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# Polymorphism of Cytotoxic T-Lymphocyte Clones That Recognize a Defined Nine-Amino-Acid Immunodominant Domain of Lymphocytic Choriomeningitis Virus Glycoprotein†

ETIENNE JOLY,* MARIA SALVATO, J. LINDSAY WHITTON, AND MICHAEL B. A. OLDSTONE

*Department of Immunology, Research Institute of Scripps, 10666 North Torrey Pines Road, La Jolla, California 92037*

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**To assess the heterogeneity of cytotoxic T lymphocytes (CTLs) directed against viral epitopes, we studied six class I major histocompatibility complex-restricted (*H-2D^b*) CTL clones that recognize the same 9-amino-acid immunodominant epitope, amino acids 278 to 286 from envelope glycoprotein 2 (GP2) of lymphocytic choriomeningitis virus (LCMV). Using Southern blot analysis of β-chain rearrangements, we found that each clone has a unique restriction pattern, providing evidence of the independent derivation of the clones and suggesting that the clones express different β-chain sequences for their T-cell receptor. All these clones killed syngeneic target cells infected with strain Armstrong or WE of LCMV; however, two of the six clones failed to recognize target cells infected with the Pasteur strain of LCMV. Sequence analysis of LCMV Armstrong, WE, and Pasteur GP in the region of amino acids 272 to 293 demonstrated a single-amino-acid substitution at amino acid 278 in the region of the defined epitope in the Pasteur strain. Interestingly, one of the two CTL clones that failed to lyse LCMV Pasteur-infected target cells nevertheless efficiently and specifically killed uninfected target cells coated with the appropriate LCMV Pasteur peptide, while the other clone failed to do so. This indicated a dichotomy between processing of the synthesized protein initiated by infection and a peptide exogenously applied. Dose-response studies utilizing several peptides with substitutions in GP amino acid 278 indicate that CTL recognition occurs at the level of a single amino acid and suggest that this difference is likely recognized at the level of the T-cell receptor.**

Virus-specific *H-2*-restricted cytotoxic T lymphocytes (CTLs) play an important role in controlling certain viral infections (2, 5, 18, 31). Technical advances in CTL cloning (4), viral genetics (25), molecular approaches to expressing unique components of viral genes (19, 29), and use of peptides to coat target cells (27) allow the fine dissection of epitopes needed for CTL recognition and the molecular mapping of cell-cell recognition. Recently, we documented that the glycoprotein (GP) of the Armstrong strain (ARM) of lymphocytic choriomeningitis virus (LCMV) was a major recognition protein of *H-2^b* mice (28). Further, of 18 independently derived CTL clones, 17 (95%) recognized this viral GP and 15 of these 17 (87% of the total) recognized a single immunodominant epitope that was restricted by the major histocompatibility complex (MHC) $D^b$. The recognition site, a 9-amino-acid sequence from 278 to 286 (VENPG GYCL), was characterized by using both molecular genetics to bracket the sequence needed for recognition and a series of truncated peptides to obtain the minimal sequence (23). Two issues, however, remained unresolved: first, the degree of diversity of CTL clones recognizing this immunodominant domain, and second, whether these CTL clones recognize targets infected with other LCMV strains. From our results, 6 CTL clones selected at random from the 15 clones that recognized one immunodominant GP epitope possess unique T-cell receptor β-gene rearrangements. Although all six individual CTL clones lysed targets infected with LCMV ARM and WE strains, both of which have identical GP sequences from amino acids 272 to 293, two of the six clones failed to lyse syngeneic target cells infected with the Pasteur strain (PAST) of LCMV. By sequence analysis, PAST differs from the other LCMV strains at only one amino acid in the region of amino acids 272 to 293 (8), that is, valine 278 in ARM and WE is threonine 278 in PAST.

After coating cells with peptides comprising amino acids 272 to 293 corresponding to either the ARM or PAST sequences, we found that one CTL clone that kills cells infected with LCMV ARM, but not PAST, will kill cells coated with either peptide. In contrast, a different CTL clone maintained discrimination, as it killed neither target cells infected by PAST nor coated with peptide originated from the PAST sequence. Hence, CTLs can recognize single-amino-acid substitutions, and the processing of antigen can be different for viral proteins synthesized by the cell compared with that for its corresponding peptide used to coat uninfected cells.

## MATERIALS AND METHODS

**Generation of CTL clones.** CTLs obtained from C57BL/6 (*H-2^b*) mice were cloned by limiting dilution as described previously (4). They were maintained for 32 months by weekly passages in the presence of irradiated LCMV ARM-infected syngeneic macrophages and spleen cells. The medium, changed three times a week, was RPMI 1640 supplemented with 12% fetal calf serum, 1% glutamine, antibiotics, and 5% T-cell growth factor (interleukin-2) obtained from Lewis rat lymphocytes treated with concanavalin A, HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) buffer, and β-mercaptoethanol (4). The first character(s) of each

* Corresponding author.

† Publication 5558-IMM of the Department of Immunology, Scripps Clinic and Research Foundation, La Jolla, Calif.

clone (2, 3, or RG) refers to the cloning experiment from which it was derived.

**CTL assay.** LCMV target cells were infected at a multiplicity of infection (MOI) of 1 48 h before standard $^{51}$Cr release assay was performed; MC57 fibroblast-macrophages were used for the *H-2*$^b$ background, and BALB clone 7 fibroblast cells were used for the *H-2*$^d$ background. Target cells were infected at a MOI of 3 with recombinant vaccinia virus (VV) 12 h before the assay. Construction and biochemical analysis of the recombinant virus are described elsewhere (29). Peptide coating of uninfected target cells was accomplished by incubating the cells with 40 or 4 μg of peptide (final concentration of 80 or 8 nmol/ml, respectively) during the 5-h incubation of the assay. After labeling and counting were done, all target cells were distributed in 96-well plates at a concentration of $2 \times 10^4$ cells per well. All samples were run in triplicate with a variance of less than 10% for $^{51}$Cr release data. The technical details for the CTL assay as to labeling, coating, analyzing, etc., have been described previously (29).

Effector cells were either bulk splenocyte CTLs or cloned CTLs. Primary LCMV-specific splenocytes were obtained from mice inoculated intraperitoneally 7 days earlier with $2 \times 10^5$ PFU of virus. These splenocytes were placed into single-cell suspensions and used at effector-to-target ratios of 50:1 and 25:1. CTL clones maintained under standard protocol (4) were collected the day of the assay. The effector-to-target ratios for CTL clones were 5:1 and 2.5:1.

Results shown were reproducible in three experiments or more. $^{51}$Cr release was calculated as: 100 × [(sample-released radioactivity − spontaneously released radioactivity)/(total released radioactivity − spontaneously released radioactivity)].

**Biochemical characterization of CTL recognition and specificity.** The mapping of the 9-amino-acid CTL immunodominant GP2 epitope was carried out by generating truncated proteins expressed in recombinant VV (29). In brief, several constructs were derived from a parental plasmid carrying the cDNA sequence coding for the first 363 amino acids of the GP of LCMV ARM. The entire GP molecule is 498 amino acids long and is cut posttranslationally to yield GP1 and GP2 subunits. Three truncated mutants were subsequently obtained by using unique restriction sites in the cDNA sequence, resulting in the expression of proteins truncated at amino acid positions 293, 271, and 218. Recombinant VV was obtained by homologous recombination in the VV TK gene, as previously described (19). Insertion of the desired sequence in the viral TK gene provided a TK-deficient phenotype selectable by bromodeoxyuridine. In addition, a β-galactosidase gene present in the plasmid vector allowed discrimination between recombinant VV and spontaneously occurring thymidine kinase (TK)-deficient mutants by a blue-plaque selection in X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside)–0.5% agar medium. Recombinant VV expressing the viral nucleoprotein (NP) were obtained by the same strategy (29, 30).

**Sequences for LCMV ARM, WE, and PAST.** The sequences for ARM, WF, and PAST were obtained by oligonucleotide primer extension by using viral RNA purified from LCMV virion particles as the template (E. M. Shimomaye and M. S. Salvato, Gene Anal. Tech., in press; M. S. Salvato, K. Schweighofer, and E. M. Shimomaye, manuscript in preparation).

**Peptide synthesis.** Peptide synthesis was conducted by the solid-phase method described by Merrifield (22) with an automated peptide synthesizer (model 430A; Applied Biosystems, Foster City, Calif.) and manually by the method of Houghten (14). Peptides were analyzed or subsequently purified by high-pressure liquid chromatography to ensure >85% purity.

**Molecular analysis of CTL receptor genes.** The β-subunit-specific probe was the 86T5 cDNA (9, 11). This 700-base-pair *Eco*RI fragment was kindly provided by N. Gascoigne (Research Institute of Scripps Clinic, La Jolla, Calif.). It contains the $C_{\beta 1}$ coding sequence, which represents greater than 80% of the used fragment, and since $C_{\beta 1}$ and $C_{\beta 2}$ are more than 90% homologous (20), the probe hybridized strongly with both sequences.

Northern (RNA) blotting was performed on RNA extracted from CTL clones by lysing $5 \times 10^6$ to $2 \times 10^7$ cells directly in 2.7 ml of GTC (5 M guanidium isothiocyanate, 0.1 M β-mercaptoethanol, 0.5% Sarkosyl) (6) and sedimenting the RNA through a 2-ml 5.7 M CsCl cushion, at 100,000 × *g* for 16 h at 14°C. RNA was resuspended in GTC, ethanol precipitated, and suspended in diethylpyrocarbonate-treated water. After the RNA concentration was determined by spectroscopy, 4 μg of each sample was run on a 1.5% agarose gel with morpholinepropanesulfonic acid (MOPS)-formaldehyde buffer (21). The gel was transferred in 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) onto a Hybond membrane (Amersham Corp., Arlington Heights, Ill.). Hybridization proceeded in a solution consisting of 5× SSC, 50% formamide, 2.5× Denhardt solution, 0.1 mg of salmon sperm DNA per ml, and 0.1% sodium dodecyl sulfate, with $10^6$ cpm of $^{32}$P-labeled 86T5 probe per ml. The labeling was done by random hexanucleotide priming (7), providing a specific activity of $10^9$ cpm/μg of DNA or greater. After washes, the filter was exposed for 48 h at −70°C with an intensifying screen.

Southern blots were performed on DNA extracted from CTL clones by using the same GTC preparation for collecting the RNA. The lowest milliliter of the CsCl cushion, found just above the RNA pellet, in which DNA was detectable as a very faint cloud, was collected, diluted to 5 ml with TE buffer (21), and ethanol precipitated. After two 70% ethanol rinses, DNA was suspended in TE, and 4-μg samples were digested overnight with the appropriate enzyme. The DNA was then loaded on 1% agarose gels with standard TBE buffer (21). After electrophoresis, the gels were treated for 10 min with 0.25 M HCl, rinsed twice in water, soaked for 10 min in 0.4 M NaOH, and transferred in 0.4 M NaOH for 4 h onto a Zetaprobe membrane (Bio-Rad Laboratories, Richmond, Calif.).

Prehybridization and hybridization were done in a solution consisting of 5× SSC, 50% formamide, 0.5% nonfat dry milk, 0.5% sodium dodecyl sulfate, and 0.1 mg of sheared salmon sperm DNA per ml, with $10^6$ cpm/ml of 86T5 probe labeled as described for the Northern blots. Filters were exposed for 72 h at −70°C with an intensifying screen.

## RESULTS

**Biological characterization of LCMV CTL clones. (i) Viral strain, viral protein, and peptide recognition profile.** Six CTL clones known to recognize an epitope comprising residues 278 to 286 (VENPGGYCL) on the LCMV ARM GP were used (23). CTL clones 232, 228, 3-9, 3-1, RG9, and 3-6 were assayed for their ability to recognize *H-2*$^b$ syngeneic targets infected with two other LCMV strains, WE and PAST. Two of the clones, 232 and 228, failed to lyse LCMV PAST-infected target cells but lysed LCMV WE target cells (Table 1). In contrast, CTL clones 3-9, 3-1, RG9, and 3-6 were able

TABLE 1. Recognition of viral proteins and LCMV strains by six LCMV ARM-specific clones[a]

| CTL | Mean[b] % specific $^{51}$Cr released in a 5-h CTL assay from: | | | | | | |
|---|---|---|---|---|---|---|---|
| | LCMV ARM infected with target | | $H$-$2^b$ target infected with: | | | | |
| | $H$-$2^b$ | $H$-$2^d$ | WE | PAST | VV expressing LCMV GP | VV expressing LCMV NP | VV |
| Clones | | | | | | | |
| 232 | 69 | 6 | 60 | <5 | 55 | <5 | <5 |
| 228 | 59 | 6 | 54 | <5 | 51 | <5 | <5 |
| 3-9 | 37 | <5 | 42 | 46 | 32 | <5 | <5 |
| 3-1 | 51 | <5 | 56 | 42 | 41 | <5 | <5 |
| RG9 | 68 | <5 | 54 | 50 | 60 | <5 | <5 |
| 3-6 | 27 | <5 | 26 | 23 | 43 | <5 | <5 |
| 21 | 42 | <5 | ND[c] | ND | <5 | 38 | <5 |
| Splenocytes (day 7) | | | | | | | |
| $H$-$2^b$ | 55 | 5 | 58 | 43 | 42 | 30 | <5 |
| $H$-$2^d$ | <5 | 56 | ND | ND | ND | ND | ND |

[a] LCMV CTL clones were generated as previously reported (29). Target cells (MC57 [$H$-$2^b$] and BALB/c17 [$H$-$2^d$]) were infected with either LCVM ARM, WE, or PAST at an MOI of 1 for 48 h or with VV expressing LCMV ARM GP, VV expressing LCMV ARM NP, or VV at an MOI of 5 for 12 h prior to labeling with $^{52}$Cr. CTL clones were used at effector-to-target cell ratios of 5 and 2.5:1, while splenic CTLs were employed at ratios of 50:1 and 25:1. Data shown are for ratios of 5:1 and 50:1, respectively. Samples were run in triplicate with a variance of less than 10% between experiments.

[b] Mean of data from one of several experiments with comparable results in each; variance between experiments was less than 20%.

[c] ND, Not determined.

to lyse both WE- and PAST-infected target cells. Other analyses (Table 1) using recombinant VV expressing either LCMV GP or LCMV NP confirmed that all six of these clones recognized LCMV GP but not NP. Target cells expressing LCMV NP could be lysed by a different CTL clone 21 known to recognize NP (29, 30) and bulk splenic CTLs that recognize both GP and NP (30).

The ARM GP domains recognized by CTL clones 232, 228, 3-9, 3-1, RG9, and 3-6 were evaluated. None of the six CTL clones recognized LCMV ARM GP amino acids 1 to 272, but all recognized residues 1 to 293 and 1 to 363 (Table 2). For direct confirmation that this indicated recognition of GP between residues 272 to 293, a peptide representing these residues was synthesized and used to coat uninfected syngeneic target cells. Only $H$-$2^b$ targets coated with the peptide were lysed by the six CTL clones (Table 2). Further, in dose-response studies, a 10-fold dilution of peptide made no difference in lysis, although the endpoint for lysis of 66% of target cells varied among individual clones (Table 2).

**(ii) Characterization of the mRNA for a β subunit of a T-cell receptor for the six selected anti-LCMV CTL clones.** MHC class I-restricted CTLs routinely express α/β-type T-cell receptors, whereas γ/δ-type receptors have been found only on CTLs for which no MHC restriction has been demonstrated (15). To confirm that the detected genomic rearrangements were functional for translation, we determined whether T-cell receptor β-subunit mRNA was present by Northern blotting. As expected, all clones expressed a 1.3-kilobase (kb) mRNA for the β subunit in roughly comparable amounts (Fig. 1). The additional 1.0-kb bands detected in clones RG9 and 3-6 are likely to be incomplete $D_\beta$-$J_\beta$-$C_\beta$ RNA lacking the $V_\beta$ region (17, 26).

**Individual CTL clones present unique patterns for the genomic rearrangements of the β-subunit gene.** The preferential use of certain V (variable) regions has been noted for several MHC class II-restricted epitopes like cytochrome *c* (10), haptens (12, 13), and myelin basic protein (1, 28). To learn whether a limited number of CTL receptors can recognize a precisely defined MHC class I-restricted viral epitope, we analyzed the restriction-digestion pattern of the rearranged β-subunit gene by Southern blotting. The probe 86T5 hybridized strongly to both $C_{\beta1}$ and $C_{\beta2}$ regions (see Materials and Methods). Each of the six CTL clones studied differed in restriction patterns following digestion with three restriction enzymes, indicating that the clones are clearly independent and most likely do not express identical T-cell receptor β-chain proteins (Fig. 2).

The *Hin*dIII digestion (Fig. 2, center gel) provides information on the $C_{\beta1}$ rearrangements. The original unmodified structure gave rise to two bands: a 7.6-kb *Hin*dIII fragment containing regions $D_{\beta1}$, $J_{\beta1}$, and $C_{\beta1}$, and therefore likely to be rearranged, and a 3-kb band including little more than the

TABLE 2. Recognition of amino acids 272 to 293 by six LCMV GP-specific CTL clones[a]

| CTL clones | Mean % speciifc $^{51}$Cr released in a 5-h CTL assay from: | | | | | | | | Ng/ml to cause 66% of maximal lysis of $H$-$2^b$ target coated with LCMV GP amino acids 272–293[c] |
|---|---|---|---|---|---|---|---|---|---|
| | $H$-$2^b$ target infected with VV-LCMV GP truncated gene expressing amino acid residues | | | | VV | $H$-$2^d$ target coated with 40 μg of LCMV GP amino acids 272–293 | $H$-$2^b$ target coated with LCMV GP amino acids 272–293 showing lysis[b] at: | | |
| | 1–218 | 1–272 | 1–293 | 1–363 | | | 40 μg | 4 μg | |
| 232 | <5 | <5 | 52 | 49 | <5 | <5 | 40 ± 4 | 36 ± 3 | <25 |
| 228 | <5 | 7 | 55 | 52 | 8 | <5 | 46 ± 6 | 41 ± 5 | 25 |
| 3-9 | 7 | 8 | 59 | 53 | 9 | <5 | 46 ± 1 | 42 ± 4 | 80 |
| 3-1 | <5 | 9 | 41 | 43 | <5 | <5 | 36 ± 5 | 30 ± 5 | 60 |
| RG9 | 12 | 15 | 38 | 29 | <5 | <5 | 60 ± 3 | 46 ± 6 | 640 |
| 3-6 | <5 | 6 | 43 | 45 | <5 | <5 | 25 ± 2 | 21 ± 5 | ND |

[a] See footnote *a* of Table 1 and Materials and Methods for protocols to generate CTL clones and assay CTL activity against LCMV ARM, VV, and VV-LCMV recombinant-infected targets. The $H$-$2^d$ target was BALB/c17, and the $H$-$2^b$ target was MC57 cells. CTL clones were used in effector-to-target cell ratios of 5:1 and 2.5:1; data given for 2.5:1. Samples were run in triplicate with a variance of 10% among samples.

[b] $H$-$2^b$ target was coated with peptide at a concentration of 40 or 4 μg/ml. Data display mean ± standard error from at least three separate experiments. Variance within a single experiment, done in triplicate, was <10%.

[c] Concentration of GP amino acids 272 to 293 peptide in nanograms per milliliter required for 66% lysis. Data was taken from a dose-response curve utilizing fivefold dilutions of peptide beginning at 40 μg/ml. ND, Not determined.

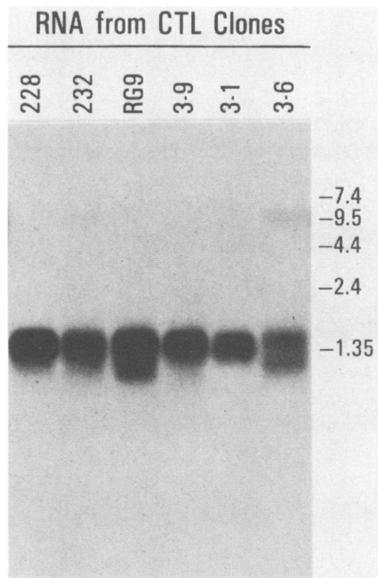


FIG. 1. Detection of T-cell receptor β-subunit mRNA by Northern blot analysis of RNA from cloned CTLs. Total RNA of CTL clones was harvested as described in Materials and Methods, and 4 μg of RNA was loaded on a 1.5% agarose gel in MOPS-formaldehyde buffer. RNA was transferred on a Hybond membrane and hybridized with a $^{32}$P-labeled β-subunit cDNA (86T5) probe. Molecular weights were determined with a 0.24- to 9.5-kb RNA ladder (Bethesda Research Laboratories, Inc., Gaithersburg, Md.).

$C_{\beta 2}$ region and therefore very unlikely to be modified after genomic rearrangements. As noted with the *Hin*dIII digest, clones 3-1 and RG9 showed no hybridization bands for the $C_{\beta 1}$ region, suggesting that these clones have deletions in both $C_{\beta 1}$ regions of their diploid genome during rearrangement and express a β2-type subunit for their receptor. Clones 3-1 and RG9 presented clearly different restriction patterns with the *Pvu*II enzyme (Fig. 2, left gel). Clone 3-1 has two bands, one at 6 kb and one at 5 kb. In contrast, clone RG9 contains only one 2.5-kb band. Clones 3-6, 232, and 228 exhibited different sizes of *Hin*dIII fragments for the $C_{\beta 1}$ gene (respectively 7, 9, and 5 kb). Although this last observation does not indicate that these clones express a β1-type subunit, it does indicate different recombination events that must lead to expression of different β-subunit molecules.

Clone 3-9 showed a unique pattern, especially with the *Bam*HI enzyme (Fig. 2, right gel), i.e., a 20-kb band was generated that none of the other five clones produced.

**Chemical characterization of sequence differences among different LCMV strains and their CTL recognition patterns.** The six LCMV ARM CTL clones have unique CTL receptors (Fig. 2). All six clones recognized LCMV ARM GP residues 272 to 293, as well as WE-infected syngeneic target cells (Table 1). Two clones, 232 and 228, failed to recognize $H\text{-}2^b$ targets infected with LCMV PAST, whereas the other four clones (clones 3-9, 3-1, RG9, and 3-6) did (Table 1). The amino acid sequence for GP 272 to 293 of both WE and PAST was obtained by oligonucleotide primer extension. Within this area, the deduced amino acid sequences from 272

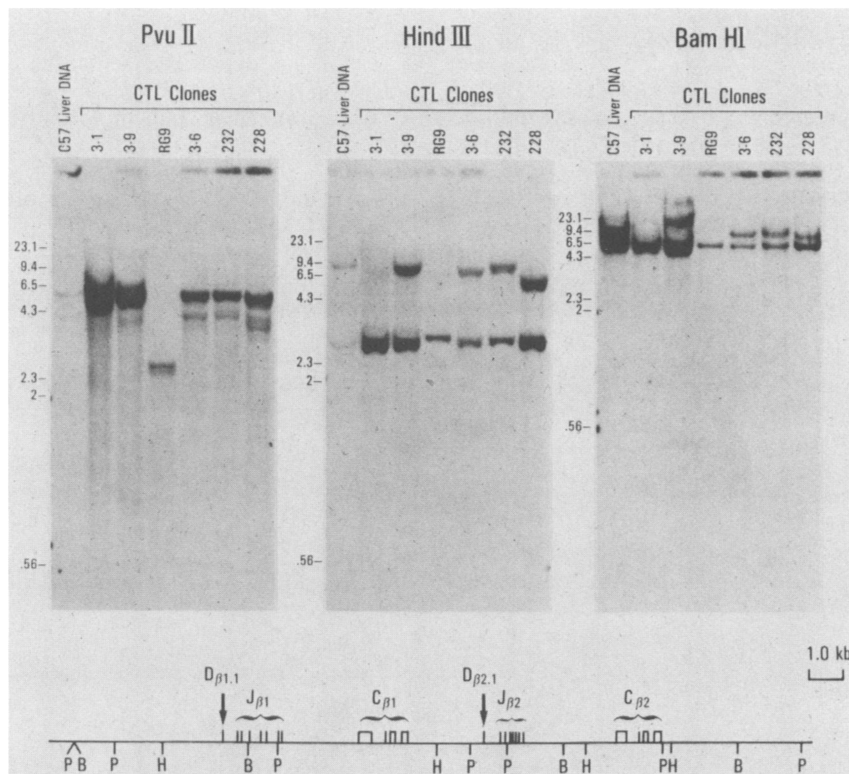


FIG. 2. Southern blot analysis of genomic rearrangement patterns of the T-cell receptor β-subunit gene. CTL DNA was collected from a CsCl centrifugation, as described in Materials and Methods. Samples (3 μg) were digested overnight with the appropriate enzyme and loaded on a 0.8 (*Pvu*II and *Hin*dIII) or 1% (*Bam*HI) agarose gel with TBE buffer. After migration, the gels were transferred to a Zetaprobe nylon membrane (Bio-Rad) and hybridized with $^{32}$P-labeled β-subunit cDNA 86T5 probe. Molecular weights were determined according to λ-*Hin*dIII marker. The restriction map was adapted from that of Kronenberg et al. (16). P, *Pvu*II; B, *Bam*HI; H, *Hin*dIII.

TABLE 3. Ability of GP peptides 272 to 293 of LCMV ARM and PAST to act as CTL recognition epitopes

| $H$-$2^b$-restricted CTL clones | Mean % $^{51}$Cr released from in a 5-h CTL assay from $H$-$2^b$ targets[a] | | | |
|---|---|---|---|---|
| | Infected with: | | Coated with GP amino acids 272–293 | |
| | ARM | PAST | ARM (V at amino acid 278) | PAST (T at amino acid 278) |
| 232 | 72 | 2 | 48 | 47 |
| 228 | 73 | <1 | 41 | 3 |
| 3-1 | 67 | 46 | 61 | 58 |
| RG9 | 60 | 37 | 57 | 55 |

[a] The 5-h $^{51}$Cr release assay used the CTL clones shown and $H$-$2^b$ (MC57) targets either infected with LCMV ARM or PAST (MOI = 1) or $H$-$2^b$ uninfected targets coated with LCMV ARM GP amino acids 272 to 293 or LCMV PAST GP amino acids 272 to 293 (200 μg/ml) (see Materials and Methods). Mean of triplicate samples. The variance among triplicates was less than 10%.

to 293 for three LCMV strains were found to be the following:

ARM LSDSSG<u>V</u>ENPGGYCLTKWMILA
PAST LSDSSG<u>T</u>ENPGGYCLTKWMILA
WE LSDSSG<u>V</u>ENPGGYCLTKWMILA

ARM and WE have identical sequences. In contrast, LCMV PAST displayed a single-amino-acid substitution (indicated by underlining) in position 278, where the valine (V) in ARM and WE is replaced by a threonine (T). We then synthesized two peptides corresponding to either amino acids 272 to 293 of ARM or PAST and used to coat $H$-$2^b$ uninfected targets. Once coated, these targets were tested against CTL clones. Clone 228 failed to lyse both LCMV PAST-infected targets and uninfected targets coated with PAST GP peptide residues 272 to 293 (Table 3). In contrast, clone 232, which was unable to lyse PAST-infected cells, nevertheless efficiently and effectively lysed uninfected $H$-$2^b$ targets coated with the corresponding PAST peptide. Analysis of two other CTL clones, 3-7 and 221, showed a similar dichotomy between lysis of PAST-infected targets and peptide-coated uninfected target cells (data not shown). CTL clones 3-1 and RG9 lysed both $H$-$2^b$ PAST-infected target cells and $H$-$2^b$ uninfected target cells coated with the relevant PAST peptide.

**CTL clones discriminate a single-amino-acid substitution.** The final experiments confirmed the observation that substitution of threonine for valine in GP amino acid position 278 segregated CTL clone 228 from clone 232. Recently, we reported experiments utilizing peptides with amino acid substitutions at position 278 to coat either $H$-$2^b$ (restricted) or $H$-$2^d$ (unrestricted) target cells (23). In current studies, we noted that CTL clone 228 maintained its inability to recognize the GP peptide when the original valine found in ARM at position 278 was replaced by a threonine, as in PAST GP (specific $^{51}$Cr release of V peptide, 41%; T substitution, 3%). Clone 228 also failed to recognize target cells coated with a peptide in which another polar group was substituted (for serine, $^{51}$Cr release, <1%). In contrast, CTL clone 232, which also failed to recognize $H$-$2^b$ targets infected with LCMV PAST, nevertheless recognized target cells coated with peptides in which either threonine (47%) or serine (42%) was substituted for valine ($^{51}$Cr release, 45%) in amino acid position 278. These results were upheld over peptide doses ranging from 40 to 1 μg/ml.

## DISCUSSION

In this report, we characterized six CTL clones that all recognize a particular epitope on LCMV-infected target cells yet are distinguishable in their T-cell receptor β-chain rearrangements and in their abilities to discriminate target cells coated with peptides containing single-amino-acid sequence variations from the major LCMV recognition epitope. Currently, we are isolating the α- and β-chain genes and proteins of these CTL clones with the anticipation of using such reagents in the study of their molecular association with MHC and viral epitopes.

CTL clones were obtained in separate experiments and cloned three times. They recognized an immunodominant domain of GP amino acids 272 to 293 with a minimal epitope of GP amino acids 278 to 286 (23). It was desirable, however, to obtain stringent chemical evidence that they were truly independent CTL clones. This was accomplished by investigating the enzyme restriction patterns of the region coding the β subunit of the T-cell receptor. Each of the clones demonstrated a unique profile defined by the three restriction enzymes used. Such results clearly proved the independent nature of each clone and suggested that the clones express different T-cell receptor β chains. Although heterogeneity of anti-LCMV, class I-restricted ($H$-$2D^b$) T-cell clones had been noted in one laboratory, the epitope specificities of the seven T-cell clones reported (24) were not defined, and hence the described heterogeneity may well have represented a variety of different viral epitope specificities. Thus, we show compelling evidence for the heterogeneity of clones that recognize the same 9-amino-acid minimal epitope indicating that diversity of CTL responses, like antibody responses, reflects a unique array of clones able to discriminate between single-amino-acid substitutions. Furthermore, biological characterization of CTL clones, using different viral strains and peptides, shows that it is possible to discriminate between clones by their cytotoxicity specificities and that viral strain-specific clones can be generated.

Armed with the results showing that these different CTL clones recognize the same LCMV ARM epitope, we can now address two important issues. First, as CTL clearly discriminates between a single-amino-acid difference in epitopes and this discrimination is likely reflected at the CTL recognition level, it should be possible to selectively generate viral mutants for CTL recognition. The mutations will likely affect the CTL recognition and lysis by modifying the way the viral peptide is presented by the MHC, either by reducing the avidity of the peptide for the MHC groove or by affecting the processing of the protein so that different peptides would be generated. Generation of mutants, especially in an immunodominant domain, may lead to the selection of a viral variant that can alter the expected pathogenicity in a host. Our experiments provide a framework for testing this hypothesis. Others have reported that a single-amino-acid substitution in an influenza virus NP epitope can alter the avidity of CTL binding (3). The development of such CTL-escaping variants must proceed in the context of a restricted MHC background, such as in inbred laboratory mice used to grow virus strains. In fact, different regions of viral proteins correspond to CTL epitopes depending on the MHC context of the infected animal. Hence, we have found for LCMV that the major CTL epitope for $H$-$2^b$ (C57BL/6 mice) consists of GP amino acids 272 to 293 (23, 29, 30), while for $H$-$2^d$ (BALB mice), the immunodominant epitope consists of NP amino acids 119

to 127 (unpublished observations; J. L. Whitton and M. B. A. Oldstone, manuscript submitted for publication). Corresponding results have been noted with influenza virus epitopes and various *H-2*-restricting molecules (3). Hence, MHC heterogeneity or polymorphism of mammalian populations can be a defense against the ability of viruses to generate mutants that escape CTL recognition.

A second point to emerge from this report is that processing or presentation of peptides used to coat uninfected cells may, in some instances, vary from the natural epitope presentation in a viral infection. For example, we demonstrated that a CTL clone directed against LCMV ARM GP 278 to 286 failed to lyse target cells infected with LCMV PAST but could lyse target cells coated with a PAST GP peptide to amino acids 278 to 286. At least, these findings suggest caution in interpreting experiments using only peptides as a probe to dissect viral epitopes. Further, the findings offer evidence that, in some instances, antigen processing may differ between viral peptide and whole-virus proteins.

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