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Immunology

In vitro selection of lymphocytic choriomeningitis virus escape mutants by cytotoxic T lymphocytes

(virus selection/virus mutants/T-cell epitope)

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ABSTRACT **Cytotoxic T lymphocyte (CTL)-mediated cytolysis is induced via the interaction of the specific T-cell antigen receptor and the peptidic viral antigen associated with the major histocompatibility complex class I antigen. Here we demonstrate *in vitro* that lymphocytic choriomeningitis virus (LCMV) can escape the cytotoxic activity of LCMV-specific cloned CTLs by single amino acid changes within the recognized T-cell epitope defined by residues 275–289 of the LCMV glycoprotein [LCMV-GP-(275–289)]. LCMV-infected fibroblasts at a multiplicity of infection of 10^{-3} exposed to virus-specific CTL at an effector-to-target cell ratio of 4:1 4 hr after infection was optimal for virus mutant selection. The selections were carried out with three LCMV-GP-(275–289)-specific CTL clones expressing T-cell antigen receptors containing the identical variable gene segments $V_\alpha 4$ and $V_\beta 10$ but different junctional regions; selection was also possible with LCMV-GP-(275–289)-specific cytotoxic polyclonal T cells. The most common escape mutation was an amino acid change of asparagine (AAT) to aspartic acid (GAT) at position 280; an additional mutation was glycine (GGT) to aspartic acid (GAT) at position 282. The results presented show that relevant point mutations within the T-cell epitope of LCMV-GP-(275–289) occur frequently and that they are selectable *in vitro* by CTLs.**

Viruses may escape immune surveillance by altering antigenic determinants recognized by neutralizing antibodies. This has been documented by the classical studies on influenza virus epidemiology; selected virus determinants may shift and drift antigenically to escape neutralizing antibody-mediated immunity. Also, neutralizing monoclonal antibodies (mAbs) against rabies, influenza viruses, and other viruses have been shown to rapidly select escape mutants *in vitro* (1–5).

Recently evidence that viruses may also escape immune surveillance by mutating a crucial T-cell epitope has been found *in vivo* in T-cell antigen receptor (TCR) transgenic mice acutely infected with lymphocytic choriomeningitis virus (LCMV) (6). Many unpublished attempts have been made over the past several years to select cytotoxic T-cell escape mutant viruses *in vitro* without success by using cytotoxic T-cell clones. Several observations suggest that such an escape from cytotoxic T-cell surveillance by the loss of the relevant T-cell epitope might exist (7–9). The rapid isolation of mutant viruses using T cells might prove useful to study immunodominance and flexibility of defined T-cell epitopes or to analyze TCR repertoires for particular epitopes. Therefore a method for the isolation of mutant viruses by using cytotoxic T cells *in vitro* would be highly desirable.

The present study shows that selection of virus variants *in vitro* by cytotoxic T lymphocytes (CTLs) is readily feasible. LCMV epitope-specific CTLs, either cloned or polyclonal, were used successfully in a mixed culture with LCMV-infected target cells to select mutant virus; the efficiency of selection was comparable to that reported with neutralizing antibodies. Isolated mutant viruses show point mutations in the relevant T-cell epitopes and were no longer recognized by the CTLs present during the selection.

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MATERIALS AND METHODS

Animals and Virus Strains. C57BL/6 mice were purchased from the Institut für Zuchthygiene (Abteilung Labortierkunde, Tierspital Zürich, Switzerland). The WE strain of LCMV (LCMV-WE) had been originally obtained from F. Lehmann-Grube (Hamburg, F.R.G.). Stock virus was grown on L929 cells from a triple-plaque-purified virus (10).

Cells and Culture Conditions. Cells used as target cells were MC57G (H-2^b), D2 (H-2^d), and simian virus 40 (SV40) transformed C57BL/6 (H-2^b) (referred to as B6-SV40) primary fibroblasts. They were cultured in Eagle's minimal essential medium containing 5% fetal calf serum.

The generation, maintenance, and specificity of the CTL clones 50.1, B23.35, B12, and B4, which are specific for the T-cell epitope defined by residues 275–289 of the LCMV glycoprotein [LCMV-GP-(275–289)], have been described in detail elsewhere (11, 12). The H-$2D^b$-restricted CTL clone 3D5, which is specific for the T-cell epitope defined by residues 32–42 of the LCMV glycoprotein [LCMV-GP-(32–42)], was kindly provided by D. Brändle (University of Zürich). This clone was established from a C57BL/6 mouse carrying the TCR β-chain transgene of the LCMV-GP-(32–42)-specific CTL clone P14 (13, 14).

Primary immune spleen cells were prepared as single-cell suspensions from animals infected i.v. with 200 plaque-forming units (pfu) of LCMV-WE 8 days before testing. LCMV-GP-(275–289)-specific polyclonal CTLs were obtained by restimulating LCMV-immune spleen cells with LCMV-GP-(275–289) peptide- (Neolab, Strasbourg, France) coated stimulator spleen cells or macrophages (6).

Virus Selection and Purification. B6-SV40 primary fibroblasts were seeded at 3×10^5 cells per well into 6-well tissue culture plates (Costar) and incubated overnight at 37°C. Cell monolayers were infected with LCMV-WE at varying multiplicities of infection (moi). Virus was allowed to absorb for 1 h at 37°C. Cells were then washed three times with 2 ml of medium to remove nonadherent virus. Finally cells were

Abbreviations: moi, multiplicity of infection; CTL, cytotoxic T lymphocyte; pfu, plaque-forming unit(s); LCMV, lymphocytic choriomeningitis virus; TCR, T-cell antigen receptor; SV40, simian virus 40; mAb, monoclonal antibody; V, variable; J, junctional; C, constant; LCMV-GP-(275–289), residues 275–289 of the LCMV glycoprotein.

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overlaid with 3 ml of Eagle's minimal essential medium containing 5% fetal calf serum. CTLs (2.4×10^6) were added 1 or 4 h later unless otherwise indicated (see Table 1). Supernatants containing virus were collected after 48 h, titrated on L929 cells as described (15), and tested in a standard cytotoxicity assay. Virus isolates were cloned twice by plaque purification as described (16). On occasions where the cytotoxicity of the selector CTLs toward targets infected with the selected virus was reduced by only 50%, a second selection cycle was carried out. Titrated virus supernatant after the first selection cycle was used, and the selection with cloned CTLs or LCMV-GP-(275–289)-specific splenocytes was repeated.

Cytotoxicity Assay. Target cells were infected with virus (moi = 0.1) for 40 h. The lytic activity of the different effector cells was determined in a standard ^{51}Cr-release assay for 4–5 h as described in detail elsewhere (12).

Sequencing of the Relevant CTL Epitopes of the LCMV Isolates. LCMV was purified from infected L929 cells (16), and viral RNA was extracted as described elsewhere (17). Viral RNA (1 μg) was used as a template for cDNA synthesis with reverse transcriptase (Moloney murine leukemia virus; Pharmacia) and a specific primer (5′-TCGTAGCATGTCA-CAGAATTCTTC-3′). One-fiftieth of the cDNA mixture was amplified by PCR using *Taq* polymerase (Boehringer Mannheim) with a second specific primer (5′-GAATTCTATC-CAGTAAAAGGA-3′) (18). PCR was run for 42 cycles using a thermal cycler (Perkin–Elmer/Cetus). Denaturation was for 1 min at 94°C, annealing was for 1.5 min at 42°C, and extension was for 3 min at 72°C. The amplified 980-base-pair fragment was digested with *Eco*RI, and the resulting 473- and 487-base-pair fragments were cloned into pUC18. Colonies were sequenced in both directions by the dideoxynucleotide chain-termination method using Sequenase (United States Biochemical). The DNA comparison and homology search was done with the PC program DNA* (DNA*, Madison, WI). The nucleotide sequence for the glycoprotein is given as the viral-sense DNA (18).

mAbs. Hybridoma supernatants of 141-51.2 (anti-H-2D^b) (19) and B8-24-3 (anti-H-2K^b) (20) were used at a 1:4 dilution.

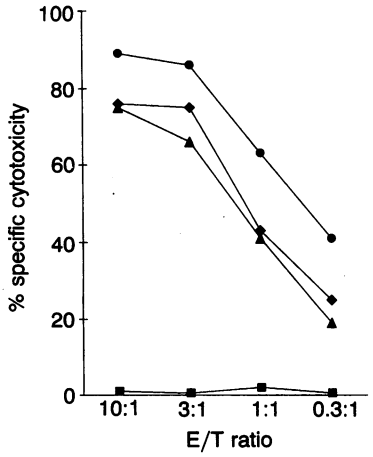

FIG. 1. Cytotoxic activity of LCMV-specific splenocytes and representative T-cell clones against wild-type (WE) and *in vitro*-selected LCMV. CTL clones 50.1 (■) and 3D5 (▲) and LCMV-WE day 8 immune splenocytes (●) were tested on MC57G target cells infected with titrated but not cloned virus from the supernatant after the selection with clone 50.1. Cytotoxic activity of clone 50.1 was also tested on MC57G target cells infected with the original parental LCMV-WE strain (◆). Effector-to-target cell (E/T) ratios for LCMV-WE immune spleen cells were 70:1, 20:1, 7:1, and 2:1, respectively. Spontaneous release was always <23% with infected and <2% with uninfected MC57G target cells.

PCR and DNA Sequencing of TCR Variable (V) Chains $V_\alpha 4$ and $V_\beta 10$. Total RNA from cloned CTL B23.35 and B12 cells was extracted by the guanidium/cesium chloride method. RNA (20 μg) was then used as templates for cDNA synthesis with reverse transcriptase (Moloney murine leukemia virus) using the manufacturer's conditions and an oligo(dT) (12- to 18-mer) primer. One-third of each cDNA mixture was amplified by PCR using *Taq* DNA polymerase in 100 μl of standard buffer and one of the following primer pairs: 5′-CAGTATCCCGGGGAAGGTCCACAGTTC (with a *Pst* I restriction site) for $V_\alpha 4$ plus 5′-CGAGGATCCTTTAACTG-GTACACAGCAGG (*Bam*HI) for constant region (C) C_α and 5′-GAATCTGCAGATCAAGTCTGTAGAGCCGG-3′ (*Pst* I) for $V_\beta 10$ or plus 5′-GTTTGTTTGCGAGCTCTGCTTTT-GATGGCTC-3′ (*Sac* I) for C_β, respectively. PCR was run for 30 cycles using a thermal cycler. Denaturation was performed for 1 min at 95°C, annealing was performed for 1 min at 48°C, and extension was performed for 3 min at 72°C. The amplified α-chain fragment and the β-chain fragments were digested with *Pst* I plus *Bam*HI and *Pst* I plus *Sac* I, respectively, and the resulting fragments were cloned into pUC18. Positive colonies were sequenced by the dideoxynucleotide method.

RESULTS

Parameters Influencing the *in Vitro* Selection of LCMV Variants by CTLs. Cloned LCMV-GP-(275–289)-specific H-2D^b-restricted CTLs (11, 12) were used with LCMV-infected B6-SV40 fibroblasts in a mixed culture system to select virus variants. These cloned CTLs are highly cytotoxic at an effector-to-target cell ratio of >3:1 as shown for a representative clone, 50.1, in Fig. 1. To select virus variants, CTL clone 50.1 was added at an effector-to-target cell ratio of 4:1 to B6-SV40 fibroblasts infected at an moi of 1 and 10^{-3}, respectively, to ensure a high selective pressure. The results summarized in Fig. 1 show that variant virus that was no longer recognized by CTL clone 50.1 seemed to predominate in the supernatant of these cultures. Clone 3D5 with a specificity for another T-cell epitope on the LCMV glycoprotein (amino acid residues 32–42) (14) was still able to efficiently lyse virus variant-infected target cells (Figs. 1 and 2).

It is possible that mutant virus could be present at a low frequency in the initial inoculum despite the fact it had been triple plaque purified before the selection procedure (16) or that these could arise during the replication cycle in the infected host cell. To evaluate these two possibilities and to select such variants, the moi and the time between the

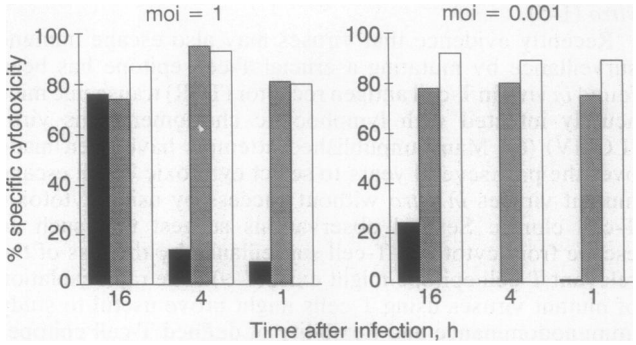

FIG. 2. Dependence of the *in vitro* virus variant selection upon time and moi of the virus. Mutant selection by CTL clone 50.1 was started at an effector-to-target cell ratio of 4:1 16, 4, and 1 h after LCMV infection of B6-SV40 fibroblasts at moi values of 1 and 10^{-3}. MC57G target cells were infected with titrated supernatant of CTL-selected virus at an moi of 0.1 for 40 h. Values indicated represent the percent specific cytotoxicity at an effector-to-target cell ratio of 10:1 for clone 50.1 (■) and 3D5 (▨) and of 70:1 for LCMV-WE immune primary B6 splenocytes (□) in a 5-h assay.

infection and the addition of CTLs were varied. Supernatants of cultures where CTLs had been added either immediately after infection or 4 h later contained virtually exclusively variant virus (Fig. 2). When CTLs were added 16 h after the infection, leaving time for a complete first replication cycle of the virus (12–14 h), selection was less complete when cells were infected at a moi of 10^{-3} and was ineffective at an moi of 1. The degree of cell-mediated lysis of target cells infected with identical moi of virus from supernatants after selection indirectly represents the efficiency of virus variant selection (solid bars in Fig. 2).

The changes in antigenicity of the variant virus were T-cell epitope specific. Target cells infected with mutant virus were still recognized by 3D5, a LCMV-GP-(32–42)-specific CTL clone as well as by polyclonal (and polyspecific) primary anti-LCMV immune spleen cells. Interestingly, the efficient selection seen at an moi of 10^{-3} was not found at very low moi values, when fibroblasts were infected at an moi of 10^{-5} (data not shown).

The importance of a sufficiently strong selective pressure is documented in Fig. 3, where the number of CTLs added to the infected fibroblasts was titrated. Selection of variant virus did not occur at an effector-to-target cell ratio of <0.7:1. This corresponded to a cytolytic activity of the CTLs below 50% in a standard ^{51}Cr-release assay (Fig. 1). It has to be noted, on the other hand, that selection was not prevented by adding a 20-fold excess of CTLs. From these results, it was concluded that under the experimental conditions used addition of CTLs at a 4:1 excess over infected fibroblasts, 4 h after infection with LCMV at an moi of 10^{-3}, appeared to be optimal for virus variant selection. Therefore the following experiments were performed using these standard parameters.

Selection of LCMV Variants *in Vitro* by Other CTLs and on Other Target Cells. When the LCMV-GP-(275–289)-specific CTL clones B23.35 or B12 were used to select variants, the results were identical to the selection with 50.1 (Fig. 4). However, two cycles of selection were necessary for the complete loss of the parental LCMV-WE phenotype in virus from the supernatant of cultures when selecting with CTL clone B23.35 (data not shown). Again the other CTL epitope monitored was not changed (Fig. 4). Not only were the selected variants no longer recognized by the CTLs present during the selection (data not shown), but in addition they were not recognized by other CTL clones with the same fine specificity (Fig. 4). Although these CTL clones express all $V_\alpha 4$ and $V_\beta 10$ TCR variable regions (21), they were heterogeneous in the primary sequence of their TCR junctional (J) regions as determined by sequencing (Fig. 5). Whereas clone

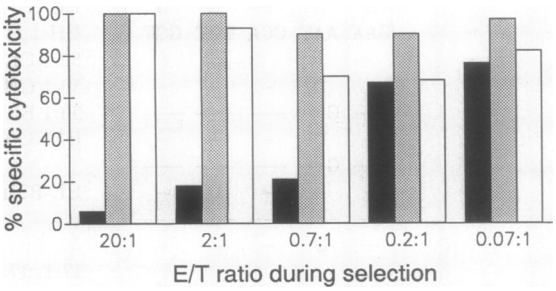

FIG. 3. The efficiency of the LCMV-WE variant selection is dependent on the ratio of the number of effectors to the number of target cells. B6-SV40 fibroblasts were infected with LCMV-WE at an moi of 10^{-3} 4 h before CTL clone 50.1 was added at the indicated ratios. Titrated virus in the supernatants was used to infect MC57G cells as described in Fig. 2. Values indicated represent the percent specific cytotoxicity at an effector-to-target cell (E/T) ratio of 10:1 for clones 50.1 (■) and 3D5 (▩) and of 70:1 for LCMV-WE immune primary B6 splenocytes. Spontaneous release was <26%.

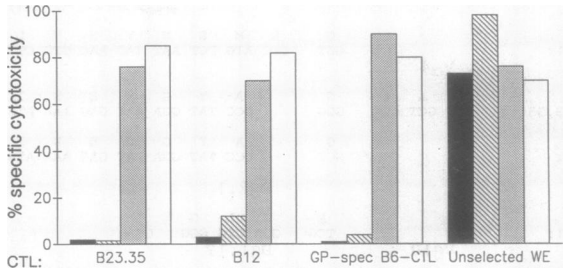

FIG. 4. Analysis of LCMV-WE variants selected by different CTL populations. Virus variants were selected by clones B23.35 and B12 and for cloned LCMV-GP-(275–289) peptide-specific CTLs (GP-spec B6 CTLs) as described in Fig. 3. The specific cytotoxicity against the titrated virus variant supernatants was measured analogously to Fig. 3 with virus-infected MC57G at an effector-to-target cell ratio of 10:1 for CTL clones 50.1 (■), B23.35 (▩), and 3D5 (■) and of 70:1 with LCMV-WE immune primary B6 splenocytes (□). Normal LCMV-WE-infected target cells (unselected WE) served as positive controls for CTL activity. Spontaneous release was <23%, and lysis of uninfected cells was <8%.

50.1 has rearranged $V_\alpha 4$ to J_αAN14.4 and $V_\beta 10$ to $D_\beta 2.1$–$J_\beta 2.5$, clones B23.35 and B12 both express $V_\alpha 4$–J_αTA31–C_α TCR α chains and show conserved amino acids in the N region of the D_β–J_β junction (Fig. 5). Even when polyclonal but monospecific CTLs of C57BL/6 origin were used for selection [established by restimulating immune spleen cells once *in vitro* with LCMV-GP-(275–289) peptide-coated stimulators], virus variants could easily be detected after two selection cycles (Fig. 4).

Attempts to select virus variants with an altered LCMV-GP-(32–42) T-cell epitope have failed so far with two cytotoxic T-cell clones or lines available.

Thus, virus variant selection *in vitro* is feasible. The efficiency of the selection might be higher or lower depending upon the CTL clone used.

To formally prove that selection depended upon cognate interaction of the CTL and its TCR with the infected target cell and the relevant major histocompatibility complex–antigen complex, selection was carried out as follows. Target cells of H-2^d haplotype (D2) were infected with LCMV-WE stock virus at an moi of 10^{-3}. No variant virus could be detected in supernatants when the LCMV-GP-(275–289)-specific H-2D^b-restricted CTL clone 50.1 was added to these cells. In contrast, when MC57G cells (H-2^b) were infected, variants could be selected with the same efficiency as with the routinely used B6-SV40 fibroblasts. In a second set of experiments, mAbs specific for H-2D^b or H-2K^b were added to the cultures. Virus from the supernatant of infected fibroblasts selected with CTL clone 50.1 in the presence of an H-2D^b-specific mAb (141-51.2) was still recognized by 50.1, whereas virus selected in the presence of an H-2K^b-specific mAb (B8-24-3) was not (data not shown).

Variants Show a Point Mutation Leading to an Amino Acid Substitution in the Relevant CTL Epitope. To determine the amino acid sequence of the relevant CTL epitopes from the phenotypically mutant viruses, variants were cloned by double plaque purifications and tested in a ^{51}Cr-release assay. Finally viral RNA was prepared, and the cDNA corresponding to the relevant CTL epitope region was sequenced. The results are summarized in Table 1. Nine independently selected variant virus isolates were subcloned and retested for their phenotypes. At least two subcloned isolates of each original plaque-purified variant were sequenced in both directions. All of the LCMV-GP-(275–289) variants examined except one were of identical genotype despite the fact that they had been selected in separate experiments and by different CTL populations (Table 1).

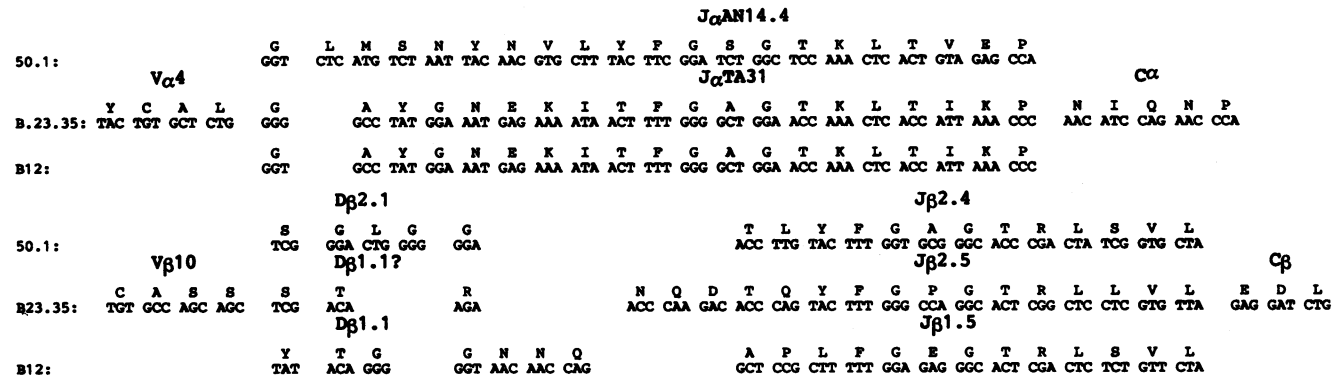

FIG. 5. Nucleotide and deduced amino acid sequences of TCR α- and β-chain junctional regions of the LCMV-GP-(275–289)-specific H-2D^b-restricted CTL clones 50.1 (21), B23.35, and B12.

A point mutation from adenosine to guanosine leading to an amino acid substitution at position 280 of the LCMV glycoprotein, asparagine replaced by aspartic acid [variant LCMV-WE(280D)], was the common virus variant genotype. This was detected in virus isolates after selection with CTL clones 50.1 and B23.35 or LCMV-GP-(275–289)-specific splenocytes. These variants exhibited no mutation in the LCMV-GP-(32–42) epitope (Fig. 4 and data not shown).

This uniform pattern of sequences in the LCMV-GP-(275–289) variants could be explained by selection of a preexisting mutant present at a very low frequency in the stock virus. To control for this possibility, selection was repeated with a freshly triple-plaque-purified isolate, which also served as a control virus exhibiting the correct wild-type LCMV-GP-(275–289) sequence (see above) EL 1.1 (Table 1). When B6-SV40 fibroblasts were infected with LCMV-WE at an moi of 10^{-3} and B23.35 was added in a first selection cycle and 50.1 CTL was added in a second selection cycle at the standard ratio of 4:1, mutant virus EL-B23(3.2) was detected in the supernatant (Table 1). In contrast to the virus selected out of the original LCMV-WE stock, a variant plaque-purified isolate showed a point mutation leading to a substitution of aspartic acid for glycine at position 282 [variant LCMV-WE(282D); Table 1].

Control virus passaged on B6-SV40 fibroblasts and plaque purified was phenotypically and genotypically of parental LCMV-WE type (Fig. 4). The selection procedure was not only applied to our regularly used LCMV-WE virus stock, but virus variants were also successfully isolated from a distinct subclone of LCMV-WE and from LCMV strain Armstrong (data not shown).

DISCUSSION

CTLs are capable of selecting mutant virus *in vitro* with great efficiency, comparable to that reported for neutralizing mAbs used in other systems (1–5). The mechanism by which the virus escaped CTL attack seems to be identical *in vitro* and *in vivo* (6). Point mutations within the epitope recognized by specific CTLs led to amino acid substitution and consequently to a failure of recognition (22) by specific CTLs.

A prerequisite for the selection of virus variants *in vitro* was the availability of monospecific, highly cytotoxic T lymphocytes. However, cloned CTLs were not absolutely necessary; more important was the specific cytolytic activity of the CTLs, which had to be equivalent to 50% specific lysis in a standard ^{51}Cr-release assay.

The failure of selection of LCMV-WE variants expressing an altered LCMV-GP-(32–42) T-cell epitope by using CTLs specific for this epitope may reflect a lower frequency of mutations within this epitope of a biologically very distinct region. This frequency of biologically relevant mutations is possibly lower than that estimated from the mistake rate of the viral RNA polymerase (23–26). *In vivo* selection against

Table 1. Phenotypic and genotypic analysis of plaque-purified LCMV variants

Selection*	Time†, h	moi‡	Cytotoxicity§ 3D5	Cytotoxicity§ 50.1	Nucleotide and glycoprotein sequences (Glu-279 to Gly-238)¶	Virus isolate
Not selected	—	10^{-3}	+	+	E N P G G	EL 1.1
Not selected	—	1	+	+	GAA AAT CCA GGT GGT	EH 1.1
50.1	1	10^{-3}	+	−		50.1 CL
50.1	1	1	+	−	– D – – –	50.1 CH
50.1	4	10^{-3}	+	−	—— G— —— —— ——	50.1 BL
B23.35‖	1	10^{-3}	+	−	– D – – –	B23.35 CL
					—— G— —— —— ——	
50.1/B23.35**	1	10^{-3}	+	−	– – – D –	EL-B23(3.2)
					—— —— —— -A- ——	
					– D – – –	
LCMV-GP-(275–289)-specific splenocytes‖	4	10^{-3}	+	−		17.1, 17.2
LCMV-GP-(275–289)-specific splenocytes‖	4	10^{-3}	+	−	—— G— —— —— ——	19.1, 19.2

*CTLs used for *in vitro* selection of virus variants at an effector-to-target cell ratio of 4:1.
†Time after virus infection, at which the selecting CTLs were added.
‡Infectivity of LCMV-WE used to infect B6-SV40 fibroblasts.
§Cytotoxicity of clones 3D5 and 50.1 against MC57G infected with double-plaque-purified virus isolates after the selection at the indicated conditions: at an effector-to-target cell ratio of 10:1, >60% killed is indicated by a "+" and <5% killed is indicated by a "−".
¶At least two independent cDNA clones were sequenced in both directions per virus isolate.
‖The selection was carried out in two cycles as described in *Materials and Methods*.
**Two selection cycles—first with clone 50.1, then with clone B23.35—were performed.

the same epitope in transgenic mice may have been considerably more efficient because replication of virus occurs over several orders of magnitude. The highest virus dose used to infect target cells *in vitro* was 6×10^5 pfu (moi = 1); in contrast, mice infected with 10^6 pfu are actually infected with 10^7 infectious units, which probably go through at least one round of replication before CTLs attack. If the frequency of biologically relevant mutations within the epitope is $<10^{-6}$, we would have simply missed this event *in vitro*, whereas it may have occurred *in vivo*. The biological effect of the mutations detected among the escape variants in the transgenic mouse model has been shown to be limited to LCMV-GP-(32–42)-specific T cells expressing the transgenic TCR. The mutated glycoprotein epitope mimicked by synthetic peptides could still be recognized by polyclonal LCMV-GP-(32–42)-specific CTL from a normal C57BL/6 mouse (6). In contrast, the LCMV-WE(280D) variant reported here was not recognized by CTL clones with identical fine specificity but expressing different joining regions in their TCR, the TCR region thought to interact intimately with the antigen presented on the major histocompatibility complex molecule (27). Variability in this part of the TCR molecule has been shown to counterbalance variation in the epitope sequence (28–32). In addition this virus variant could be selected by polyclonal LCMV-GP-(275–289)-specific CTLs. In other words, the LCMV-WE(280D) mutation seemed to drastically affect the antigenicity of the LCMV-GP-(275–289) epitope and possibly also its immunogenicity.

Whether the variants selected *in vitro* actually arose during the selective process in a directed manner under the driving force of the CTLs or were present in a surprisingly high but undetected frequency among the initial inoculum cannot be answered absolutely. The following considerations may apply. First, a very high excess in the number of CTLs over infectable target cells did not prevent selection. In this situation, virus-infected cells should presumably be eliminated before any virus progeny would emerge. Second, no virus variants could be selected when target cells were infected at a very limiting moi. However, a mutated virus could be selected from a freshly plaque-purified and sequenced control LCMV-WE isolate using an moi of 10^{-3}.

It may, however, be safely stated that among the 6×10^2 pfu applied at least 1 mutant virus (i.e., 1 pfu) should have been present; therefore, one might have expected to find such frequent mutants more easily. Replication of 1 virus results in at least 1000 progeny virus. Since one replication cycle may occur in one selection round, true frequencies of relevant mutations cannot be defined in this system.

We therefore should like to argue that the more likely sequence of events is that the mutant virus arose during the first round after infection of the target cells in the selection assay or was contained in the virus stock, unless all experiments can be done with the same virus particle. From a practical point of view, the results indicate that the relevant mutations occur frequently and that they are selectable *in vitro* by CTLs. As suggested by our experience, this may not always happen in a single round of selection. The described methods should open up experimental analysis of T-cell epitopes and TCR specificities by using specific CTL-directed mutation selection of biologically relevant CTL epitopes in a nonrandom fashion.

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