopes. One could speculate then that Tyr residues in K^d-restricted epitopes might always be at position two, as is the case with the natural K^d-restricted epitope TYQRTRALV. Under this assumption, comparison of the latter epitope with other peptides containing K^d-restricted epitopes suggests a consensus sequence for K^d-restricted T cell epitopes (Table 3). Peptides presented by K^d molecules generally might be nonapeptides, might have a Tyr residue at position 2, and an amino acid residue containing a side chain methyl group (Val, Ile, Thr, Ala, or Leu) at position 9 (Table 4). The other residues appear to be more variable, although there seem to be preferential occupancies at positions 3, 5, or 6. This hypothetical consensus motif is consistent with replacement studies carried out by Maryanski et al. (19), who showed that in derivatives of the K^d-restricted peptide RYLENGKETLQRA, all amino acids

Table 4. *Consensus for K^d-restricted Epitopes*

Positions	1	2	3	4	5	6	7	8	9
Residues	*	**Y**	Q L V *	*	V N *	A G *	*	*	V I T A L

* Indicates variable occupancy. Size of letters corresponds to the importance of the respective residue.

between Tyr and Thr can be replaced by Pro residues without abrogation of binding capacity to K^d. In addition, replacement of Tyr at both the above-mentioned peptide and the peptide TYQRTRALVTG for Ala severely reduced binding capacity to K^d. The consensus motif hypothesized here has now essentially been confirmed by sequencing the mixture of self peptides eluted from K^d molecules (20). It should be emphasized here, however, that binding to MHC is only one requirement a peptide has to fulfill to be naturally presented by MHC; other constraints may include intracellular transport mechanisms (11) of proteins/peptides and the intracellular proteolysis of protein yielding the final peptide. The comparison in Table 3 leading to the consensus motif in Table 4 is based only on epitopes actually presented by infected or transfected cells; peptides shown to just bind to K^d were not considered in both tables.

The natural D^b-restricted influenza peptide ASNENMETM does not fit into the K^d-restricted consensus sequence; neither does the natural K^b-restricted VSV sequence RGYVYQGL. Thus, it may emerge that each MHC allelic product has its own peptide motif that it likes to present to T cells. This notion has been confirmed by recent work from this laboratory (20). Similar speculations have been formulated earlier (19–22); the notion, however, is significantly strengthened by the identification of naturally presented peptides. It may be useful, therefore, to refine any T cell epitope forecasts to allele-specific ones, like the one offered in Table 4 for K^d-restricted epitopes.

The presentation of nonrandom, relatively rare allele-specific peptide motifs by MHC molecules could be a way for cells to ensure that not too many peptides from a single abundant protein are presented on the expense of peptides from less abundant proteins. The relatively rare occurrence of allele-specific peptide motifs (for example, a Tyr residue followed by either of Val, Ile, Thr, Ala, or Leu exactly seven positions later for a K^d-restricted peptide; the chance for this is 1 in 80 random nonapeptides) also implies that it is of advantage for an individual to express several different MHC molecules to increase the chance that one of the peptide motifs covered is contained in a given foreign protein, that is, to be heterozygous for MHC alleles.

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