Differential T Cell Receptor Photoaffinity Labeling among H-2K^d Restricted Cytotoxic T Lymphocyte Clones Specific for a Photoreactive Peptide Derivative. Labeling of the α-Chain Correlates with Jα Segment Usage

By Pedro Romero, Jean-Laurent Casanova, Jean-Charles Cerottini, Janet L. Maryanski, and Immanuel F. Luescher

From the Ludwig Institute for Cancer Research, Lausanne Branch, 1066 Epalinges, Switzerland

Summary

Using a direct binding assay based on photoaffinity labeling, we studied the interaction of T cell receptor (TCR) with a K^d-bound photoreactive peptide derivative on living cells. The K^d-restricted *Plasmodium berghei* circumsporozoite (PbCS) peptide 253-260 (YIPSAEKI) was reacted NH_2-terminally with biotin and at the TCR contact residue Lys_{259} with photoreactive iodo, 4-azido salicylic acid (IASA) to make biotin-YIPSAEK(IASA)I. Cytotoxic T lymphocyte (CTL) clones derived from mice immunized with this derivative recognized this conjugate, but not a related one lacking the IASA group nor the parental PbCS peptide. The clones were K^d restricted. Recognition experiments with variant conjugates, lacking substituents from IASA, revealed a diverse fine specificity pattern and indicated that this group interacted directly with the TCR. The TCR of four clones could be photoaffinity labeled by biotin-YIPSAEK(^{125}IASA)I. This labeling was dependent on the conjugates binding to the K^d molecule and was selective for the TCR α (2 clones) or β chain (1 clone), or was common for both chains (1 clone). TCR sequence analysis showed a preferential usage of JαTA28 containing α chains that were paired with Vβ1 expressing β chains. The TCR that were photoaffinity labeled at the α chain expressed these Jα and Vβ segments. The tryptophan encoded by the JαTA28 segment is rarely found in other Jα segments. Moreover, we show that the IASA group interacts preferentially with tryptophan in aqueous solution. We thus propose that for these CTL clones, labeling of the α chain occurs via the Jα-encoded tryptophan residue.

In general, CD8$^+$ CTL recognize antigenic peptides bound to MHC class I molecules on the surface of target cells (1). The specificity of the interaction is determined principally by the TCR-α/β of the CTL that binds the peptide–MHC complex. Recently, the structures of several MHC class I molecules and defined peptide–MHC complexes have been elucidated by x-ray crystallography (2–5). These studies showed that whereas certain amino acid side chains of the bound peptide intruded into allele-specific pockets of the MHC molecule, others were oriented towards the solvent phase, potentially accessible to the TCR.

The precise structure of the TCR is unknown, but the variable domains are presumed to resemble those of Ig based on overall sequence homology and a conservation of certain residues that are critical for the structure of the Ig molecule (6–8). On this basis, a model was proposed for the interaction of the TCR with the peptide–MHC complex in which the most variable, "CDR3-equivalent," regions of the TCR α and β chains interact with peptide residues, and the less variable regions, CDR1 and CDR2, interact with MHC residues (6). Indirect evidence for a role of the CDR3 region in contacting the peptide comes from studies involving TCR repertoire analysis (7–10), site-directed mutagenesis of the TCR (11) or immunization of single-chain TCR transgenic mice with variant peptides (12). The latter study also suggested an orientation of the peptide with respect to the TCR α and β chains. However, it is not known whether this orientation applies generally.

Our earlier studies on the K^d-binding antigenic peptide *Plasmodium berghei* circumsporozoite (PbCS)[1] 253-260 (YIPSAEKI) showed that Tyr_{253} and Ile_{260} were engaged in peptide binding to the K^d molecule, whereas Lys_{259} appeared to be in contact with the TCR for most CTL clones analyzed (13). Molecular modeling suggested that the side chain of

[1] *Abbreviations used in this paper:* ABA, 4-azido benzoic acid; ASA, 4-azido salicylic acid; BA, benzoic acid; IASA, iodo, 4-azido salicylic acid; PbCS, *Plasmodium berghei* circumsporozoite; pI, isoelectric point.

 J. Exp. Med. © The Rockefeller University Press • 0022-1007/93/05/1247/10 $2.00
Volume 177 May 1993 1247–1256

the Lys_{259} residue was indeed oriented away from the K^d molecule (14). Furthermore, we found that a derivative of the PbCS peptide containing an NH_2-terminal photoreactive iodo, 4-azido salicylic acid (IASA) moiety, [IASA-YIPSAEK-(biotin)I] selectively labeled the K^d molecules, whereas an isomeric derivative with the IASA group coupled to the Lys_{259} side chain, biotin-YIPSAEK(IASA)I, could not, even though it bound equally efficiently to K^d molecules (15). Realizing that the IASA group of the latter derivative might be available for TCR interaction, we immunized mice with this material and obtained CTL clones that recognized this photoreactive PbCS derivative. We recently demonstrated that the TCR of one of these clones could be specifically photoaffinity labeled with this antigenic peptide derivative (15). In the present study, eight different K^d-restricted CTL clones that recognize biotin-YIPSAEK(IASA)I were compared in terms of photoaffinity labeling, fine specificity of recognition, and TCR sequences. The results are discussed in the context of current models of antigen recognition by TCR. This approach provides new possibilities for the study of the molecular principles of antigen recognition by T cells.

Materials and Methods

Peptide and Conjugate Synthesis. Reagents for peptide and conjugate synthesis were obtained from Bachem Finechemicals (Bubendorf, Switzerland) and from Sigma Chemie (Buchs, Switzerland). Peptides and NH_2-terminal conjugates were synthesized on Wang resin using Fmoc for transient NH_2-terminal protection on a manual peptide synthesizer (RAMPS; Du Pont, Regensdorf, Switzerland) as described by the supplier. The deprotected peptides or NH_2-terminal conjugates were purified by C-18 reverse phase HPLC using a Waters 600 E system on line with a 1000 S diode array UV detector (Applied Biosystems, Inc., Foster City, CA). The HPLC column was eluted by a linear gradient of acetonitrile on 0.1% trifluoroacetic acid in water, rising within 1 h from 0–75%. The amino acid compositions of all peptides were assessed according to the DABSYL method and were ± 10% in agreement with the expected ratios.

Photoreactive derivatives were prepared under dimmed light as previously described (15, 16). The structures of the different conjugated groups are shown in Figure 1. In brief, the bifunctional conjugates (*a*) biotin-YIPSAEK(IASA)I, (*b*) biotin-YIPSAEK(4-azido salicylic acid [ASA])I, (*c*) biotin-YIPSAEK (4-azido benzoic acid [ABA]I, and (*d*) biotin-YIPSAEK(benzoic acid [BA])I were obtained by reacting biotin-YIPSAEKI in anhydrous DMSO with the corresponding *N*-hydroxysuccinimidyl esters in the presence of 1-hydroxybenzotriazole and diisopropylethylamine. The products were purified by precipitation with −20°C ethyl acetate and by HPLC. The products eluted at (*a*) 50.2, (*b*) 46.4, (*c*) 43.8, and (*d*) 42.7% acetonitrile and displayed UV absorption maxima at: (*a*) 214, 275, and 325 nm; (*b*) 214, 272, and 310 nm; (*c*) 214 and 270 nm; and (*d*) 214 and 275 nm. YIPSAEK(IASA)I was prepared by reacting likewise Fmoc-YIPSAEKI with *N*-hydroxysuccinimidyl iodo, 4-azido salicylate (IASA-ONSu). After *N*-deprotection and ethyl acetate precipitation, this product eluted from the HPLC column at 45.4% acetonitrile and showed the same UV absorptions as (*a*). IASA-YIPSAEK(biotin)I was obtained by biotinylation of IASA-YIPSAEKI as previously described (16, 17). The purified materials were reconstituted in PBS as 1 mM solutions and stored in the dark at −20°C.

Radioactive ^{125}IASA-YIPSAEK(biotin)I and biotin-YIPSAEK-(^{125}IASA)I were prepared by reacting freshly radioiodinated ^{125}IASA-ONSu with the corresponding biotin derivatives as described (15). After ethyl acetate precipitation and HPLC purification, the photoprobes were reconstituted in PBS at ~2 × 10^9 cpm/ml. The specific radioactivities were ~2,000 Ci/mmol. The photoprobes were kept in the dark at 4°C and were used within 4 d.

Photoaffinity Labeling. Cells were washed once in DMEM supplemented with 0.5% FCS and 10 mM Hepes and resuspended in the same medium at 6 × 10^6 cells/ml. Cells (6 × 10^6) were incubated with biotin-YIPSAEK(^{125}IASA)I (6 × 10^7 cpm in 30–50 μl PBS) and human β_2m (2,4 μg/ml; Sigma Chemie) or ^{125}IASA-YIPSAEK(biotin) (10^7 cpm in 10–20 μl PBS) in 6-well plates (Costar Corp., Cambridge, MA) at 37°C, 5% CO_2 for 4–5 h and then UV irradiated at 4°C for 5 min (15-W lamp with emission maximum at 312 nm).

The labeled cells were washed once in PBS and solubilized with NP-40 (0.7%) in the presence of leupeptin (10 μg/ml), PMSF (1 mM) and iodoacetamide (10 mM). K^d was immunoprecipitated with mAb 20-8-4S and the TCR-α/β with mAb H57-597 (American Type Culture Collection [ATCC], Rockville, MD) and analyzed on 10% linear SDS-PAGE under reducing conditions as previously described (15). Two-dimensional gel electrophoresis of TCR precipitated with α-CD3 mAb (145-2C11; ATCC) from digitonin lysates was performed as described (15, 18).

The relative extent of photolabeling of single amino acids by IASA was determined by a solid phase assay. N_ϵ Lys IASA was prepared by reacting Fmoc-Lys with iodinated *N*-hydroxysuccinimidyl ASA and, after Fmoc removal, was purified by HPLC. The purified Lys(IASA) was reacted with CNBr-activated Sepharose 4B (Sigma Chemie) in 50 mM sodium bicarbonate in water/acetonitrite (1:1). The degree of coupling was 18 μmol IASA per ml sedimented Sepharose. 300 pmol of ^{3}H-labeled amino acids (New England Nuclear, Boston, MA) dissolved in 10 μl PBS were incubated with 20 μl sedimented Lys (IASA)-Sepharose under agitation at 4°C for 1 h. After 5 min UV irradiation (15-W lamp with emission maximum at 365 nm), the agarose was washed extensively with PBS containing 0.5 M NaCl, 10 mM EDTA, and 0.5% NP-40, with PBS containing 1% SDS, 75% acetonitrile, and DMSO and the bound radioactivity was determined by liquid scintillation counting. In parallel, the same experiments were performed in the absence of UV irradiation. After subtraction of the latter from the former radioactivity values, the amount of the photolabeled amino acids was calculated via the specific radioactivity. All experiments were performed at least in triplicates and the SDs were calculated according to the Student's *t* method.

mAbs. All mAbs were produced by hybridomas obtained from the ATCC and were purified by affinity chromatography on protein A–Sepharose.

Generation of CTL Clones and CTL Assays. The N and O CTL clones were derived from two (BALB/c × C57BL/6)F_1 mice immunized with biotin-YIPSAEK(IASA)I by a previously described method (13). The mice were injected subcutaneously at the base of the tail with 50 μg of biotin-YIPSAEK(IASA)I emulsified in IFA. Cell suspensions from draining LNs were prepared 9 d later. LN cells (4 × 10^6) were cocultured with 1.5 × 10^5 P815-HTR cells (clone 444/A.1.1) that had been pulsed for 1 h at 37°C with 1 μM of peptide biotin-YIPSAEK(IASA)I and irradiated (10,000 rads) in a volume of 2 ml in 12-well plates (Costar Corp.). The medium used was DMEM supplemented with 10% FCS, 10 mM Hepes, 1% Gln, 5 × 10^{-5} M 2-ME, and EL-4 supernatant as a source of IL-2 (30 U/ml). 7 d later, 5 × 10^5 viable cells were restimulated with 2 × 10^5 peptide pulsed and irradiated P815 and

5×10^6 irradiated BALB/c spleen cells in 2 ml of the same culture medium in 12-well plates. The two cultures were cloned by limiting dilution 9 d after the second in vitro stimulation. A total of 11 clones were analyzed for TCR-α/β expression by cDNA-PCR. Two clones were eliminated as probable sister clones, based on Vβ segments. Clone 08 grew poorly and thus was not included in all of the experiments reported here. Details of the recognition assay are described (13, 19). The ^{51}Cr-release assay was terminated after 4 h incubation at 37°C. The specific lysis was calculated as 100 × [(experimental − spontaneous release)/(total − spontaneous release)].

cDNA-PCR and Direct Sequencing. RNA extraction, cDNA synthesis, and PCR with Vα, Vβ, Cα, and Cβ primers were carried out on CTL clones as previously described (20). A detailed description including the nucleotide sequences will be reported elsewhere (Casanova, J.-L., P. Romero, and I. F. Luescher, manuscript in preparation).

Results

CTL Recognition of Biotin-YIPSAEK(IASA)I. Eight independent CTL clones, derived from (BALB/c × C57BL/6)F_1 mice immunized with biotin-YIPSAEK(IASA)I, specifically recognized the PbCS derivative conjugate, as illustrated in Fig. 2 for CTL clones N9 and O1. The concentration of biotin-YIPSAEK(IASA)I required for half maximal lysis varied for the different clones in the range of 10^{-9}–10^{-11} M (Table 1). All of the six clones tested were K^d restricted as assessed by recognition of the antigenic peptide derivative on L cell transfectants that expressed cell surface K^d molecules but not on untransfected controls (data not shown). The IASA group appeared to form a critical part of the antigenic epitope, since the CTL clones failed to recognize the peptide derivative lacking the IASA group (biotin-YIPSAEKI) (Fig. 1) or the unmodified PbCS peptide (15 and data not shown). None of the clones recognized the isomeric derivative IASA-YIPSAEK(biotin)I (data not shown).

As previously shown (15), deletion of the NH_2-terminal biotin or of substituents from the IASA group had no significant effect on the binding efficiency of the peptide derivatives to the K^d molecule. In contrast, these variant peptide derivatives were differentially recognized by the CTL clones (Table 1). The biotin group that was included in the derivative for technical reasons (ease of synthesis of the derivative and reactivity with streptavidin for biochemical studies) was not required for recognition by most (six out of eight tested) of the CTL clones (Table 1). Removal of the iodine from the IASA group [biotin-YIPSAEK(ASA)I] clearly decreased (by 30–1,000-fold) the efficiency of recognition for three of the clones (N1, N4, and O1), but actually improved recognition for the other four by 6–12-fold. Only two clones (N6 and N9) efficiently recognized a derivative in which both the iodine and the hydroxy residues were deleted [(biotin-YIPSAEK(ABA)]. One of these clones, N9, recognized this particular derivative about 50 times more efficiently than the original photoprobe. It was also the only clone among the eight tested to recognize the variant derivative lacking all three substituents of the IASA group [biotin-YIPSAEK(BA)I]. It

IASA

Table 1. *Recognition of PbCS Peptide Derivative Biotin-YIPSAEKI and its Variants by Eight Independent CTL Clones*

	Recognition of: *IASA* biotin-YIPSAEKI	Relative antigenic activity‡			
		IASA	*ASA*§	*ABA*	*BA*
CTL clone	(pmol) 50%	YIPSAEKI	biotin-YIPSAEKI	biotin-YIPSAEKI	biotin-YIPSAEKI
N1	20*	1.2	0.02	$<10^{-5}$	$<10^{-5}$
N4	70	1.2	0.09	$<10^{-5}$	$<10^{-4}$
N6	7	0.7	12	0.2	$<10^{-6}$
N9	100	1.4	8.3	50	1.1
N11	50	2	12	$<10^{-5}$	$<10^{-6}$
O1	18	0.9	0.0012	$<10^{-4}$	$<10^{-5}$
O7	30	0.008	6	$<10^{-4}$	$<10^{-4}$
O11	600	<0.0006	ND	<0.0006	$<10^{-5}$

* The numeric values represent the concentration (pmol, 10^{-12} M) of the photoreactive peptide derivative required for 50% maximal lysis by the corresponding CTL clones in each experiment. This concentration was determined in a ^{51}Cr-release assay (see Fig. 1). The E/T ratio was 3:1. Threefold dilutions of the peptide derivative were made covering a concentration range between 10^{-6} and 10^{-13} M. The 50% lysis value was used as the reference concentration for calculating the relative antigenic activity of the other peptide derivatives.

‡ The antigenic activity of the four variant photoreactive peptide derivatives was assayed in a ^{51}Cr-release assay as described above for the reference peptide. The relative antigenic activity is calculated as the concentration of the reference peptide biotin-YIPSAEK(IASA)I required to obtain 50% maximal lysis divided by that of the variant peptide.

§ The structural formulas of the variant *IASA* groups are as shown in Fig. 1

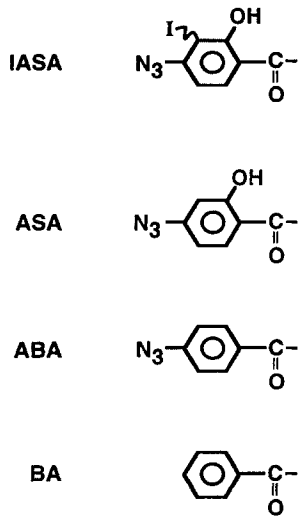


Figure 1. Structural formulas of the orthogonally conjugated groups. Iodo, 4-azido salicylic acid (*IASA*), 4-azido salicylic acid (*ASA*), 4-azido benzoic acid (*ABA*), or benzoic acid (*BA*) were coupled onto Lys_{259} via an amide bond.

is remarkable that it recognized the latter compound as efficiently as the original biotin-YIPSAEK(IASA)I conjugate.

TCR Photoaffinity Labeling. The eight biotin-YIPSAEK-(IASA)I–specific CTL clones were tested for TCR photoaffinity labeling. The different clones were incubated with the radiolabeled photoprobe. After UV irradiation, the immunoprecipitated TCR were analyzed by SDS-PAGE. In addition to the N6 clone that was previously analyzed (15), the clones N4, N9, and N11 showed TCR photoaffinity labeling (Fig. 3). No significant TCR labeling was observed under the same conditions for the other four clones tested (N1, O1, O7, and O11), although clone N1 showed a faint band on overexposure of the gel (data not shown). The labeled immunoprecipitated TCR material migrated with an apparent molecular mass of ~82–92 kD under nonreducing conditions, and 42–47 kD under reducing conditions. This corresponds to the migration pattern of conventional disulfide-linked α/β heterodimer TCR molecules (21). Two labeled species of different molecular weights were apparent for the photoaffinity labeled TCR of clone N9 under reducing conditions, but only one species was observed under nonreducing conditions (Fig. 3).

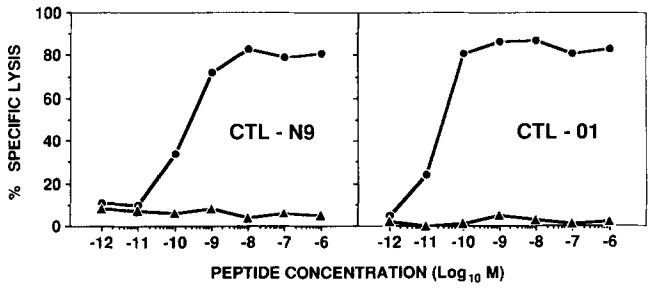


Figure 2. Antigen recognition by the N9 and O1 CTL clones. Two selected biotin-YIPSAEK(IASA)I-specific CTL clones illustrate recognition of P815 target cells in the presence of the indicated concentrations of biotin-YIPSAEK(IASA)I (●) or biotin-YIPSAEKI (▲). The unmodified parental peptide PbCS 253-260 is not recognized by CTL even at 10^{-5} M concentration (data not shown).

We verified that the TCR labeling of clones N4, N9, and N11 was dependent on the peptide derivative binding first to the K^d molecules expressed on the surface of the clones, i.e., that the photoprobe per se was unable to label directly these TCR. This has been shown previously for clone N6 (15) and is illustrated for clone N9 in Fig. 4. Thus the TCR labeling was abolished when cells were incubated with biotin-YIPSAEK(^{125}IASA)I in the presence of a 300-fold molar excess of the K^d-restricted peptides PbCS 253-260 or P198$^-$.14-22 (19), but not of the D^b-restricted adenovirus peptide 234-243 (17) (Fig. 4, lanes *2–4*). Furthermore, the TCR labeling was also inhibited in the presence of the anti-K^d mAb 20-8-4S, but not by an isotype-matched irrelevant mAb (anti-I-A^k) (Fig. 4, lanes *5* and *6*).

Since biotin-YIPSAEK(IASA)I was presented by K^d molecules expressed on the CTL clones, we compared the ability of the different clones to bind peptide by K^d photoaffinity labeling. We previously showed that the isomeric IASA-YIPSAEK(biotin)I selectively photoaffinity labels the K^d molecule on different cell types, but fails to label TCR (15–17). As shown in Fig. 5, the K^d photoaffinity labeling was efficient in the case of the N4, N6, N9, and O1 clones and slightly reduced for the N1 and N11 clones. The K^d labeling on the O7 and O11, however, was considerably weaker (~10-fold). We assessed the level of expression of several cell surface molecules that are involved in CTL target cell interactions by flow cytometric analysis. There was, however, no obvious correlation between the level of expression of K^d, CD3, CD8, LFA-1 or CD44 and TCR photoaffinity labeling (data not shown), suggesting that the lack of TCR photoaffinity labeling observed with some clones could not be accounted for by a low degree of surface expression of these molecules.

Assessment of Photoaffinity-labeled TCR Chain. We next wished to identify which TCR chain was photoaffinity labeled. The clones N4, N9, and N11 were either lactoperoxidase radioiodinated or photoaffinity labeled with biotin-YIPSAEK-(^{125}IASA)I and the immunoprecipitated TCR were analyzed by two-dimensional gel electrophoresis (Fig. 6). The lactoperoxidase-labeled TCR of the N4 clone showed a weakly labeled α chain of an ~ isoelectric point (pI) of 4.8 and an intensively labeled β chain of a pI of ~6.1, both having a molecular mass of ~43 kD (Fig. 6 *A*). The photoaffinity-labeled material from the N4 TCR migrated very similarly to the α chain, indicating that this TCR was selectively photoaffinity labeled at the α chain. The lactoperoxidase labeled N9 TCR showed a strongly labeled β chain of an ~pI of 6 and a molecular mass of ~45 kD and a weakly labeled α chain with a pI of ~4.7 and a molecular mass of 42 kD (Fig. 6 *B*). The photoaffinity-labeled N9 TCR showed a very similar pattern, with a relatively more intense labeling of the α chain. This indicated that the N9 TCR was photoaffinity labeled on both chains and explains the two labeled species seen in the SDS-PAGE under reducing conditions (Fig. 3). The lactoperoxidase-labeled N11 TCR showed a weakly labeled β chain and an intensively labeled α chain with approximate pIs of 6.6 and 4.9, respectively, both of a molecular mass of ~45 kD (Fig. 6 *C*). For clone N11, only the

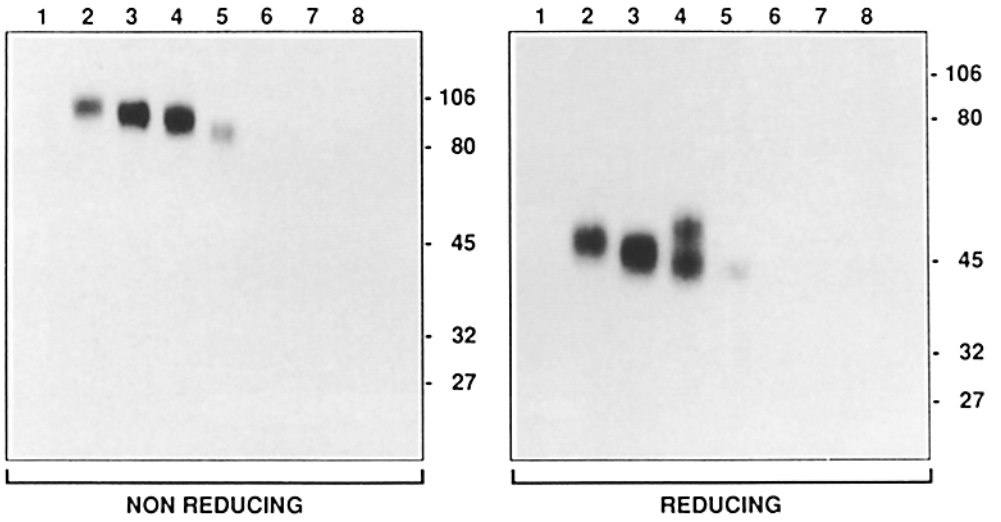


Figure 3. TCR photoaffinity labeling on biotin-YIPSAEK(IASA)-I–specific CTL clones. N1 (lane *1*), N4 (lane *2*), N6 (lane *3*), N9 (lane *4*), N11 (lane *5*), O1 (lane *6*), O7 (lane *7*), and O11 (lane *8*) were incubated with biotin-YIPSAEK-(^{125}IASA)I and after UV irradiation, the immunoprecipitated TCR were analyzed by SDS-PAGE under nonreducing or reducing conditions.

TCR β chain was photoaffinity labeled. The same analysis performed on the N6 clone showed that its TCR was selectively labeled at the α chain (15). Taken together, the data showed that biotin-YIPSAEK(^{125}IASA)I selectively labeled the α chain of the N4 and N6 TCR and the β chain of the N11 TCR, but labeled both chains of the N9 TCR.

TCR Sequence Analysis of Biotin-YIPSAEK(IASA)I–specific CTL Clones. Four out of the eight clones analyzed (N1, N4, N6, and N9) were found to express a Vβ1-containing TCR. Moreover, all four Vβ1-expressing clones display α locus rearrangements that use the same Jα segment (TA28) (Table 2). The same pairing was observed for the additional O8 clone. This clone also recognized biotin-YIPSAEK(IASA), but not biotin-YIPSAEKI (data not shown). Because of its slow growth, this clone was not further analyzed. It should be noted that for one of the latter clones (N9), a second in-frame α chain transcript was also found, so that the functional α chain cannot be identified with certainty. The occurrence of two rearranged α loci, each encoding potentially functional chains, is a common finding among T cell clones (20). A different pair of Vβ/Jα segments (Vβ8.3/JαC7) is used by two other CTL clones, N11 and O1. There is no apparent preferential usage of Vα or Jβ segments among the nine clones studied.

For the β chains, there is no obvious CDR3 sequence similarity, except for the two clones that used the same Jβ element, N11 and O8. The CDR3β lengths are variable, from 6 to 11 amino acid residues long, although those of the five Vβ1 TCR are only six or seven residues long. The CDR3 regions of the α chains show more sequence similarity, in part because of the common Jα usage, and the CDR3α lengths are less variable than those of the β chains.

Comparison of the Relative Photolabeling of Individual Amino Acids by the IASA Group. The preferential expression of the Trp containing Jα segment prompted us to assess whether this amino acid preferentially interacts with the IASA group. To test this possibility, we compared the relative photolabeling of various radiolabeled amino acids by Sepharose-coupled IASA in aqueous solution (Fig. 7). The highest degree of labeling was observed for Trp (32%), followed by Cys (17%) and Tyr (13%). The other amino acids tested gave lower values. Thus,

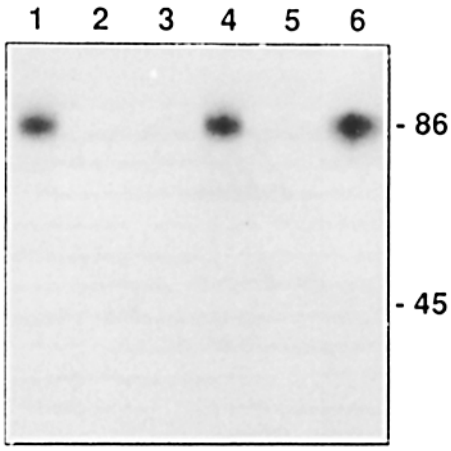


Figure 4. The N9 TCR photoaffinity labeling is K^d restricted. N9 cells were incubated with biotin-YIPSAEK(^{125}IASA)I in the absence (lane *1*) or presence of a 300-fold molar excess of PbCS 253-260 (lane *2*), p198-.14–22 (lane *3*), Ad5 E1a (lane *4*), anti-K^d mAb (20-8-4S; 10 μg/ml) (lane *5*), or anti-IA^k mAb (10.3.6.2; 10 μg/ml). After UV irradiation, the immunoprecipitated TCR were analyzed by SDS-PAGE (10%, nonreducing).

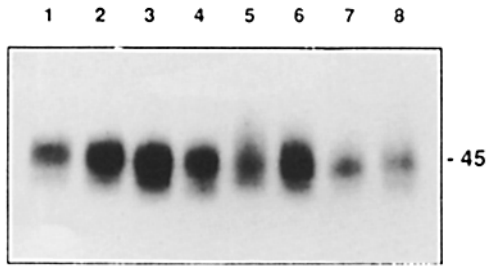
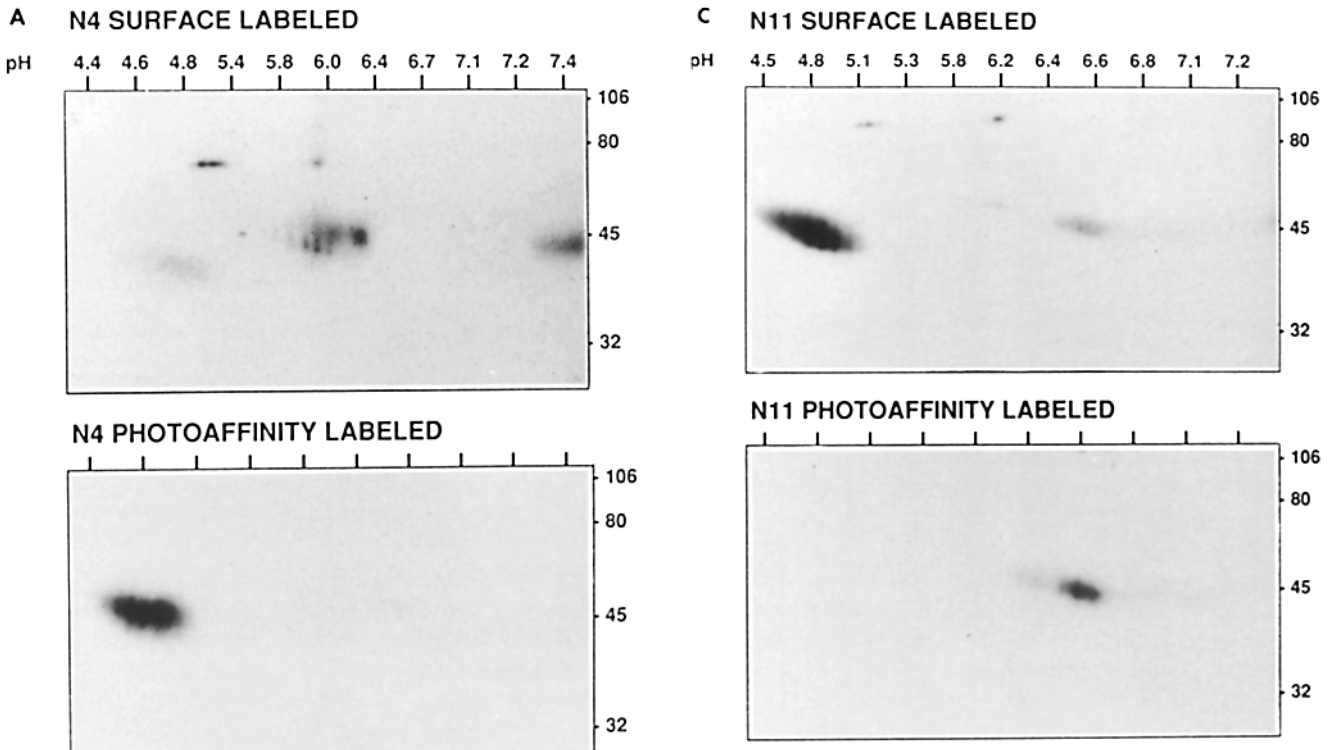


Figure 5. K^d photoaffinity labeling by ^{125}IASA-YIPSAEK(biotin)I on CTL clones N1 (lane *1*), N4 (lane *2*), N6 (lane *3*), N9 (lane *4*), N11 (lane *5*), O1 (lane *6*), O7 (lane *7*), and O11 (lane *8*) cells were incubated with ^{125}IASA-YIPSAEK(biotin)I and after UV irradiation, the immunoprecipitated K^d was analyzed by SDS-PAGE (10%, reducing).



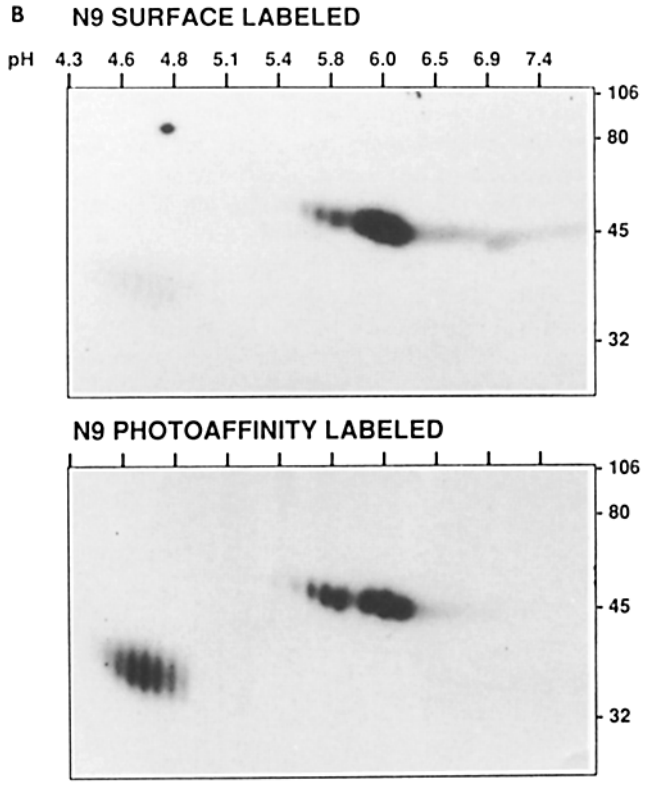


Figure 6. Identification of the photoaffinity-labeled TCR subunit chain. The TCR of N4 (*A*), N9 (*B*), and N11 (*C*) cells were lactoperoxidase radioiodinated (*top*) or photoaffinity labeled (*bottom*) and analyzed by two-dimensional gel electrophoresis. The first dimension was isoelectric focusing (the pH gradient increases from left to right) and the second dimension SDS-PAGE (molecular weight increases from bottom to top).

among the 15 amino acids tested, Trp was labeled by the IASA group with a high preference. Since the UV irradiation-induced intermediate nitrenes are short-lived (22, 23) and capable of undergoing diverse chemical reactions with virtually any amino acid side chain (23, 24) the preferential photolabeling of Trp suggests a physical interaction of Trp with the IASA group in aqueous medium.

Discussion

We investigated antigen recognition by CTL using a new approach based on two strategies. The first was to modify a potential TCR contact residue of a MHC-restricted antigenic peptide with a photoreactive group and to generate CTL clones that specifically recognized this peptide derivative. The second strategy was to study the TCR–ligand interactions in such systems by TCR photoaffinity labeling. We have previously demonstrated the feasibility of this approach for one CTL clone that specifically recognized the biotin-YIPSAEK-(IASA)I derivative of the PbCS 253-260 peptide (15). In this report, eight independent biotin-YIPSAEK(IASA)I–specific CTL clones were studied for recognition of their photoreactive ligand, the K^d molecule–biotin-YIPSAEK(IASA)I complex.

Our results demonstrate that MHC class I–restricted CTL specific for a chemically modified peptide can be induced by

Table 2. *TCR α and β Junctional Amino Acid Sequences*

CTL clone	TCR-β					TCR-α				
	Vβ	FW	CDR3	FW	Jβ	Vα	FW	CDR3	FW	Jα
N1	1	CAS	S Q G N S D Y	TFG	1.2	8.F3.4	CAL	S V P S G S W Q L	IFG	TA28
						2.3				C9
N4	1	CAS	S Q G G I Q	YFG	2.7	BMA	CAT	V P S S G S W Q L	IFG	TA28
						4.3				14.4
N6	1	CAS	S S G R E V	FFG	1.1	4.BDFLII	CVL	G D S S G S W Q L	IFG	TA28
						8.F3.3				TA28
N9	1	CAS	S Q A P S E Q	YFG	2.7	5.TA72	CAV	A S S G S W Q L	IFG	TA28
						1.11-3	CAM	S P D Y S N N R L	TFG	N9
O8	1	CAS	S G G N T L	YFG	1.3	13.1	CAA	S A P S G S W Q L	IFG	TA28
						A10				TT11
N11	8.3	CAS	S E V S G N T L	YFG	1.3	4.PJR-25	CAL	R I T G N T R K L	IFG	C7
						34s281				TA61
O1	8.3	CAS	S E G G G G E R L	FFG	1.4	4.TA65	CAL	G I T G N T R K L	IFG	C7
						3.01				TA37
O7	10	CAS	S S N R V S Y N S P L	YFA	1.6	8.F3.4	CAL	S D R D N N R I	FFG	MD-13
						BMB	CAM	R E R P G T G S N R L	TFG	07
O11	13	CAS	S P G R Y E Q	YFG	2.7	4.TA65	CAL	I H T G N Y K Y	VFG	TA19
						10.FN1-18				TA65

Nine biotin-YIPSAEK(*IASA*)I–specific CTL clones are listed on the vertical axis. The TCR-β (*left*) and α (*right*) only segments consistent with an open reading frame, encoding for junctional, hypervariable, and putatively CDR3-like regions are shown.

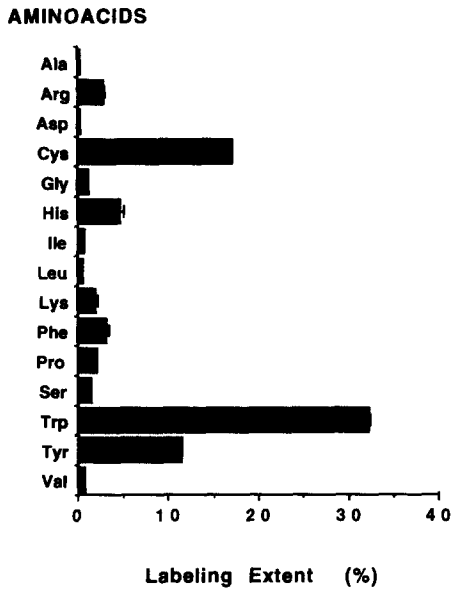


Figure 7. IASA labeling of individual amino acids in PBS. The fraction of the IASA photolabeled amino acids is shown as percent of the offered amino acid. The highest degree of labeling was 32% for Trp and the lowest was 0.33% for Ala.

direct immunization with the peptide derivative, much as has been previously shown for the parental PbCS peptide (13). It is noteworthy that the in vivo induction of T cell reactivities against chemically modified antigenic peptides may play a role in certain diseases. It is known that naturally processed peptides derived from autologous proteins efficiently bind to MHC class I and II histocompatibility molecules (25, 26) and that various chemical agents, including many drugs, can modify autologous proteins (27). It is thus conceivable that the induction of T cells specific for chemically modified self-peptides is a primary event in the development of disorders such as drug allergies or delayed-type hypersensitivities. Indeed, it has been shown recently that in vivo-induced trinitrophenyl-reactive CTLs specifically recognize trinitrobenzene sulfonic acid-modified self-peptides in an MHC-restricted manner (28).

The *biotin*-YIPSAEK(*IASA*) I–specific CTL clones displayed all the hallmarks of conventional antigen recognition by CTL, including specific peptide binding to MHC molecules and involvement of auxiliary molecules (15 and Fig. 4). The photoreactive IASA group was an essential part of this CTL epitope, since its removal abrogated recognition and deletions of its substituents affected recognition, in a clone-specific

manner, but not K^d binding (15 and Table 1). This suggested that the TCR of these clones directly interact with the conjugated photoreactive group. For some of the clones, this interaction was indeed directly demonstrated by photoaffinity labeling (Fig. 3).

It is not clear why only some of these CTL clones displayed detectable TCR photoaffinity labeling. It is conceivable that TCR labeling requires a certain TCR ligand affinity, which may not be achieved by some of the TCR studied. TCR ligand interactions, where measured, were reported to be of low affinity (10^{-4}–10^{-5} M; 29, 30) and thus may not be detectable in the present TCR photoaffinity labeling experiments, in which the photoprobe concentration is low ($\sim 10^{-8}$ M). The photoaffinity-labeled TCR may indeed have a higher affinity than the others, since the recognition by the clones that displayed TCR labeling generally required lower antigen concentrations and/or was less susceptible to deletions of substituents from biotin-YIPSAEK(IASA)I (Table 1).

Other factors, however, may also be important for TCR photoaffinity labeling. For example, the TCR labeling in these experiments depended on the presentation of the photoprobe by the K^d molecules of the CTL clones. The relatively inefficient presentation by the N1, O7, and O11 clones, as reflected by the reduced K^d photoaffinity labeling (Fig. 5) thus could account, at least in part, for the lack of their TCR labeling. Furthermore, the possibility also exists that the photoaffinity labeling of some TCR results in unstable covalent bonds that decay during subsequent procedures (23).

TCR sequence analysis of the biotin-YIPSAEK(IASA)-I–specific CTL clones showed a preferential usage of TCR containing the Vβ1 and JαTA28 segments (Table 2). It is remarkable that all the clones, except N11, that displayed TCR labeling, expressed such TCR. Preliminary results from a currently ongoing study showed an even higher preference. In that study, six out of eight independent CTL clones recognizing a PbCS derivative, in which the IASA group was replaced with the related ABA group and that were selected for efficient TCR photoaffinity labeling, also expressed TCR using the Vβ1 and JαTA28 paired elements (Luescher, I. F., P. Romero, and J.-L. Casanova, unpublished results).

A different pairing (Vβ8.3/JαC7) was expressed by two of the biotin-YIPSAEK(IASA)I–specific clones, N11 and O1 (Table 2). A pairing of Vβ and Jα segments is not unique to these responses against modified peptides since we found that all of 23 H-$2K^d$-restricted CTL specific for the CW3 170-179 antigenic peptide express TCR that use the Vβ10 and JαpHDS58 segments in combination with a variety of different Jβ and Vα elements (31). It is interesting that a reciprocal pairing in which TCR express the Vα10 and Jβ2.6 elements was described for H-2^b-restricted TNP-specific CTL clones (32). One possible explanation for this pairing of TCR elements is provided by the molecular model of the TCR proposed by Chothia et al. (7), where the Jα-encoded residues of the CDR3α would be in close proximity to the CDR2 region of the β chain. Similarly, those of the CDR3β would be close to the CDR2α region.

The preferential Vβ1/JαTA28 usage by the biotin-YIPSAEK(IASA)I–specific TCR is remarkable when compared with the TCR repertoire found in the response to the parental peptide, PbCS 253-260 (20). In that case, none of the TCR expressed by 28 different CTL clones used the Vβ1 or JαTA28 segments, and no preferential Vβ/Jα pairing was observed. Since all of the Vβ1/JαTA28 expressing CTL clones specific for the biotin-YIPSAEK(IASA)I compound recognized the YIPSAEK(IASA)I derivative but not biotin-YIPSAEKI, it appears that recognition of the IASA-modified Lys_{259} residue and the preferential usage of Vβ1/JαTA28 expressing TCR are directly related. The mouse genome contains at least 50 distinct Jα gene segments (6), and the JαTA28 element has thus far not been found in analyses of other K^d-restricted CTL clones (Casanova, J.-L., unpublished results). The high occurrence of this particular Jα segment among the biotin-YIPSAEK(IASA)I–specific TCR thus argues strongly for its direct involvement in TCR selection and ligand recognition.

It is now generally accepted that the CDR3 loops of TCR-α/β interact mainly with amino acid side chains of MHC-bound antigenic peptides (6–12). The JαTA28 segment contains Trp, an amino acid rarely found in other CDR3 Jα sequences. From our analysis of the photolabeling of different amino acids by IASA (Fig. 7), it appears that the IASA group preferentially associates with Trp. It is therefore conceivable that the CDR3α Trp of the JαTA28 segment is directly involved in an efficient binding of the photoreactive group of the ligand. This interpretation is supported by the finding that all the photoaffinity-labeled TCR expressing the JαTA28 segment were labeled at the α chain. The finding that the N9 TCR was also labeled at the β chain does not discount this interpretation since β chain residues, located in close proximity to the JαTA28 Trp, could also become photocrosslinked. The specific cross-linking of the IASA group to two distinct residues in a target protein has already been observed (33).

A recent study using immunization of TCR single chain transgenic mice with variants of the I-E^k-restricted mouse cytochrome peptide 88-103 and TCR analysis indicated that NH_2-terminal peptide residues interacted with the CDR3α and COOH-terminal residues with the CDR3β (12). In contrast, the present study suggests that a residue next to the COOH-terminal end of the peptide derivative preferentially interacts with the CDR3α region. The possibility has thus to be considered that TCR–ligand interactions follow no simple, general rules and may involve considerable variation in topology and relative orientation. This may be true for TCR recognizing the same MHC–peptide complex. In the present system, for example, the finding that the N11 TCR, which expresses different CDR3α and β sequences, was selectively labeled at the β chain, suggests that this TCR may interact with the IASA group preferentially via the β chain.

Further analysis, including the characterization of additional systems, will be required before the general molecular principles of TCR–ligand interactions become apparent. The approach described here should facilitate such studies and also

provide new possibilities. For example, because of the high chemical reactivity and short life span of phenylnitrenes, photoaffinity labeling often takes place in a site-specific manner (23, 33). In principle, the photoaffinity labeled residue(s) on a target protein can be identified by amino acid sequencing of the labeled digest fragment (33–35). Tryptic peptide maps of the photoaffinity-labeled N6 TCR indeed showed reproducibly one major labeled digest fragment (15). This should now allow us to test directly the hypothesis that the Trp residue of the JαTA28 segment interacts with the IASA group of the antigenic peptide derivative presented by the K^d molecule.

We thank P. A. Cazenave and E. Barbier for advice with the two-dimensional gel electrophoresis. Expert technical assistance by A.-S. Cordy and A. Layer, and secretarial assistance by A. Zoppi are gratefully acknowledged.

P. Romero was supported, in part, by a grant from the Roche Research Foundation, and J.-L. Casanova, by the Ligue Suisse contre le Cancer.

Address correspondence to Dr. P. Romero, Ludwig Institute for Cancer Research, Ch. des Boveresses 155, 1066 Epalinges, Switzerland. J.-L. Casanova is currently at Unité de développement normal et pathologique du système immunitaire, INSERM 132, Hôpital des Enfants Malades, 149 rue de Sèvres, 75743 Paris Cedex 15, France.

Received for publication 31 December 1992.

References

1. Townsend, A., and H. Bodmer. 1989. Antigen recognition by class I–restricted T lymphocytes. *Annu. Rev. Immunol.* 7:601.
2. Madden, D.R., J.C. Gorga, J.L. Strominger, and D.C. Wiley. 1992. The three dimensional structure of HLA-B27 at 2.1 Å resolution suggests a general mechanism for tight peptide binding to MHC. *Cell.* 70:1035.
3. Zhang, W., A.C.M. Young, M. Imarai, S.G. Nathenson, and J.C. Sacchettini. 1992. Crystal structure of the major histocompatibility complex class I H-2K^b molecule containing a single viral peptide: implications for peptide binding and T-cell receptor recognition. *Proc. Natl. Acad. Sci. USA.* 89:8403.
4. Matsumura, M., D.H. Fremont, P.A. Peterson, and I.A. Wilson. 1992. Emerging principles for the recognition of peptide antigens by MHC class I molecules. *Science (Wash. DC).* 257:927.
5. Fremont, D.H., M. Matsumura, E.A. Stura, P.A. Peterson, and I.A. Wilson. 1992. Crystal structures of two viral peptides in complex with murine MHC class I H-2K^b. *Science (Wash. DC).* 257:919.
6. Davis, M.M., and P.J. Bjorkman. 1988. T cell receptor genes and T cell recognition. *Nature (Lond.).* 334:395.
7. Chothia, C., D.R. Boswell, and A.M. Lesk. 1988. The outline structure of the T cell αβ receptor. *EMBO (Eur. Mol. Biol. Organ.) J.* 7:3745.
8. Claverie, J.-M., A. Prochnicka-Chalfour, and L. Bougueleret. 1989. Implications of a Fab like structure for the T cell receptor. *Immunol. Today.* 10:10.
7. Danska, J.S., A.M. Livingstone, V. Paragas, T. Ishiara, and C.G. Fathman. 1990. The presumptive CDR3 regions of both T cell receptor α and β chains determine T cell specificity for myoglobin peptides. *J. Exp. Med.* 172:27.
8. Wither, J., J. Pawling, L. Phillips, T. Delovitch, and N. Hozumi. 1991. Amino acid residues in the T cell receptor CDR3 determine the antigenic reactivity patterns of insulin-reactive hybridomas. *J. Immunol.* 146:3513.
9. Winoto, A., J.L. Urban, N.C. Lan, J. Goverman, L. Hood, and D. Hansburg. 1986. Predominant use of Vα gene segment in mouse T-cell receptors for cytochrome c. *Nature (Lond.).* 324:679.
10. Fink, P.J., L.A. Matis, D.L. McElligott, M. Bookman, and S.M. Hedrick. 1986. Correlation between T-cell specificity and the structure of the antigen receptor. *Nature (Lond.).* 321:219.
11. Engel, I., and S.M. Hedrick. Site-directed mutations in the VDJ junctional region of a T cell receptor β chain cause changes in antigenic peptide recognition. 1988. *Cell.* 54:473.
12. Jorgensen, J.L., U. Esser, B. Fazekas de Groth, P.A. Reay, and M.M. Davis. 1992. Mapping T-cell receptor-peptide contacts by variant peptide immunization of single-chain transgenics. *Nature (Lond.).* 255:224.
13. Romero, P., G. Eberl, J.-L. Casanova, A.-S. Cordey, C. Widmann, I.F. Luescher, G. Corradin, and J.L. Maryanski. 1992. Immunization with synthetic peptides containing a defined malaria epitope induces a highly diverse cytotoxic T lymphocyte response. *J. Immunol.* 148:1871.
14. Maryanski, J.L., R. Lüthy, R. Romero, F. Healy, C. Drouet, J.-L. Casanova, C. Jaulin, P. Kourilsky, and G. Corradin. 1993. The interaction of antigenic peptides with the H-2K^d MHC class I molecule. *Sem. Immunol.* In press.
15. Romero, P., J.L. Maryanski, and I.F. Luescher. 1993. Photoaffinity labeling of the T cell receptor on living cytotoxic T lymphocytes. *J. Immunol.* In press.
16. Luescher, I.F., P. Romero, J.-C. Cerottini, and J.L. Maryanski. 1991. Specific binding of antigenic peptides to cell-associated MHC class I molecules. *Nature (Lond.).* 351:72.
17. Luescher, I.F., J.A. López, B. Malissen, and J.-C. Cerottini. 1991. Interaction of antigenic peptides with MHC class I molecules on living cells studied by photoaffinity labeling. *J. Immunol.* 148:1003.
18. O'Farrell, P.H. 1975. High resolution two-dimensional electrophoresis of proteins. *J. Biol. Chem.* 250:4007.
19. Romero, P., G. Corradin, I.F. Luescher, and J.L. Maryanski. H-2K^d restricted antigenic peptides share a simple binding

motif. *J. Exp. Med.* 174:603.
20. Casanova, J.-L., P. Romero, C. Widmann, P. Kourilsky, and J.L. Maryanski. 1991. T cell receptor genes, in a series of class I MHC restricted CTL clones specific for a single *Plasmodium berghei* nonapeptide: implications for T cell allelic exclusion and antigen-specific receptor. *J. Exp. Med.* 174:1371.
21. Samelson, L.E. 1985. Analysis of the structure of the antigen receptor on a pigeon cytochrome specific T cell hybridoma. *J. Immunol.* 134:2529.
22. De Graff, B.A., D.V. Gillepsie, and R.J. Sundberg. 1974. Phenylnitrene. A flash photolytic investigation of the reaction with secondary amines. *J. Am. Chem. Soc.* 96:7491.
23. Baley, H., and J.V. Staros. 1984. In Azides and Nitrenes—Reactivity and Utility. E.F.V. Scriven, editor. Academic Press, London. 434.
24. Matheson, R.R., H.E. Van Wart, A.W. Burgess, and L.I. Weinstein. 1977. Study of protein topography with flash photolytically generated nonspecific surface-labeling reagents: surface labeling of ribonuclease A. *Biochemistry.* 16:396.
25. Falk, K., O. Rötzschke, S. Stevanivoc, G. Jung, and H.-G. Rammensee. 1991. Allele-specific motifs revealed by sequencing of self-peptides eluted from MHC molecules. *Nature (Lond.).* 351:290.
26. Chicz, M.R., R.G. Urban, W.S. Lane, J.C. Gorga, L.J. Stern, D.A. Vignali, and J.L. Strominger. 1992. Predominant naturally processed peptides bound to HLA-DR1 are derived from MHC-related molecules and are heterogenous in size. *Nature (Lond.).* 358:764.
27. Bell, S.J., and W.J. Pichler. 1989. Penicillin-allergic patients react to penicillin-modified "self". *Allergy.* 44:199.
28. Ortmann, B., S. Martin, A. von Bonin, E. Schlitz, H. Hoschuetzky, and H.U. Weltzien. 1992. Synthetic peptides anchor T cell-specific TNP epitopes to MHC antigens. *J. Immunol.* 148:1445.
29. Weber, S., A. Traunecher, F. Oliversi, W. Gerhard, K. Karjalainen. 1992. Specific low-affinity recognition of major histocompatibility complex plus peptide by soluble T-cell receptor. *Nature (Lond.).* 356:793.
30. Matsui, K., J.L. Boniface, P.A. Reay, H. Schild, B. Fazekas-de St. Groth, and M.M. Davis. 1991. Low affinity interaction of peptide-MHC complexes with T cell receptors. *Science (Wash. DC).* 254:1788.
31. Casanova, J.-L., J.-C. Cerottini, M. Matthes, A. Necker, H. Gournier, C. Barra, C. Widmann, H.R. MacDonald, F. Lemonnier, B. Malissen, and J.L. Maryanski. 1992. H-2-restricted cytolytic T lymphocytes specific for HLA display T cell receptors of limited diversity. *J. Exp. Med.* 176:439.
32. Kempkes, B., E. Palmer, S. Martin, A. Bonin, K. Eichmann, B. Ortmann, and H.U. Weltzien. 1991. Predominant T cell receptor gene elements in TNP-specific cytotoxic T cells. *J. Immunol.* 147:2467.
33. Leuscher, I.F., D.L. Crimmins, B.D. Schwartz, and E.R. Unanue. 1990. The sites in the I-A^k histocompatibility molecule photoaffinity labeled by an immunogenic lysozyme peptide. *J. Biol. Chem.* 265:11177.
34. Brinegar, A.C., G. Cooper, A. Stevens, C.R. Haver, J. Shabanowitz, D.F. Unt, and J.E. Fox. 1988. Characterization of a benzyladenine binding-site peptide isolated from a wheat cytokinin-binding protein: sequence analysis and identification of a single affinity-labeled histidine residue by mass spectrometry. *Proc. Natl. Acad. Sci USA.* 85:5927.
35. Pandey, V.N., K.R. Williams, K.L. Stone, and M.J. Modak. 1987. Photoaffinity labeling of the thymidine triphosphate binding domain in *Escherichia coli* DNA polymerase I: identification-881 as the site of cross-linking. *Biochemistry.* 26:7744.