In Vivo Treatment with a MHC Class I-Restricted Blocking Peptide Can Prevent Virus-Induced Autoimmune Diabetes

Matthias G. von Herrath, Bryan Coon, Hanna Lewicki, Honore Mazarguil, Jean Edouard Gairin and Michael B. A. Oldstone

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In Vivo Treatment with a MHC Class I-Restricted Blocking Peptide Can Prevent Virus-Induced Autoimmune Diabetes


We tested the in vivo potential of a MHC class I-restricted blocking peptide to sufficiently lower an anti-viral CTL response for preventing virus-induced CTL-mediated autoimmune diabetes (insulin-dependent diabetes mellitus (IDDM)) in vivo without affecting systemic viral clearance. By designing and screening several peptides with high binding affinities to MHC class I H-2D\(^b\) for best efficiency in blocking killing of target cells by lymphocytic choriomeningitis virus (LCMV) and other viral CTL, we identified the peptide for this study. In vitro, it selectively lowered CTL killing restricted to the D\(^b\) allele, which correlated directly with the affinity of the respective epitopes. Expression of the blocking peptide in the target cell lowered recognition of all DG- restricted LCMV epitopes. In addition, in vitro expansion of LCMV memory CTL was prevented, resulting in decreased IFN-\(\gamma\) secretion. In vivo, a 2-wk treatment with this peptide lowered the LCMV DG-restricted CTL response by over threefold without affecting viral clearance. However, the CTL reduction by the peptide treatment was sufficient to prevent LCMV-induced IDDM in rat insulin promoter-LCMV-glycoprotein transgenic mice. Following LCMV infection, these mice develop IDDM, which depends on DG-restricted anti-self (viral) CTL. Precursor numbers of splenic LCMV-CTL in peptide-treated mice were reduced, but their cytokine profile was not altered, indicating that the peptide did not induce regulatory cells. Further, non-LCMV-CTL recognizing the blocking peptide secreted IFN-\(\gamma\) and did not protect from IDDM. This study demonstrates that in vivo treatment with a MHC class I blocking peptide can prevent autoimmune disease by directly affecting expansion of autoreactive CTL. The Journal of Immunology, 1998, 161: 5087–5096.

Abbreviations used in this paper: IDDM, insulin dependent diabetes mellitus; aa, amino acid; ARM, Armstrong strain; GP, glycoprotein; IC\(_{50}\), 50% inhibiting concentration; LCMV, lymphocytic choriomeningitis virus; MOLT, multiplicity of infection; NP, nucleoprotein, pfu, plaque-forming unit; RIP, rat insulin promoter; SOM, SMILEYME peptide; tg, transgenic; vv, vaccinia virus.
276–286 (GP2) and a minor K\(^{\text{a}}\)-restricted response to GP aa 34–41/43 (GP1) (24–26). The K\(^{\text{b}}\) response becomes prominent only after the D\(^{\text{b}}\) allele has been knocked out (24). Because interactions between the T cell receptor (TCR), the MHC class I molecule, and the peptide are highly specific, and the affinities of peptide binding to the MHC and MHC-peptide complex to the TCR can determine whether a specific peptide is presented efficiently by a MHC class I allele and whether a CTL is activated (27–31), alteration of the peptide are highly specific, and the affinities of peptide binding to the viral nucleoprotein (NP) in the islets and in the thymus (6)

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Materials and Methods

Transgenic lines

Generation and characterization of RIP-LCMV tg mice with S9M prevented LCMV-induced diabetes in up to 100% of mice. Concurrently, the anti-viral (self) CTL precursor generation was reduced 26- to 46-fold and the expansion reduced 7-fold, which was sufficient to prevent IDDM but, at the same time, clear the viral infection. One implication of our data is that the association of autoimmune diseases with specific MHC class I molecules and epitopes (9–11, 38), knowledge of the anchor residues in the peptide motif (33), and function of the flanking amino acid sequences (32, 35, 36) restricted by these MHC molecules potentially allows the precise manufacturing of peptides with blocking properties to treat these disorders. However, in practice, we are still far away from designing such peptides for human IDDM, because the self-Ags have not been defined and mapping of MHC class I alleles that could predispose for disease has just begun. Further, blocking of the MHC class II alleles that are associated with human diabetes is much more difficult than blocking MHC class I due to the higher likelihood to induce (potentially regulatory) immunity rather than block or tolerize. This has been shown very convincingly in the nonobese diabetic mouse model (39).

Virus

Virus stocks consisted of LCMV-ARM (clone 53b) and vaccinia virus (vv) recombinants that express the LCMV strain Armstrong (ARM) GP only in the β2m of the islets and another line that expressed the viral nucleoprotein (NP) in the islets and in the thymus (6) were used for this report.

RNA analysis

RNA was extracted from tissues using the guanidinium isothiocyanate method, and specific mRNA transcripts detected by RT-PCR as described (6).

Analysis of blood glucose and pancreatic insulin levels

Blood samples were obtained from the retro-orbital plexus of mice and plasma glucose concentration determined using Accuchek II (Boehringer Mannheim, Indianapolis, IN). Mice with blood glucose values >350 mg% were considered diabetic (13).

Peptides and binding studies

Peptides were synthesized on an automated peptide synthesizer (Model 430A, Applied Biosystems, Foster City, CA) by the solid-phase method using t-butyl or N-(9-fluorenyl)methoxycarbonyl (Fmoc) chemistry, purified by HPLC on a RP300-C8 reversed-phase column (Brownlee; Perkin-Elmer, Norwalk, CT) and identified by fast atom bombardment of electrospray mass spectrometry. The H-2\(^{\text{D}}\)-selective radioactive probe 125I-YAINEAEL (sp. act., 40–80 TBq/mmol) was prepared and purified as described (32). Binding studies were performed on T-2D\(^{\text{b}}\) or T-2K\(^{\text{b}}\) cells as described (32). Briefly, in competition assays, T-2D\(^{\text{b}}\) or T-2K\(^{\text{b}}\) cells (1 × 10\(^{5}\) cells/well) were incubated in 96-well filtration plates (0.45 μm; Millipore, Bedford, MA) for 90 min at 37°C with 10 nM 125I-YAINEAEL and increasing concentrations (10\(^{-10}\) M to 10\(^{-8}\) M) of unlabeled competitors. Cells were then washed three times with BSA-PBS, and the filters were counted for radioactivity. Total and nonspecific binding was measured in the absence or presence of 1 μM unlabeled YAINEAEL. Specific binding to H-2D\(^{\text{b}}\) was defined as the difference between total and nonspecific bindings. Percent inhibition of binding was calculated as 100 × (1 – cpm in presence of competitor – cpm for nonspecific binding/cpm for specific binding. IC\(_{50}\) represents the peptide concentration inhibiting 50% of the specific binding of the radioactive probe. In both binding experiments, protease inhibitors (0.1 mM bestatin, 1 mM EDTA, 1 mM PMSF, and 1 mM aprotonin) were added during incubation at 37°C to prevent a possible degradation of the peptides. Values are mean ± SE of at least three independent experiments.

CTL and Ab assays

CTL activity was measured in a 5 to 6 h in vitro 51Cr release assay (13). Briefly, to judge CTL recognition and lysis, syngeneic or allogeneic target cells were either infected with LCMV-ARM (multiplicity of infection (MOI) = 1), or with recombinant vv expressing the full length LCMV-ARM-GP or -NP (MOI = 3). Uninfected cells coated with LCMV peptides GP 33–41 (KAVYNFATC), GA aa 276–286 (SGVVENPQGYCL), NP 396–404 (FQPQNGQFI), NP 118–126 (RPQAGSYVM), or blocking peptide (S9M) were also used as targets (25, 26, 40). Epitopes GP 33–41, GP 276–286, and NP 396–404 of LCMV are all H-2\(^{\text{D}}\) restricted for CTL recognition, while NP 118–126 is restricted by the L\(^{\text{d}}\) allele. Assays used splenocytes at E/T ratios of 50:1, 25:1, and 12:5:1 or CTL clones and secondary CTL lines at E/T ratios of 10:1, 5:1, 2.5:1, and 1:1. To determine CTL activity after secondary stimulation, spleen cells harvested from mice 30 to 180 days after primary inoculation with 1 × 10\(^{6}\) plaque-forming units (pfu) of LCMV i.p. were incubated with MHC-matched, irradiated, LCMV-infected, or peptide-coated (10\(^{-4}\) M) macrophages in the presence of T cell growth factor containing IL-2 and irradiated syngeneic spleen feeder cells (41) for 5 to 12 days. MC57 (H-2K\(^{\text{d}}\)) and BALB/c I7 (H-2D\(^{\text{d}}\)) cells used as CTL targets were grown as reported (13). T-2 cells stably transfected with D\(^{\text{b}}\) or K\(^{\text{b}}\) were generated as described (25). Precursor frequencies of LCMV-specific CTL were determined as described (42).

Assessment of lymphocyte proliferation in LCMV-memory splenocyte cultures

LCMV-specific memory splenocytes were isolated from C57BL6/J mice and cultivated in the presence or absence of 10\(^{-4}\) M blocking peptide and IL-2. Cells were then harvested and 5 × 10\(^{6}\) cells/ml were incubated at 37°C for 10 min in a 1:500 dilution of CFDA-SE (5-(and-6)-carboxyfluorescein diacetate-succinimidyl ester; C-1157, Molecular Probes, Eugene, OR), washed twice, and cultured on a fresh LCMV-infected and irradiated macrophage plate. After 4 days, cells were harvested and FACs staining was performed as described above. CFDA-SE is activated by intracellular binding to amino groups and can be detected by FACs. After cell division, CFDA-SE is distributed in roughly equal amounts into daughter cells, which consequently exhibit reduced fluorescence intensity. Thus, the degree of fluorescence intensity correlates inversely with the number of cell divisions.

Assessment of cytokine production by lymphoid cells (ELISPOT and ELISA)

Cytokines (IL-4 and IFN-γ) produced by lymphocytes in the spleen were detected using the ELISA assay. All Abs were purchased from Phar-Mingen, San Diego, CA). Briefly, 96-well Millititer HA plates (Millipore) were coated with capture Abs for IL-4 and IFN-γ diluted at optimal concentrations. IFN-γ and IL-4 production was measured in tissue culture supernatants from splenocytes obtained from peptide-treated or -untreated RIP-GP tg or C57BL6/J non-tg mice infected with LCMV and stimulated in vitro for 3 days in the presence of LCMV peptides. Cytokine
ELISA assays were conducted as proposed by PharMingen, who supplied the capture and detection Abs.

**Histologic and immunohistochemical analysis of tissues**

Tissues taken for histologic and immunohistochemical analysis were fixed in 10% zinc formalin and stained with hematoxylin and eosin. Immunohistochemical studies were conducted on 6- to 10-μm freshly frozen cryosections for immunostaining of islets to detect expression of MHC class I and II, Dd, insulin, CD4, CD8, B220, and F4/80. Primary Abs were applied for 1 h. These consisted of rat anti-mouse CD4 (clone RM 4-5), anti-CD8 (clone 53-6-7), anti-B220 (clone RA3 6B2), anti-F4/80 (clone A3-1), anti-MAC-1 (clone M 1/70), anti-MHC class I (clone M 1/42), and anti-class II (clone M5/114), (PharMingen and Boehringer Mannheim). (6) After washing in PBS, the secondary Ab (biotinylated goat anti-rat (or anti-mouse)) IgG, Vector Laboratories, Burlingame, CA) was applied for 1 h. Color reaction was developed with sequential treatment using avidin-horseradish peroxidase conjugate (Boehringer Mannheim) and diaminobenzidine-hydrogen peroxide (41).

**Results**

**S9M peptide inhibits CTL killing of viral peptide-coated Db target cells—the efficacy correlates with the affinity of the epitope**

Binding affinities (IC50 values) to Dd and Kb were assessed as described (32), which showed that S9M bound with high affinity to Dd-expressing (IC50Db = 11 nM), but not to Kb-expressing cells (IC50Kb > 10,000 nM) T-2 cells. In comparison, the binding affinities for the LCMV peptides were as follows: NP (IC50Db = 15 nM; IC50Kb > 10,000 nM), GP1 (IC50Db = 330 nM; IC50Kb = 940 nM), and GP2 (IC50Db = 31 nM; IC50Kb > 10,000 nM). Thus, the binding affinity of the blocking peptide to Dd is ~30 times higher than the LCMV-GP1 peptide, 2 times higher than the LCMV-GP2 peptide, and equivalent to the LCMV-NP peptide. The affinities correlate well with the minimum molar amounts of the LCMV-GP and -NP agonist peptides required for CTL killing of target cells (10^{-8.5} M for the NP compared with 10^{-7} to 10^{-8.3} M for the GP epitopes).

We then studied whether LCMV-specific killing of targets by activated CTL generated on the H-2b background was affected by S9M. Data presented in Table I show that three different LCMV clones are efficiently inhibited by this peptide in an affinity-dependent way. Preincubation of CTL with S9M did not reveal any antagonistic properties for LCMV (Table I). The results shown in Table II demonstrate that Dh but not Kbh restricted LCMV-specific CTL killing can be blocked in vitro by S9M. Earlier studies had shown that Dh and Kbh restrict the LCMV-GP1 epitope, whereas the LCMV-GP2 and -NP epitopes are restricted only by Dh (25). These studies indicate that S9M is specific to the Dh allele and does not affect Kbh-restricted responses. Although responses to all three Dh-restricted LCMV epitopes (data for GP1 and NP shown, not shown for GP2) are inhibited by this single peptide, inhibition for targets coated with the GP1 (Table I) or GP2 (data not shown) peptide is better than for the NP peptide. These findings correlate well with the 30-fold higher binding affinity of the NP peptide compared with the GP1 peptide (see previous paragraph). As a control, Dh-restricted killing by LCMV-CTL was not affected by addition of an Lbh-restricted peptide (RPQASGVMY).

The K4 analogue (SMIKLENYM) of S9M was then expressed using a vv recombinant. Our previous studies have shown that this analogue is as efficient in inhibiting LCMV-CTL as S9M (23). The vv recombinant infected target cells, thereby achieving intracellular production and introduction of the blocking peptide in the Ag presentation pathway. As shown in Table III, targets infected with LCMV-GP or -NP expressing vv recombinants were recognized efficiently by LCMV-CTL. In contrast, coinfection of these targets with SMIKLENYM expressing vv recombinant, but not wild-type vv, resulted in complete abrogation of their recognition by LCMV-CTL. In conclusion, studies shown in Tables I and II demonstrate that S9M acts as an allele-specific (Db), Ag-nonspecific blocking peptide without evidence for any antagonist properties in an affinity-dependent manner.

**S9M selectively inhibits expansion of LCMV-CTL in vitro**

We determined whether the blocking peptide was able to inhibit activation and expansion of LCMV memory CTL in vitro. We found that whereas good expansion and recovery of CTL activities was observed after an 8-day stimulation of memory splenocytes in the presence of IL-2 with LCMV peptides, and some expansion even without LCMV peptides, S9M abrogated this process (Table IV) and no CTL activities could be detected in cultures grown in the presence of S9M. In good correlation with these findings, precursors of LCMV-CTL recovered from cultures with the blocking peptide were up to sevenfold lower than those found in untreated cultures and fivefold lower than precursors detected in cultures stimulated with LCMV agonist GP1 and NP peptides and S9M compared with cultures expanded in the presence of GP1 and NP alone (Table IV). In parallel, production of IFN-γ by LCMV-CTL was reduced in these S9M-treated cultures. Additional experiments using a fluorescent dye showed that expansion of CD8 lymphocytes was reduced by threefold in the presence of the blocking peptide over a 5-day observation period (data not shown). Thus, S9M effectively inhibits expansion and IFN-γ production by LCMV-CTL in vitro.

**In vivo administration of S9M reduces LCMV-specific Dh-restricted CTL generation and prevents IDDM in RIP-LCMV-GP Tg mice**

Non-tg C57BL6/J (H-2b) and tg RIP-GP (H-2b) mice were injected with various amounts of S9M i.p. or i.v. to determine the optimal

<table>
<thead>
<tr>
<th>LCMV-CTL</th>
<th>Epitope</th>
<th>Minimum Concentration (M) of Agonist Peptide for No S9M</th>
<th>S9M 10^{-6} M</th>
</tr>
</thead>
<tbody>
<tr>
<td>LCMV-GP-1 clone</td>
<td>KAVYNFATC</td>
<td>10^{-7}</td>
<td>10^{-4} to 10^{-5}</td>
</tr>
<tr>
<td>LCMV-GP-2 clone</td>
<td>SGVENPGGYCL</td>
<td>10^{-8}</td>
<td>10^{-3}</td>
</tr>
<tr>
<td>LCMV-NP clone</td>
<td>FQPQNGQFI</td>
<td>10^{-13.5}</td>
<td>ND</td>
</tr>
</tbody>
</table>

*Viral clones were maintained in the presence of irradiated syngeneic APCs, IL-2, and irradiated feeder cells (see Materials and Methods) coated with the respective peptide or infected with LCMV (for the LCMV-GP-1, -GP-2, and -NP clones). CTL assays were carried out as described in Materials and Methods using a constant amount of S9M (10^{-6} M) in 50% of the wells and by making serial dilutions of the agonist peptides, respectively. The minimum molar amount of agonist peptide required to generate >10% lysis of syngeneic MC57 H-2b fibroblasts in a 5-h^{51}Cr release assay is displayed in the presence and absence of S9M. Preincubation of LCMV-specific clones with S9M (10^{-6} M) was performed in vitro over a 48-h period in 24-well plates in the presence of APCs (irradiated). Effector lymphocytes were washed three times before being introduced into the CTL assay. In additional studies, bulk LCMV splenocytes harvested 7 days postinfection were preincubated in the same way, and no effect on CTL killing was observed (data not shown). Furthermore, comparison of the actual^{51}Cr release values from S9M treated or untreated clones did not yield any significant differences (data not shown). Similarly, preincubation of effector CTL with “cold” targets coated with S9M did not affect CTL killing. These findings show no evidence for an antagonistic effect of S9M on LCMV-CTL.*
protocol for reduction of CTL and prevention of IDDM. Optimal success was achieved in RIP-GP mice by administering 1 mg, but not lower amounts, of S9M i.p. without adjuvant daily for 2 wk. All mice were injected 1 day after beginning peptide therapy with 1 × 10⁵ pfu LCMV i.p., and 7 days later LCMV-specific CTL activities were measured in a ⁵¹Cr release assay using splenocytes obtained by hemisplenectomy (13). The data are shown in Table V. Robust CTL responses were noted in untreated control groups receiving no peptide treatment (Table V) or receiving H-2Ld-restricted peptides and prevention of IDDM in RIP-GP mice (Fig. 1). This effect is specific to the blocking effect of this peptide, because parallel treatment with the LCMV-GP peptide, or an unrelated LCMV L⁹-restricted peptide, did not result in CTL or IDDM reduction (data not shown).

When RIP-GP tg mice were treated in vivo with S9M using the optimal protocol (Table V) and incidence of IDDM was monitored over an 8-mo observation period, IDDM was prevented at a rate of 100% (Fig. 1). Treatment was more effective when given i.p. than i.v., likely because the i.v.-administered peptide is eliminated more rapidly through the kidneys (data not shown). In contrast, control groups receiving no peptide treatment (Table V) or receiving H-2Ld-restricted LCMV-NP peptide RPQASGVYM all developed IDDM within 2 wk. Thus, in our model system, virus-induced autoimmune diabetes can be prevented in vivo by treatment with an allele-specific blocking peptide. The next series of studies focus on the mechanism by which S9M prevents IDDM in RIP-GP mice in vivo.

S9M selectively inhibits generation and expansion of LCMV-CTL in vivo

Precursor frequency analysis of LCMV-specific CTL showed that in vivo treatment with S9M reduced primary expansion of LCMV-CTL, usually observed 7 days postinfection, by up to 26- to 46-fold, resulting in significantly decreased numbers of LCMV-precursor CTL (Table V and Fig. 1). Stimulation of these in vivo blockaded day 7 splenocyte populations in the presence of IL-2 and LCMV Ag or peptides (see Materials and Methods) did not result in an increase in precursors of CTL, indicating that anergy was not induced by the peptide treatment (data not shown). This is paralleled by our observation that in vitro cultures with S9M loose LCMV-specific memory CTL activity (Table IV), which cannot be recovered in the presence of IL-2.

Thus, our results indicate that treatment with S9M results in an overall reduction in the number of activated LCMV-specific CTL. This lack of expansion of LCMV-specific precursors likely demonstrates an in vivo blocking effect of the peptide.

Prevention of IDDM in RIP-GP mice treated with S9M is associated with reduction of islet infiltration

Immunohistochemical analysis was performed on sections obtained 28 days after LCMV infection from islets of RIP-GP tg mice that did or did not receive treatment with S9M. As shown in Figure 2, infiltration into the islets was only observed in untreated control groups (E–H). In contrast, lymphocytes were

Table III. In vitro expression of H-2D⁹ blocking peptide in target cells prevents their recognition by D⁹-restricted LCMV-CTL

<table>
<thead>
<tr>
<th>Specific Cr⁵¹ Release (%) from Targets</th>
<th>H-2D⁹K² (MC57)</th>
<th>H-2⁹ (BALB)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LCMV-infected</td>
<td>vv-LCMV-GP</td>
</tr>
<tr>
<td>vV SMIKNLEYM Effector CTL</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>H-2⁹ spleen day 7 LCMV</td>
<td>23</td>
<td>5</td>
</tr>
<tr>
<td>H-2⁹ GP-2 CTL clone</td>
<td>59</td>
<td>0</td>
</tr>
<tr>
<td>H-2⁹ NP CTL clone</td>
<td>38</td>
<td>12</td>
</tr>
</tbody>
</table>

* MC57 H-2⁹ target cells were infected with vV recombinants expressing LCMV-GP or NP (52) or SMIKNLEYM (23). CTL assays were performed as described for Table II. Earlier studies showed that SMIKNLEYM is equally as effective as S9M in blocking CTL responses in vivo (25). As a negative control, infection of targets with wild-type vV together with vaccinia recombinants expressing LCMV proteins did not affect the amount of target killing.
Materials and Methods

Peptide stimulation or coating was performed as described in Materials and Methods. CTL activities were assayed from splenocytes obtained by hemisplenectomy 7 days post-LCMV infection on syngeneic and allogeneic target cells infected with LCMV. Memory splenocytes were harvested 6 mo post-LCMV infection as described above. Precursor CTL activities were determined after an 8 to 12-day in vitro stimulation with LCMV-GP1 and -NP peptides. S9M-specific precursors were stimulated on macrophages coated with the S9M peptide. Note that S9M-specific CTLs were present only in mice treated with the S9M and infected with LCMV. In parallel, tissue culture supernatants were tested for IFN-γ and IL-4 production.

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Table IV. H-2D<sup>b</sup>-blocking peptide S9M inhibits expansion of primary D<sup>b</sup>-restricted LCMV-CTL in vitro

<table>
<thead>
<tr>
<th>Splenocytes Post-LCMV Infection of H-2&lt;sup&gt;b&lt;/sup&gt; mice</th>
<th>8-Day In vitro Stimulation with IL-2 ± Peptide</th>
<th>Specific 51Cr Release (%) from Targets H-2&lt;sup&gt;b&lt;/sup&gt; (D&lt;sup&gt;b&lt;/sup&gt;,K&lt;sup&gt;b&lt;/sup&gt;)&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57BL6/J, day 180</td>
<td>No peptide</td>
<td>44 ± 3 38 ± 4 48 ± 12</td>
</tr>
<tr>
<td></td>
<td>GP-1 and NP (H-2&lt;sup&gt;b&lt;/sup&gt;)</td>
<td>70 ± 8 60 ± 8 62 ± 6</td>
</tr>
<tr>
<td></td>
<td>S9M (H-2&lt;sup&gt;b&lt;/sup&gt;)</td>
<td>5 ± 2 0 0</td>
</tr>
<tr>
<td></td>
<td>No peptide</td>
<td>49 ± 7 58 ± 12 46 ± 11</td>
</tr>
<tr>
<td>C57BL6/J, day 7 control</td>
<td>No peptide</td>
<td></td>
</tr>
<tr>
<td><strong>Precursor CTL and Cytokine Production</strong>&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C57BL6/J, day 180</td>
<td>No peptide</td>
<td>1:3000</td>
</tr>
<tr>
<td></td>
<td>S9M (H-2&lt;sup&gt;b&lt;/sup&gt;)</td>
<td>1:2000</td>
</tr>
<tr>
<td></td>
<td>GP-1 and NP (H-2&lt;sup&gt;b&lt;/sup&gt;)</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1:180</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>N/A</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>1:940</td>
<td>+</td>
</tr>
<tr>
<td>C57BL6/J, day 7 control</td>
<td>No peptide</td>
<td>1:120</td>
</tr>
<tr>
<td></td>
<td>N/A</td>
<td>++</td>
</tr>
</tbody>
</table>

* C57BL6/J H-2<sup>b</sup> mice were immunized with 1 × 10<sup>6</sup> pfu LCMV. Splenocytes were harvested 180 days following immunization and stimulated for 8 days in vitro in the presence of syngeneic APCs, T cell growth factor (IL-2), and no peptide, or LCMV-GP1/LCMV-NP or S9M peptides (see Materials and Methods). CTL activities were then tested in a 51Cr release assay using the stimulated splenocytes at an E:T ratio of 5:1.

* Memory splenocytes were harvested 6 mo post-LCMV infection as described above. Precursor CTL activities were determined after an 8 to 12-day in vitro stimulation with or without peptides (10<sup>-6</sup> M), as indicated, in the presence of APCs and TCGF (see Materials and Methods). IFN-γ and IL-4 was assessed in culture supernatants by ELISA after 8 days (see Materials and Methods). ++, >2000 U/ml; +, 40–1000 U/ml; –, not detectable (<20 U/ml).

found around the islets in S9M-treated mice without IDDM (Fig. 2, A–D). Fewer CD8<sup>+</sup> lymphocytes were detected in S9M-treated tg mice (Fig. 2C) compared with diabetic mice without treatment (Fig. 2G). However, in both groups of mice up-regulation of MHC class I molecules (Fig. 2, D and H), CD4 (Fig. 2, B and F), and B lymphocytes (data not shown) were observed. Despite this inflammation, IDDM did not occur in S9M-treated mice. In conclusion, administration of S9M results in reduction of insulitis and CD8<sup>+</sup> lymphocytes in islets.

We then evaluated the mechanism by which the peptide treatment influenced the local milieu in the islets of Langerhans and determined whether this occurred directly through lowering the numbers of autoreactive lymphocytes or indirectly through the generation of immunoregulatory cells.

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Table V. H-2D<sup>b</sup>-blocking peptide S9M inhibits expansion of LCMV-CTL in vivo<sup>a</sup>

<table>
<thead>
<tr>
<th>Effector (LCMV GP1 NP)</th>
<th>8-Day In vitro stimulation</th>
<th>Specific 51Cr Release (%) from Targets H-2&lt;sup&gt;b&lt;/sup&gt; (D&lt;sup&gt;b&lt;/sup&gt;,K&lt;sup&gt;b&lt;/sup&gt;)&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group</td>
<td>E:T</td>
<td>LCMV GP1 GP2 T-2(D&lt;sup&gt;b&lt;/sup&gt;)&lt;sup&gt;b&lt;/sup&gt; T-2(K&lt;sup&gt;b&lt;/sup&gt;)&lt;sup&gt;b&lt;/sup&gt; H-2&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Splenocytes from H-2&lt;sup&gt;b&lt;/sup&gt; mice day 7 post-LCMV</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C57BL6/J</td>
<td>50:1</td>
<td>60 ± 7 50 ± 6 20 ± 8</td>
</tr>
<tr>
<td>RIP-GP</td>
<td>25:1</td>
<td>38 ± 6 28 ± 7 12 ± 5</td>
</tr>
<tr>
<td>SMIELEYM 1 mg i.p.</td>
<td>(daily for 2 wk)</td>
<td>60 ± 8 42 ± 9 23 ± 7</td>
</tr>
<tr>
<td></td>
<td>25:1</td>
<td>34 ± 7 22 ± 5 10 ± 3</td>
</tr>
<tr>
<td></td>
<td>50:1</td>
<td>12 ± 6 13 ± 5 5 ± 4</td>
</tr>
<tr>
<td></td>
<td>25:1</td>
<td>9 ± 5 10 ± 4 0</td>
</tr>
</tbody>
</table>

Precursor CTL and Cytokine Production

<table>
<thead>
<tr>
<th>Effector (LCMV GP1 NP)</th>
<th>8-Day In vitro stimulation</th>
<th>Specific 51Cr Release (%) from Targets H-2&lt;sup&gt;b&lt;/sup&gt; (D&lt;sup&gt;b&lt;/sup&gt;,K&lt;sup&gt;b&lt;/sup&gt;)&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group</td>
<td>E:T</td>
<td>LCMV GP1 GP2 T-2(D&lt;sup&gt;b&lt;/sup&gt;)&lt;sup&gt;b&lt;/sup&gt; T-2(K&lt;sup&gt;b&lt;/sup&gt;)&lt;sup&gt;b&lt;/sup&gt; H-2&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Splenocytes from H-2&lt;sup&gt;b&lt;/sup&gt; mice day 7 or 45 post-LCMV</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C57BL6/J</td>
<td>None</td>
<td>1/900 1/3,000 &lt;1/10^5 &lt;1/40,000 2,000 ± 670 &lt;200</td>
</tr>
<tr>
<td>RIP-GP</td>
<td>None</td>
<td>1/120 ND &lt;1/10^5 &lt;1/40,000 2,300 ± 560 &lt;200</td>
</tr>
<tr>
<td>SMIELEYM 1 mg i.p.</td>
<td>(daily for 2 wk)</td>
<td>1/3,200 1/2,800 &lt;1/10^5 &lt;1/10,000 750 ± 80 &lt;200</td>
</tr>
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</table>

* Groups of 3 to 6 C57BL6/J (H-2<sup>b</sup>), RIP-LCMV-GP gp mice were infected with 1 × 10<sup>6</sup> pfu LCMV-ARM i.p., and some groups were additionally treated with S9M as indicated. CTL activities were assayed from splenocytes obtained by hemisplenectomy 7 days post-LCMV infection on syngeneic and allogeneic target cells infected with LCMV or coated with LCMV-GP1, -GP2, -NP, or S9M peptides (see Materials and Methods). Specific killing is displayed as percent specific 51Cr release from target cells ± 1 SE. Groups of two mice were tested for killing of T-2 cells transfected with either D<sup>b</sup> or K<sup>b</sup> and coated with LCMV-GP1 peptide. Results demonstrate the specificity of the S9M effect in vivo for D<sup>b</sup>. IDDM was assessed by Accucheck (blood glucose >350 mg/dl). Precursors of LCMV- or S9M-specific CTL in spleens obtained 7 or 45 days postinfection were determined as described in Materials and Methods. Stimulation for LCMV-specific precursors was carried out in the presence of T cell growth factor on macrophages coated with LCMV-GP1 and -NP peptides. S9M-specific precursors were stimulated on macrophages coated with the S9M peptide. Note that S9M-specific CTLs were present only in mice treated with the S9M and infected with LCMV. In parallel, tissue culture supernatants were tested for IFN-γ and IL-4 production.
H-2b-CTL repertoire in vivo when administered daily for 2 wk (Table VI). Thus, S9M is not capable of significantly activating the induced by immunization with LCMV-GP1 and -GP2 peptides. In contrast, no detectable levels of CTL against S9M after stimulation of memory splenocytes in the presence of S9M (Tables I and II). S9M is only effective for treating IDDM initiated by CTL and levels of LCMV-specific memory CTL are not decreased.

Viral titers and levels of LCMV-specific CTL were quantitated in mice that did or did not receive treatment with S9M to assess whether this treatment led to a persistent infection. Kinetics of viral clearance were not delayed with complete clearance of virus from the serum in both groups by 14 ± 3 days. Further, as shown in Table V, only levels of LCMV-primary (Table V) but not LCMV-memory CTL were affected, likely due to the relatively short duration of the treatment. Thus, S9M therapy was safe and prohibited the development of a persistent viral infection, likely due to K b-restricted LCMV-GP1-specific CTL and remaining D b-restricted CTL directed mainly to LCMV-NP.

**FIGURE 1.** Treatment with D b blocking S9M peptide prevents IDDM in RIP-GP tg mice. Groups of eight to 10 RIP-GP tg mice were infected with 1 × 10^9 pfu LCMV-ARM i.p. and incidence of IDDM was measured at weekly intervals for 1 mo and thereafter once every month for a total of 8 mo S9M treatment was conducted as indicated. Mice with blood glucose values exceeding 350 mg/dl were considered diabetic and sacrificed for further analysis (see Materials and Methods). Lower dosages of S9M, as well as treatment with an L2-restricted peptide, did not lead to prevention of IDDM.

**Discussion**

Our study shows that a blocking peptide engineered to selectively bind to one MHC class I allele but not react with LCMV-specific (“anti-self”) CTL, can inhibit anti-viral CTL activity, modulate T cell-mediated injury, and prevent diabetes in a virally triggered tg model in vivo, without affecting overall immune competence. For effective therapy, the affinity of the targeted viral peptides to MHC class I has to be lower than that of the blocking peptide to the same MHC allele. Such treatment effectively reduces expansion of CTL that recognize an epitope at 10^{-7} to 10^{-8.5} M minimal peptide concentrations. By contrast, this approach was less effective in vitro and in vivo for CTL that still recognized and lysed targets coated only with 10^{-13.5} M of the viral peptide. The blocking peptide inhibited activation, expansion, and IFN-γ production of anti-viral LCMV-CTL in vitro and in vivo but did not induce regulatory cells or exert detectable antagonistic effects. The in vivo effect resulted in complete abrogation of LCMV-induced, CTL-mediated IDDM in RIP-LCMV mice. These findings raise the possibility that diseases associated with a MHC class I allele can be influenced with peptide therapy, if the affinity for the targeted epitope is lower than that of the blocking peptide, without affecting the hosts ability to clear viral infections relying on an immune response restricted to this allele. However, MHC class I restriction and self-Ags have to be known for each disease to be able to apply this strategy.

Virus is cleared in LCMV-infected mice treated with S9M and levels of LCMV-specific memory CTL are not decreased. Our MHC class I-restricted RIP-LCMV model system for autoimmune diabetes is well suited to study the possibility to abort CD8+ CTL and, in this way, prevent IDDM. We asked whether a blocking peptide that binds to the MHC class I allele of interest (D b) but does not activate the targeted LCMV-CTL repertoire could be used for this purpose. Earlier studies using mapping strategies with viral reassortants, vv recombinants expressing LCMV proteins, and synthetic peptides precisely defined the fine specificity and peptide components recognized by LCMV-specific CTL.
Results showed that the normal LCMV-CTL repertoire of H-2b mice recognizes three D\(^b\)-restricted epitopes located in GP1 (aa 33–41/43), GP2 (aa 276–286), and NP (aa 396–404). Using the D\(^b\)-restricted blocking peptide, we noted that anti-LCMV-CTL responses generated to the three distinct peptide segments on both viral proteins were all reduced, but quantitatively to different degrees. The GP1 and GP2 epitopes were blocked more efficiently than the NP epitope. The effect was allele specific, because Kb-restricted LCMV-CTL responses were not affected (Table II). The blocking peptide was initially designed on the basis of the amino acids most commonly found in the sequenced pool of endogenous peptides eluted from H-2Db molecules (28, 33) by testing peptides synthesized from the LCMV-GP and -NP proteins that fitted the Db motif (32) and by defining those amino acids in the peptide that bind to the TCR (35, 36, 45). When the optimal designed peptide, S9M, was given alone or with IFA, it was unable to induce CTL responses in vivo (Table VI). However, IFN-\(\gamma\) producing CTL directed against the LCMV-CTL blocking peptide could be induced when it was administered together with replicating LCMV in vivo. These observations suggested that the LCMV-CTL blocker could function as an agonist for other CTL and that the virus probably functions as a strong “adjuvant,” perhaps through activation of APC. Most importantly, when S9M was given to RIP-LCMV-GP tg mice, it prevented development of virus-induced autoimmune diabetes. This was predominantly accomplished by quantitatively reducing the amount and/or expansion of LCMV-GP-specific anti-self CTL required for the destruction of \(\beta\) cells (Table V). Some additional effect by directly locally blocking D\(^b\)-MHC molecules expressed on \(\beta\) cells to prevent CTL recognition cannot be ruled out. No evidence was found supporting the assumption that the S9M peptide could induce lymphoid cells able to prevent IDDM upon adoptive transfer or exert antagonistic effects on LCMV-CTL. This notion was also supported by the finding that S9M blocked other viral CTL (influenza, SV40) equally as well as LCMV-CTL and in an affinity-dependent manner (J.E.G., M.G.v.H., H.L., and M.O., manuscript in preparation). If S9M would exert antagonistic effects, one would expect differential effects on other CTL, and preincubation with S9M should abrogate or lower CTL killing, both effects that we did not observe.

Thus, our data support the hypothesis that prevention of IDDM in RIP-GP mice occurs via a MHC-specific, allele-restricted effect. LCMV-specific D\(^b\)-restricted CTL responses that are required for induction of IDDM are quantitatively reduced over 26- to 46-fold (Tables V and VI, Fig. 1). This results in less infiltration of islets (Fig. 2). In this context, direct effects on CTL recognition of the MHC-presented viral peptides likely occur. A parallel could be drawn between the in vitro and in vivo results (Tables II and V), which would occur at the level of the infected cells. In this scenario, S9M would directly compete with the viral peptides in binding to the MHC-H-2Db molecules to a point below the threshold of inhibition.
Table VI. *H-2D<sup>b</sup>-blocking peptide S9M does not activate LCMV-CTL in vivo but induces S9M-specific IFN-γ-producing CTL when given together with LCMV*

<table>
<thead>
<tr>
<th>Peptide alone&lt;sup&gt;a&lt;/sup&gt;</th>
<th>8-Day In Vitro Stimulation with IL-2 ± Peptide</th>
<th>Specific 51Cr Release (%) from Targets H-2&lt;sup&gt;b&lt;/sup&gt;(D&lt;sup&gt;b,K&lt;/sup&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No peptide</td>
<td>GP-1 and NP (H-2&lt;sup&gt;b&lt;/sup&gt;)</td>
<td>LCMV: 1,100, SMILEYM: ND, IFN-γ: +, IL-4: –</td>
</tr>
<tr>
<td>GP-2 peptide 100 µg/IFA i.p.</td>
<td>GP-1 and GP-2 (H-2&lt;sup&gt;b&lt;/sup&gt;)</td>
<td>GP-1: 1,180, IFN-γ: &lt;1:10,000, IL-4: –</td>
</tr>
<tr>
<td>GP-1 peptide 100 µg/IFA i.p.</td>
<td>SMILEYM (H-2&lt;sup&gt;b&lt;/sup&gt;)</td>
<td>GP-2: 1,680, IFN-γ: &lt;1:10,000, IL-4: –</td>
</tr>
<tr>
<td>SMIE peptide 100 µg/IFA i.p. (daily for 2 wk)</td>
<td>GP-1 and GP-2 (H-2&lt;sup&gt;b&lt;/sup&gt;)</td>
<td>SMILEYM (H-2&lt;sup&gt;b&lt;/sup&gt;): 1,500, IFN-γ: &lt;1:6,000, IL-4: –</td>
</tr>
<tr>
<td>GP-1 peptide 100 µg i.p. (daily for 2 wk)</td>
<td>SMILEYM (H-2&lt;sup&gt;b&lt;/sup&gt;)</td>
<td>GP-1: 1,800, IFN-γ: 1:7,000, IL-4: + + –</td>
</tr>
<tr>
<td>SMIE peptide 1.0 mg i.p. (daily for 2 wk)</td>
<td>SMILEYM (H-2&lt;sup&gt;b&lt;/sup&gt;)</td>
<td>GP-1: 1,500, SMILEYM (H-2&lt;sup&gt;b&lt;/sup&gt;): 2,500, IFN-γ: &lt;1:20,000, IL-4: –</td>
</tr>
<tr>
<td>SMIE peptide 100 µg/IFA i.p. (daily for 2 wks)</td>
<td>GP-1 and NP (H-2&lt;sup&gt;b&lt;/sup&gt;)</td>
<td>GP-1: 1,500, SMILEYM (H-2&lt;sup&gt;b&lt;/sup&gt;): 2,500, IFN-γ: &lt;1:20,000, IL-4: –</td>
</tr>
</tbody>
</table>

<sup>a</sup> C57BL/6/J H-2<sup>b</sup> mice were immunized with 100 µg of LCMV-GP1, 1 mg of S9M, or 100 µg of S9M/IFA emulsion daily for 2 wk or with GP1 and GP2 peptide or S9M (100 µg) emulsified 1:1 in IFA i.p. once. Splenocytes were harvested 35 days following immunization and stimulated for 8 to 14 days in vitro in the presence of syngeneic APCs, TCGF (IL-2), and GPl/NP or S9M peptides (see Materials and Methods). CTL activities were then tested in a 51Cr release assay using the stimulated splenocytes at an E:T ratio of 5:1.

<sup>b</sup> Memory splenocytes were harvested 6 mo post-LCMV infection from S9M-treated or untreated mice. CTL activities were determined after an 8-day in vitro stimulation with or without peptides (10<sup>−8</sup> M) as indicated in the presence of APCs and TCGF (see Materials and Methods). Note that S9M specific CTLs were present only in mice treated with the blocking peptide and infected with LCMV and produced IFN-γ but no IL-4. IFN-γ and IL-4 were assessed in culture supernatants, and standard ELISA were used as directed by the manufacturer (PharMingen, San Diego, CA), who also provided capture and detection Abs. In both cases, positive controls were run with every assay (data not shown), which showed linear correlation according to the manufacturers instructions. + + +, >2000 U/ml; + +, 1000–2000 U/ml; +, 40–1000; –, not detectable (<20 U/ml).

viral peptide necessary for activated T cells to lyse the infected cell or for naive T cells to be activated and expand. This effect has been observed in vitro (23), and, therefore, S9M can be considered as an inhibitory or “MHC blocking peptide.” An additional effect that may be relevant to the in vivo situation is the occupation of empty MHC-H-2<sup>D</sup> molecules at the cell surface and/or the displacement of low-affinity peptides. Our findings indicate that the blocking peptide can change activation profiles and distribution of CTL in vivo. It is possible that lowering the CTL response alone is sufficient to abrogate β cell destruction, because our earlier studies demonstrated that induction of IDDM in RIP-LCMV mice is dependent on the numbers of autoreactive CTL induced (41). An additional and complimentary mechanism may have a direct effect on D<sup>ρ</sup> molecules that are up-regulated on β cells in RIP-GP mice after LCMV infection (42, 46).

Up-regulation of MHC class I molecules and peri-insulitis composed mainly of CD4<sup>+</sup> and B lymphocytes was observed in islets from S9M-treated mice without IDDM (Fig. 2). This peri-insulitis persists and lymphocytes do not enter the islets. Recent data support the concept that autoimmune processes are locally maintained and/or regulated by a balance of critical cytokines such as IFN-γ (Th1) or IL-4 (Th2) (41, 42, 47). Altered peptide ligands have been proposed to modify the cytokine profile of lymphocytes after their activation (48–51). These observations led us to test whether regulatory cells were induced by the S9M treatment or whether the altered cytokine profiles found in the pancreas of treated mice (data not shown) were primarily a reflection of a less-destructive infiltrate due to lower amounts of autoreactive CTL. Immunization with S9M 35 days before LCMV challenge neither lowered LCMV-inducible CTL response nor protected against the development of IDDM. This suggests that regulatory cells did not mediate protection. The point is strengthened by the observation that the cytokine balance was not altered in the spleen. Further, CTL specific for the blocking peptide were recovered from mice that had received both LCMV and S9M treatment and expanded in vitro (Table VI). These CTL mainly produced IFN-γ and not IL-4 and IDDM after transfer into RIP-GP recipients was not prevented. Thus, short-lived regulatory cells are likely not involved in the observed peptide protection.

It is important to note that continuous treatment with S9M reduced but not completely aborted the LCMV-specific CTL response (Table V). As a consequence, a CTL response remained that was sufficient to control viral infection, but insufficient to cause IDDM. This finding is in agreement with reports by us (41) and others (12, 52, 53) that noted a direct correlation between the number of specific effector CTL and causation of the autoimmune disease. By our observation, the residual CTL response present in peptide-treated mice was directed against the K<sup>b</sup>-restricted GP1 peptide (Table IV), because the blocking peptide is allele-specific and inhibits only D<sup>ρ</sup>-restricted but not K<sup>b</sup>-restricted CTL responses (Tables II and IV). This has potentially important consequences for designing therapies to control autoimmune disorders. Because responses to most pathogens are restricted by more than one MHC allele, blocking the response to only one MHC allele or epitope linked to an autoimmune disease does not abrogate the T cell control of a viral infection.

The generation of MHC (D<sup>ρ</sup>)-restricted LCMV-specific memory CTL is not greatly affected by S9M treatment. Despite the reduction of numbers of LCMV-specific D<sup>ρ</sup>-restricted primary CTL
tested 7 days after primary infection, equivalent levels of LCMV-memory CTL were present in the S9M-treated and -untreated mice (Table V). One possible explanation for this observation is that the subset of primary CTL that develops into memory CTL may have a different TCR affinity (54, 55) and is therefore less affected by the peptide treatment. Importantly, these memory CTL are not able to induce autoimmune diabetes, presumably because they are not being activated, because the autoimmune process in the pancreas was aborted earlier by S9M treatment. Thus, S9M is effective when administered during the critical initial period for development of IDDM and after discontinuation of therapy IDDM did not develop over a 6-mo observation period.

In conclusion, the blocking peptide strategy employed here might be a very useful approach for MHC class I-associated disease and differs from earlier reports that used MHC class I-restricted agonist peptides in vivo (18). The complication of risk for immunopathology through induction of immunologic memory found when using agonist peptides (15) was removed. Our approach resulted in a quantitative reduction of expansion of MHC class I (Dp)-restricted T lymphocytes and no enhancement of immunopathology or immunologic memory. Importantly, not all autotoxic T cells need to be eliminated, but a quantitative reduction below a certain threshold level suffices to arrest the autoimmune process, suggesting that blocking peptides can be designed for the control of some autoimmune diseases or in the reduction of immune-mediated damage associated with viral or other microbial infections restricted to or associated with one MHC class I allele. However, several restrictions apply for this therapy. First, the binding affinity of the designed blocking peptide has to be equal or higher than that of the targeted epitope. In our studies, epitopes that require 10−8 M or higher concentration can be effectively blocked in vivo, while epitopes requiring less than 10−9 M peptide for recognition were not efficiently manipulated. Second, peptide analogues with an extended in vivo half-life have to be developed. Third, the disease process has to depend to a significant extent on MHC class I-restricted CTL-mediated injury. Earlier studies using MHC class II-restricted peptides to block IDDM in nonobese diabetic mice (39) showed that immunoregulatory effects (due to altered peptide ligand recognition) but not blocking of MHC class II was responsible for the prevention of IDDM. Due to the occurrence of altered peptide ligand effects, MHC class II-restricted reactivity might therefore be more difficult to block. Finally, the self-Ags recognized by CTL have to be defined. This is not yet a possibility for IDDM but might become available in the future.

Acknowledgments

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