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

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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*

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 D^b-restricted LCMV epitopes. In addition, in vitro expansion of LCMV memory CTL was prevented, resulting in decreased IFN-γ secretion. In vivo, a 2-wk treatment with this peptide lowered the LCMV D^b-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 D^b-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-γ 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.


Many autoimmune disorders involve the participation of autoreactive CD4^+ or CD8^+ T lymphocytes. For example, in individuals with autoimmune diabetes type 1 (insulin-dependent diabetes mellitus (IDDM)) both subsets of lymphocytes are found infiltrating the islets of Langerhans (1–5), and several experimental animal models have demonstrated that both are required for the destruction of β cells and the development of IDDM (6, 7). Further, several diseases are associated with distinct MHC class I or class II alleles (8–10), and viral infections both are required for the destruction of β cells and the development of IDDM (6, 7). Further, several diseases are associated with distinct MHC class I or class II alleles (8–10), and viral infections have been implicated as a cause for autoimmunity (2, 6, 11–13). Immune-mediated therapy using MHC class II- and I-restricted peptides to modify or control T lymphocyte responses involved in autoimmune diseases in vivo has elicited considerable interest (14–22). Based on this background, the goal for our present study was formed: we analyzed the potential of a MHC class I-restricted allele-specific blocking peptide (23) to prevent anti-viral CTL-mediated autoimmune diabetes (IDDM) in a transgenic (tg) mouse model (6, 13). This approach had the advantage that the MHC class I-restricted CTL response, which is directed to the virus and, at the same time, leads to IDDM, is well characterized, and the fate of autoreactive cells and the development of IDDM, as well as the course of the triggering viral infection, could all be precisely followed in the presence and absence of peptide treatment.

Autoimmune diabetes in our tg model of the rat insulin promoter (RIP)-lymphocytic choriomeningitis virus (LCMV) H-2^b mice is caused by a D^b-restricted CTL response, in addition to other contributing factors such as APC activation, and does not develop in the absence of CD8^+ cells (12, 13). In these mice, viral proteins of LCMV are expressed as tg “self” proteins in β cells of the pancreatic islets of Langerhans using RIP. Peripheral unresponsiveness to the viral (self) proteins is maintained over the course of the animal’s life unless these tg mice are infected with LCMV, after which >95% develop IDDM (13). In the fast-onset RIP-glycoprotein (GP) mouse line used for this report, IDDM occurs within 10 to 14 days after LCMV infection independently of CD8^+ help, but depending on LCMV-specific CD8^+ CTL. It is important to point out that the viral GP expressed as the transgene is identical to the infecting LCMV sequence and therefore functions as a traceable model self-Ag. LCMV itself has not been implicated in human IDDM. Therefore, the RIP-GP rapid-onset IDDM model is specifically suited to dissect the potential role of MHC class I-restricted CTL, but does not take the role of CD4 lymphocytes or the genetic predisposition for IDDM into account. Thus, the advantage is that CTL responses generated by (H-2^b) RIP-GP mice after LCMV infection are well characterized. There are two major D^b-restricted GP responses to GP amino acid (aa) 33–43 (GP1) and aa
THERAPY WITH MHC CLASS I-RESTRICTED BLOCKING PEPTIDE PREVENTS IDDM

276–286 (GP2) and a minor K\(^{b}\)-restricted response to GP aa 34–41/43 (GP1) (24–26). The K\(^{b}\) response becomes prominent only after the D\(^{b}\) 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 TCR contact residues is an attractive strategy to use peptides as immune modulators for therapy. Thus, we designed a peptide with blocking properties that binds with high affinity to the TCR and 1 D\(^{b}\) allele, but neither activates the LCMV-specific D\(^{b}\)-restricted CTL repertoire nor exerts antagonist effects on LCMV-CTL (23, 25, 28). The sequence SMIE4LYM5 (SM) fits the D\(^{b}\) binding motif (23, 32, 33), but does not contain the crucial TCR interactive epitopes (aa positions 1, 4, and 8 (29, 34) of the LCMV-CTL epitopes (26, 35–37)).

Our results show that in vivo treatment of RIP-LCMV 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).

Materials and Methods

Transgenic lines

Generation and characterization of RIP-LCMV tg mice with rapid (8–14 days) and slow onset IDDM after LCMV infection has been described (6, 13). RIP-GP 34–20 (H-2\(^{d}\)) mice that express the viral (LCMV strain Armstrong (ARM) (ARM) GP) only in the β cells 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.

Virus

Virus stocks consisted of LCMV-ARM (clone 53b) and vaccinia virus (vv) recombinants that express the LCMV-GP aa 1–398 (vv/GP), LCMV-NP aa 1–558 (vv/NP), or the blocking peptide SMKNLLEYM (vv/SMKIEY). LCMV was plaque-purified three times on Vero cells and stocks prepared by a single passage on BHK-21 cells. Stocks of recombinant vv were prepared by infection of 143 TK\(^{-}\) cells in media containing bromodeoxyuridine (26, 39).

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/dl 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-butyloxycarbonyl or N-9-fluorenylmethoxycarbonyl (Fmoc) chemistry, purified by HPLC on a RP300-C8 reversed-phase column (Brownlee; PerkinElmer, Norwalk, CT) and identified by fast atom bombardment of electrospray mass spectrometry. The H-2\(^{d}\)-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\(^{b}\) or T-2K\(^{b}\) cells as described (32). Briefly, in competition assays, T-2D\(^{b}\) or T-2K\(^{b}\) cells (1 \(\times\) 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\(^{-10}\)–10\(^{-5}\) 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 mM unlabeled YAINEAEL. Specific binding to H-2D\(^{d}\) was defined as the difference between total and nonspecific bindings. Percent inhibition of binding was calculated as 100 \(\times\) (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 aprotinin) were added during incubation at 37°C to prevent a possible degradation of the peptides. Values are mean \(\pm\) 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 (KAVVYNFATC), GA aa 276–286 (SGFVPGVGCC), NP 396–404 (FPQPQNGQFI), NP 118–126 (RPQASGVMY), or blocking peptide (S9M) were also used as targets (25, 26, 24, 40). Epitopes GP 33–41, GP 276–286, and NP 396–404 of LCMV are all H-2\(^{d}\) restricted for CTL recognition, while NP 118–126 is restricted by the L\(^{d}\) allele. Assays used splenic lymphocytes 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 \(\times\) 10\(^{7}\) plaqueforming 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\(^{d}\)) and BALB/c I7 (H-2K\(^{d}\)) cells used as CTL targets were grown as reported (13). T-2 cells stable transfected with D\(^{b}\) or K\(^{b}\) 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 C57BL/6J mice and cultivated in the presence or absence of 10\(^{-4}\) M blocking peptide and IL-2. Cells were then harvested and 5 \(\times\) 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 Pharmingen, San Diego, CA). Briefly, 96-well Millititer HA plates (Millipore) were coated with capture Abs for IL-2. Cells were then harvested and 5 \(\times\) 10\(^{4}\) cells were incubated in 96-well plates (0.45 μm, Millipore, Bedford, MA) for 90 min at 37°C with 10 nM 125I-YAINEAEL and increasing concentrations (10\(^{-10}\)–10\(^{-5}\) 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 mM unlabeled YAINEAEL. Specific binding to H-2D\(^{d}\) was defined as the difference between total and nonspecific bindings. Percent inhibition of binding was calculated as 100 \(\times\) (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 aprotinin) were added during incubation at 37°C to prevent a possible degradation of the peptides. Values are mean \(\pm\) SE of at least three independent experiments.

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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 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, D<sup>b</sup>, insulin, CD<sub>4</sub>, CD<sub>8</sub>, 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 M1/42), and anti-class II (clone M5/114), (PharMingen and Boehringer Mannheim). (6) After washing in PBS, the secondary Ab (biotinylated goat anti-(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**

**9M peptide inhibits CTL killing of viral peptide-coated D<sup>b</sup> target cells—the efficacy correlates with the affinity of the epitope**

Binding affinities (IC<sub>50</sub> values) to D<sup>b</sup> and K<sup>b</sup> were assessed as described (32), which showed that 9M bound with high affinity to D<sup>b</sup>-expressing (IC<sub>50</sub>(D<sup>b</sup>) = 11 nM), but not to K<sup>b</sup>-expressing (IC<sub>50</sub>(K<sup>b</sup>) > 10,000 nM) T-2 cells. In comparison, the binding affinities for the LCMV peptides were as follows: NP (IC<sub>50</sub>(D<sup>b</sup>) = 15 nM; IC<sub>50</sub>(K<sup>b</sup>) > 10,000 nM), GP1 (IC<sub>50</sub>(D<sup>b</sup>) = 330 nM; IC<sub>50</sub>(K<sup>b</sup>) = 940 nM), and GP2 (IC<sub>50</sub>(D<sup>b</sup>) = 31 nM; IC<sub>50</sub>(K<sup>b</sup>) > 10,000 nM). Thus, the binding affinity of the blocking peptide to D<sup>b</sup> 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<sup>−13.5</sup> M for the NP compared with 10<sup>−7</sup> to 10<sup>−8.5</sup> M for the GP epitopes).

We then studied whether LCMV-specific killing of targets by activated CTL generated on the H<sup>2</sup><sup>b</sup> background was affected by 9M. Data presented in Table I show that three different LCMV clones are efficiently inhibited by this peptide in an affinity-dependent way. Preincubation with 9M did not reveal any antagonistic properties for LCMV (Table I). The results shown in Table II demonstrate that D<sup>b</sup> but not K<sup>b</sup> restricted LCMV-specific CTL killing can be blocked in vitro by 9M. Earlier studies had shown that D<sup>b</sup> and K<sup>b</sup> restrict the LCMV-GP1 epitope, whereas the LCMV-GP2 and -NP epitopes are restricted only by D<sup>b</sup> (25). These studies indicate that 9M is specific to the D<sup>b</sup> allele and does not affect K<sup>b</sup>-restricted responses. Although responses to all three D<sup>b</sup>-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, D<sup>b</sup>-restricted killing by LCMV-CTL was not affected by addition of an L<sup>d</sup>-restricted peptide (RPQASGVYM).

The K<sup>4</sup> analogue (SMIKLEYM) of 9M was then expressed using a vv recombinant. Our previous studies have shown that this analogue is as efficient in inhibiting LCMV-CTL as 9M (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 SMIKLEYM 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 9M acts as an allele-specific (D<sup>b</sup>), Ag-nonspecific blocking peptide without evidence for any antagonist properties in an affinity-dependent manner.

**9M 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, 9M abrogated this process (Table IV) and no CTL activities could be detected in cultures grown in the presence of 9M. 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 9M 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 9M-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, 9M effectively inhibits expansion and IFN-γ production by LCMV-CTL in vitro.

**In vivo administration of 9M reduces LCMV-specific D<sup>b</sup>-restricted CTL generation and prevents IDDM in RIP-LCMV-GPtg mice**

Non-tg C57BL6J (H<sup>2</sup><sup>b</sup>) andtg RIP-GP (H<sup>2</sup><sup>b</sup>) mice were injected with various amounts of 9M i.p. or i.v. to determine the optimal

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### Table I. H-2<sup>D<sup>b</sup></sup>-blocking peptide 9M inhibits primary LCMV-CTL responses depending on epitope affinity but has no antagonistic effects on LCMV-CTL<sup>a</sup>

<table>
<thead>
<tr>
<th>LCMV-CTL (LCMV agonist)</th>
<th>Epitope</th>
<th>Minimum Concentration (M) of Agonist Peptide for 10&lt;sup&gt;10&lt;/sup&gt;&lt;sup&gt;−&lt;/sup&gt;Cr Release</th>
</tr>
</thead>
<tbody>
<tr>
<td>LCMV-GP1 clone</td>
<td>KAVYNFATC</td>
<td>10&lt;sup&gt;−7&lt;/sup&gt;</td>
</tr>
<tr>
<td>LCMV-GP2 clone</td>
<td>SGVENPGYCYL</td>
<td>10&lt;sup&gt;−8&lt;/sup&gt;</td>
</tr>
<tr>
<td>LCMV-NP clone</td>
<td>FQPQNGQFI</td>
<td>10&lt;sup&gt;−8&lt;/sup&gt;</td>
</tr>
<tr>
<td>LCMV day 7 bulk (50:1)</td>
<td>GP-1/GP-2</td>
<td>10&lt;sup&gt;−6&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>NP</td>
<td>10&lt;sup&gt;−12&lt;/sup&gt;</td>
</tr>
<tr>
<td>LCMV-GP1 clone (9M preincubated)</td>
<td>KAVYNFATC</td>
<td>10&lt;sup&gt;−7&lt;/sup&gt;</td>
</tr>
<tr>
<td>LCMV-NP clone (9M preincubated)</td>
<td>FQPQNGQFI</td>
<td>10&lt;sup&gt;−13.5&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> 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 9M (10<sup>−8</sup> 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-2<sup>d</sup> fibroblasts in a 5-h 5<sup>11</sup>Cr release assay is displayed in the presence and absence of 9M. Preincubation of LCMV-specific clones with 9M (10<sup>−6</sup> 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 5<sup>11</sup>Cr release values from 9M treated or untreated clones did not yield any significant differences (data not shown). Similarly, preincubation of effector CTL with “cold” targets coated with 9M did not affect CTL killing. These findings show no evidence for an antagonistic effect of 9M 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 × 10^5 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 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 blocked 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 mice that did or did not receive treatment with S9M. As shown in Figure 2, infiltration into the islets was only observed in untreated mice that did or did not receive treatment with S9M. As shown in Figure 2, infiltration into the islets was only observed in untreated mice that did or did not receive treatment with S9M.

In contrast, lymphocytes were

<table>
<thead>
<tr>
<th>Specific Cr51 Release (%) from Targets</th>
<th>T-2Dβ</th>
<th>T-2Kβ</th>
</tr>
</thead>
<tbody>
<tr>
<td>GP1 (10^-5 M)</td>
<td>NP (10^-5 M)</td>
<td>GP1 (10^-5 M)</td>
</tr>
<tr>
<td>SMIELENYM concentration (M)</td>
<td>0</td>
<td>10^-5</td>
</tr>
<tr>
<td>Splenocytes day 7 LCMV</td>
<td>22 ± 5</td>
<td>3 ± 1</td>
</tr>
<tr>
<td>H-2β RIP-GP</td>
<td>25 ± 6</td>
<td>5 ± 4</td>
</tr>
</tbody>
</table>

* Splenocytes were harvested from C57BL/6J and RIP-GP tg mice 7 days after infection with 1 × 10^5 pfu LCMV-ARM i.p. and tested for LCMV-specific CTL activity in a 51Cr release assay (see Materials and Methods). Target cells were T-2 cells transfected with Dβ or Kβ (see Materials and Methods) and allogeneic BALB/c17 (H-2β) cells (data not shown) that were coated or not with S9M immediately before starting the 5-h assay. Effector splenocytes were mixed with LCMV-CTL peptides GP1, GP2 (not shown), and NP immediately 1 h before the assay to test blocking properties of S9M for targets already loaded with peptides and added at an E:T ratio of 50:1. The blocking peptide (S9M) and LCMV peptides GP1, GP2 (not shown), and NP were used at a final concentration, as indicated, that induced maximal specific lysis of the target cells for the LCMV agonist peptides (25).

**Table II. In vitro administration of H-2Dβ blocking peptide S9M inhibits primary Dβ but not Kβ restricted LCMV-CTL response in (H-2β) mice**

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

* MC57 H-2β 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 vitro (23). 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.
found around the islets in S9M-treated mice without IDDM (Fig. 2, A–D). Fewer CD8+ 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+ 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.

### Table IV. H-2Db-blocking peptide S9M inhibits expansion of primary Db-restricted LCMV-CTL in vitro

<table>
<thead>
<tr>
<th>Splenocytes Post-LCMV Infection of H-2b mice</th>
<th>8-Day In vitro Stimulation with IL-2 ± Peptide</th>
<th>Specific ³¹Cr Release (%) from Targets H-2b (D¹,K¹)+</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LCMV GP1 NP</td>
<td></td>
</tr>
<tr>
<td>C57BL6/J, day 180</td>
<td>No peptide</td>
<td>44 ± 3</td>
</tr>
<tr>
<td></td>
<td>GP-1 and NP (H-2b)</td>
<td>70 ± 8</td>
</tr>
<tr>
<td></td>
<td>S9M (H-2b)</td>
<td>5 ± 2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>49 ± 7</td>
</tr>
<tr>
<td>C57BL6/J, day 7 control</td>
<td>No peptide</td>
<td></td>
</tr>
</tbody>
</table>

* C57BL6/J H-2b mice were immunized with 1 x 10⁶ 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 ³¹Cr 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 (10⁻⁴ 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).

### Table V. H-2Db-blocking peptide S9M inhibits expansion of LCMV-CTL in vivo

<table>
<thead>
<tr>
<th>Group</th>
<th>Peptide treatment</th>
<th>Effector</th>
<th>E/T</th>
<th>LCMV</th>
<th>GP1</th>
<th>GP2</th>
<th>NP</th>
<th>T-2(D¹)</th>
<th>T-2(K¹)</th>
<th>H-2b</th>
<th>Precursor CTL and Cytokine Production</th>
</tr>
</thead>
<tbody>
<tr>
<td>Splenocytes from H-2b mice day 7 post-LCMV C57BL6/J</td>
<td>None</td>
<td>50:1</td>
<td>60 ± 7</td>
<td>50 ± 6</td>
<td>20 ± 8</td>
<td>39 ± 8</td>
<td>64</td>
<td>56</td>
<td>0</td>
<td>no</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>25:1</td>
<td>38 ± 6</td>
<td>28 ± 7</td>
<td>12 ± 5</td>
<td>30 ± 6</td>
<td>52</td>
<td>53</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RIP-GP</td>
<td>No</td>
<td>50:1</td>
<td>60 ± 8</td>
<td>42 ± 9</td>
<td>23 ± 7</td>
<td>40 ± 4</td>
<td>58</td>
<td>60</td>
<td>0</td>
<td>100%</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>25:1</td>
<td>34 ± 7</td>
<td>22 ± 5</td>
<td>10 ± 3</td>
<td>20 ± 8</td>
<td>50</td>
<td>52</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SMIENLEYM 1 mg i.p. (daily for 2 wk)</td>
<td></td>
<td>50:1</td>
<td>12 ± 6</td>
<td>13 ± 5</td>
<td>5 ± 4</td>
<td>12 ± 5</td>
<td>26 ± 8</td>
<td>54 ± 9</td>
<td>0</td>
<td>0%</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>25:1</td>
<td>9 ± 5</td>
<td>10 ± 4</td>
<td>0</td>
<td>5 ± 4</td>
<td>20</td>
<td>56</td>
<td>0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

| Splenocytes from H-2b mice day 7 or 45 post-LCMV C57BL6/J | None              | 1/90 | 1/3,000 | <1/10¹⁵ | <1/40,000 | 2,000 ± 670 | <20 |
| RIP-GP                      | No                | 1/120 | ND | <1/10¹⁵ | <1/40,000 | 2,300 ± 560 | <20 |
| SMIENLEYM 1 mg i.p.         |                   | 1/3,200 | 1/2,800 | 1/9,000 | 1/10,000 | 750 ± 80 | <20 |

* Groups of 3 to 6 C57BL6/J (H-2b), RIP-LCMV-GP tg mice were infected with 1 x 10⁶ 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 ³¹Cr release from target cells ± 1 SE. Groups of two mice were tested for killing of T-2 cells transfected with either D¹ or K¹ and coated with LCMV-GP1 peptide. Results demonstrate the specificity of the S9M effect in vivo for D¹. IDDM was assessed by Accuchek (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. S9M, and T cell growth factor (containing IL-2). In contrast, de-
activities could not be recovered after such immunizations, and were not “rescued” by in vitro stimulation in the presence of APC,
levels of S9M, as well as treatment with an L2-restricted peptide, did not lead to prevention of IDDM.

Treatment with S9M does not induce regulatory cells, but H-2b-restricted, IFN-γ-producing CTL that do not protect from IDDM when adoptively transferred
Mice were immunized with S9M alone or S9M in IFA once or daily for 2 wk. Results (Table VI) show that S9M-specific CTL activities could not be recovered after such immunizations, and were not “rescued” by in vitro stimulation in the presence of APC, S9M, and T cell growth factor (containing IL-2). In contrast, detectable levels of CTL were recovered in a parallel experiment induced by immunization with LCMV-GP1 and -GP2 peptides (Table VI). Thus, S9M is not capable of significantly activating the H-2b-CTL repertoire in vivo when administered daily for 2 wk
with or without IFA.

However, CTL activity to the S9M peptide was observed in mice that received both peptide treatment and infection with LCMV (precursor CTL 1/9000 at day 7 (Table V), and 1/150 after stimulation of memory splenocytes in the presence S9M (Table VI)). In contrast, no detectable levels of CTL against S9M were found in cultures from non-S9M-treated mice (precursor CTL <1/40,000; Table VI). Next, ELISA were used to compare cytokine profiles from supernatants of cells cultures either with LCMV peptides or S9M. IL-4 production was not detected, but cultures grown in the presence of S9M produced significant amounts of IFN-γ. A secondary cell line specific for S9M was established in vitro. These CTL continued to produce IFN-γ and killed S9M-coated targets with high specificity. Adoptive transfer of 108 of these CTL that recognized S9M into RIP-LCMV-GP- infected recipients did not influence the incidence of IDDM (>95%), indicating that the S9M-specific lymphocytes were unable to regulate IDDM in vivo (data not shown). Analysis of other organs (kidney, liver, heart, lung, and brain) showed that these S9M-CTL were neither autoreactive nor pathogenic, because no significant infiltration was noted in any of the organs after adoptive transfer of 5-bromodeoxyuridine-labeled CTL.

These observations were complemented by experiments showing that preimmunization of RIP-GP mice 35 days before LCMV infection with 100 μg of S9M in IFA did not lower LCMV-induced CTL nor protect from IDDM (data not shown), suggesting that the protection is most likely due to MHC blockade but not to long-lived regulatory cells.

Virus is cleared in LCMV-infected mice treated with S9M 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 Kβ-restricted LCMV-GP1-specific CTL and remaining Dβ-restricted CTL directed mainly to LCMV-NP.

S9M is only effective for treating IDDM initiated by CTL reactive for a lower-affinity epitope
The affinity of CTL recognition in vitro is 10−13.3 M for the NP but 10−8.5 M to 10−7 M for both Dβ-restricted GP1 and GP2 epitopes, respectively, indicating that NP-CTL are probably more difficult to block by S9M. Indeed, as shown in Tables I and II, while S9M reduced CTL lysis of Dβ targets coated with GP1 or GP2 peptides by 10-fold, it only reduced NP-directed CTL by 3-fold. This finding correlated well with the 20-fold higher binding affinity of the LCMV-NP peptide to Dβ (15 nM) compared with the -GP1 (330 nM) and -GP2 epitopes (31 nM). In parallel studies, while 2 wk of daily therapy with S9M prevented IDDM in RIP-GP mice (incidence of IDDM = 0%), the same protocol did not reduce IDDM in RIP-NP mice (incidence > 90%).

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 10−13.3 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.

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 (Dβ) 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 Db-restricted epitopes located in GP1 (aa 33–41/43), GP2 (aa 276–286), and NP (aa 396–404). Using the Db-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-γ 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 β cells (Table V). Some additional effect by directly locally blocking Db-MHC molecules expressed on β 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 Db-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
viral peptide necessary for activated T cells to lyse the infected cell or for naïve 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 viral peptide necessary for activated T cells to lyse the infected cell or for naïve 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 viral peptide necessary for activated T cells to lyse the infected cell or for naïve T cells to be activated and expand. 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**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 &lt;sup&gt;31&lt;/sup&gt;Cr 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;)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Peptide alone, day 35&lt;sup&gt;b&lt;/sup&gt;</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No peptide</td>
<td>GP-1 and NP (H-2&lt;sup&gt;b&lt;/sup&gt;) 0 0 0 0 0 0 0</td>
<td></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;) 27 18 0 17 0</td>
<td></td>
</tr>
<tr>
<td>GP-1 peptide 100 μg/IFA i.p.</td>
<td>GP-1 and GP-2 (H-2&lt;sup&gt;b&lt;/sup&gt;) 20 22 0 16 0</td>
<td></td>
</tr>
<tr>
<td>S9M peptide 100 μg/IFA i.p.</td>
<td>SMIENLEYM (H-2&lt;sup&gt;b&lt;/sup&gt;) 2 3 1 1 1 0</td>
<td></td>
</tr>
<tr>
<td><strong>Peptide and LCMV infection&lt;sup&gt;c&lt;/sup&gt;</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C57Bl/6J, day 45-180 (no peptide)</td>
<td>No peptide 1:1,000 ND + −</td>
<td></td>
</tr>
<tr>
<td>GP-1 and NP (H-2&lt;sup&gt;b&lt;/sup&gt;) 1:1,800 1:140,000 + + + −</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S9M peptide (H-2&lt;sup&gt;b&lt;/sup&gt;) 1:6,800 1:140,000 − − − −</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RIP-GP, day 45-180 (SMIENLEYM 1 mg daily for 2 wk)</td>
<td>No peptide 1:5,000 1:20,000 + −</td>
<td></td>
</tr>
<tr>
<td>GP-1 and NP (H-2&lt;sup&gt;b&lt;/sup&gt;) 1:800 1:7,000 + + + −</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S9M peptide (H-2&lt;sup&gt;b&lt;/sup&gt;) &lt;1:140,000 1:500 + + + −</td>
<td></td>
<td></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 GP1/NP or S9M peptides (see Materials and Methods). CTL activities were then tested in a <sup>31</sup>Cr 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>9</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 manufactures instructions. + + +, >2000 U/ml; +, 1000–2000 U/ml; +, 40–1000; −, not detectable (<20 U/ml).

<sup>c</sup> The generation of MHC (D<sup>b</sup>)-restricted LCMV-specific memory precursor CTL and cytokines (8-day in vitro stimulation)
tested 7 days after primary infection, equivalent levels of LCVM-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 (D$^3$)-restricted T lymphocytes and no enhancement of immunopathology or immunological memory. Importantly, not all autoggressive 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

We thank Diana Frye for help with the manuscript.

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heterogeneity, modes of inheritance, and risk estimates for a joint study of Cau-

diabetes mellitus in non-obese diabetic mice by immunogenic but not tolerated

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