Immunity, Vol. 6, 519–529, May, 1997, Copyright ©1997 by Cell Press

Peptide Antigen Treatment of Naive and Virus-Immune Mice: Antigen-Specific Tolerance Versus Immunopathology

Peter Aichele,* Karin Brduscha-Riem,* Stephan Oehen,* Bernhard Odermatt,† Rolf M. Zinkernagel,* Hans Hengartner,* and Hanspeter Pircher‡
*Institute of Experimental Immunology
Department of Pathology
†Department of Pathology
University of Zürich
CH-8091 Zürich
Switzerland

Summary

Peptide-specific down-regulation of T cell responses may represent a powerful tool to intervene in autoimmune diseases or graft rejections. It is therefore important to know whether peptide treatment tolerizes both naive and antigen-experienced memory T lymphocytes. Here we show that a major histocompatibility complex class I binding peptide, derived from the glycoprotein (GP33 peptide) of lymphocytic choriomeningitis virus (LCMV), specifically tolerized naive cytotoxic T lymphocytes (CTL) when administered three times intraperitoneally in incomplete Freund's adjuvants. However, in the presence of GP33-specific memory CTL in LCMV-primed mice, the same treatment had a general immunosuppressive effect on unrelated third-party antigen-specific T cell responses and caused severe immunopathological damage to the spleen.

Introduction

The function of the immune system is to eliminate foreign pathogens while maintaining unresponsiveness against self-antigens. T cell tolerance is achieved by both thymic and peripheral processes. Thymocytes reactive for self-peptides are eliminated by clonal deletion (Kappler et al., 1987; Kappler et al., 1988; Kisielow et al., 1988; MacDonald et al., 1988; Sha et al., 1988; Pircher et al., 1989) or are functionally inactivated (Blackman et al., 1990; Roberts et al., 1990; Ramsdell et al., 1989). However, central tolerance is incomplete because of the absence of particular self-peptides in the thymus. Therefore, potentially autoreactive T cells will reach the periphery, where they may be tolerized by several mechanisms upon exposure to their specific antigen (Burkly et al., 1989; Rammensee et al., 1989; Rocha and von Boehmer, 1991; Miller and Morahan, 1992; Hämmerling et al., 1993). T cell tolerance is not established toward self-antigens located in immunologically sequestered sites (Ohashi et al., 1991; Oldstone et al., 1991) or to those expressed below a critical concentration level (Schild et al., 1990; Ferber et al., 1994; Oehen et al., 1994). Thus, although autoreactive T cells are present in normal individuals (Schluesener and Wekerle, 1985; Pette et al., 1990; Aichele et al., 1996), they are harmless as long as the self-antigen is not presented by professional antigen-presenting cells in lymphoid tissues. However, genetic predisposition, injuries, or exposure to cross-reactive antigens may activate these T cells and cause autoimmune diseases (Londei et al., 1985; Sinha et al., 1990; Wucherpfennig and Strominger, 1995).

In such instances, it would be desirable to manipulate the immune system in an antigen-specific manner by specifically down-regulating autoreactive or graft-rejecting T cells without affecting other cells of the immune system. To achieve T cell tolerance as a therapeutic goal, it is essential to identify the antigens recognized by the disease-mediating T cells (Rötzschke et al., 1990; Jardetzky et al., 1991; Rudensky et al., 1991; Rammensee et al., 1993a, 1993b) and to define protocols for antigen application that specifically tolerize naive as well as antigen-experienced T cells.

Numerous reports have shown that it is possible to tolerize naive $CD4^+$ T cells in vivo (Dresser, 1962; Mitchison, 1964; Rajewsky and Brenig, 1974; Oki and Sercarz, 1985; Ria et al., 1990; Burstein and Abbas, 1993; Romball and Weigle, 1993; Kearney et al., 1994; Vidard et al., 1994; Chen et al., 1995) and hence to prevent the development of autoimmune responses or allergic reactions in various animal models (Wraith et al., 1989; Hoyne et al., 1993; Kaufman et al., 1993; Metzler and Wraith, 1993; Miller et al., 1991; Tisch et al., 1993; Weiner et al., 1994). Peptide-specific immunotherapy in an ongoing disease by down-regulation of activated $CD4^+$ T cells has so far succeeded only in the murine model of experimental autoimmune encephalomyelitis (Smilek et al., 1991; Gaur et al., 1992). Recent studies have shown that repetitive, systemic administration of peptides results in peripheral deletion of naive $CD8^+$ cytotoxic T lymphocytes (CTL) in vivo (Kyburz et al., 1993; Mamalaki et al., 1993; Aichele et al., 1995) and can prevent a virus-induced T cell-mediated autoimmune diabetes in a transgenic mouse model (Aichele et al., 1994). However, the question of whether antigen-experienced, memory $CD8^+$ T lymphocytes can be tolerized with peptide antigens in vivo has not yet been addressed.

We therefore have used a major histocompatibility complex class I (D^b) binding peptide derived from the glycoprotein GP33–41 (GP33 peptide) of lymphocytic choriomeningitis virus (LCMV) as a model antigen, to study whether antigen-experienced, memory $CD8^+$ T lymphocytes are also susceptible to peptide-induced tolerance.

Results

Peptide Treatment of Naive and of LCMV-Primed Mice to Induce T Cell Tolerance

The effect of LCMV GP33 peptide treatment on naive and LCMV-primed CTL was compared using a protocol for peptide administration that has been established

‡Present address: Institute of Med. Microbiology and Hygiene, University of Freiburg, D-79104 Freiburg, Germany.

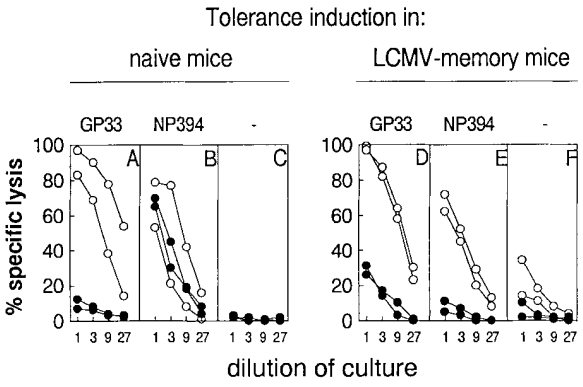

Figure 1. Peptide Treatment of Naive and LCMV-Primed Mice

In the first group, unprimed C57BL/6 mice (A–C) were treated intraperitoneally three times in 3-day intervals with IFA (open circles) or 500 μg GP33 peptide in IFA (closed circles). Five days after the last peptide treatment the mice were infected intravenously with 200 pfu LCMV-WE, and 30 days later spleen cells of these mice were restimulated in vitro for 5 days with irradiated LCMV-infected peritoneal macrophages. In the second group, LCMV-primed mice (day 30 after LCMV infection) (D–F) were injected intraperitoneally three times in 3-day intervals with IFA (open circles) or with 500 μg GP33 peptide in IFA (closed circles). Five days after the last peptide application, spleen cells of these mice were restimulated in vitro for 5 days with irradiated LCMV-infected peritoneal macrophages as described in Experimental Procedures. CTL activities of effector cells from both groups were tested on GP33-loaded (A and D), NP394-loaded (B and E), or unloaded (C and F) MC57G target cells at the indicated dilutions.

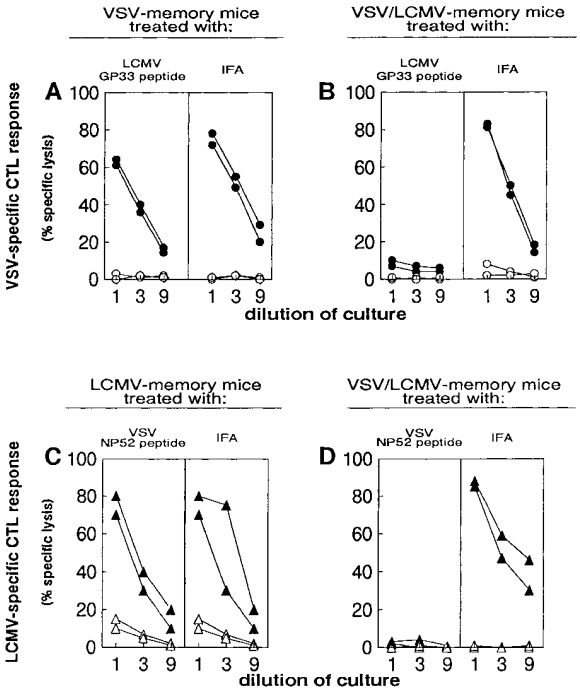

Figure 2. Immunosuppressive Effect of Peptide Treatment on Unrelated Memory CTL Responses

VSV-primed mice (A) and mice primed for both VSV and LCMV (B) were injected intraperitoneally three times at 3-day intervals with either 500 μg LCMV GP33 peptide in IFA (A and B, left) or IFA alone (A and B, right). Five days after the last peptide treatment the spleen cells were restimulated in vitro for 5 days with the VSV nucleoprotein expressing cell line N1 and tested for VSV-specific killing in a ^{51}Cr release assay on unloaded (open circles) or VSV NP52-loaded (closed circles) EL-4 target cells. LCMV-primed mice (C) and mice primed for both VSV and LCMV (D) were treated three times in 3-day intervals with either 500 μg of VSV NP52 peptide in IFA (C and D, left) intraperitoneally or IFA alone (C and D, right). Spleen cells from these mice were restimulated in vitro for 5 days with LCMV-infected peritoneal macrophages and tested then for LCMV-specific cytolysis on unloaded (open triangles) or LCMV GP33-loaded (closed triangles) EL-4 target cells.

previously to tolerize naive CTL in vivo (Aichele et al., 1995). Unprimed C57BL/6 mice (Figures 1A–1C) were treated three times at 3-day intervals with 500 μg of GP33 peptide in incomplete Freund's adjuvants (IFA) intraperitoneally. Five days after the last peptide treatment, mice were infected with LCMV to expand LCMV-specific CTL efficiently in vivo. Thirty days after infection, spleen cells from these mice were restimulated in vitro with LCMV-infected macrophages, and LCMV-specific CTL activities were determined. Control mice, pretreated with IFA alone, exhibited high CTL activities against both GP33 peptide–loaded and NP394–408 (NP394 peptide)–loaded target cells (Figures 1A and 1B, open circles). In contrast, GP33 peptide–treated mice were unable to generate GP33-specific CTL activities, while the CTL response against NP394 peptide–loaded target cells was within the normal range (Figures 1A and 1B, closed circles). Thus, GP33 peptide treatment tolerized naive CTL in vivo in an antigen-specific manner.

To examine whether GP33-specific memory CTL could be similarly tolerized, mice primed 30 days previously with LCMV were treated with GP33 peptide in IFA as described above (Figures 1D–1F). Spleen cells from these mice were restimulated in vitro, and LCMV-specific CTL activities were determined. The analysis of CTL responses specific for GP33 or NP394 epitopes revealed that both were strongly reduced in LCMV-primed mice after treatment with the GP33 peptide (Figures 1D and 1E, closed circles), whereas they were unimpaired in LCMV-primed control mice treated with IFA alone (Figures 1D and 1E, open circles). Thus, while naive CTL were efficiently rendered tolerant, GP33 peptide treatment of memory CTL in LCMV-primed mice induced an immunosuppression, since the LCMV GP33-specific and NP394-specific CTL responses were equally abrogated. Therefore, GP33 peptide treatment of virus-primed mice caused an immunosuppression rather than an antigen-specific unresponsiveness or tolerance.

Analysis of Immune Suppression after Peptide Treatment of Specific Memory CTL

To examine whether treatment of memory CTL with specific peptide antigen suppresses not only CTL responses to the same virus but also unrelated third-party antigen-specific CTL responses, mice primed for both vesicular stomatitis virus (VSV) and LCMV were treated with the LCMV GP33 peptide, and the effect of this treatment on the VSV-specific CTL response was analyzed. VSV-primed mice treated with LCMV GP33 peptide (Figure 2A, left) generated VSV NP52–59 (NP52 peptide)–specific CTL responses comparable to those of VSV-primed mice treated with IFA alone (Figure 2A, right), indicating that LCMV GP33 peptide treatment itself was neither immunosuppressive nor toxic. However,

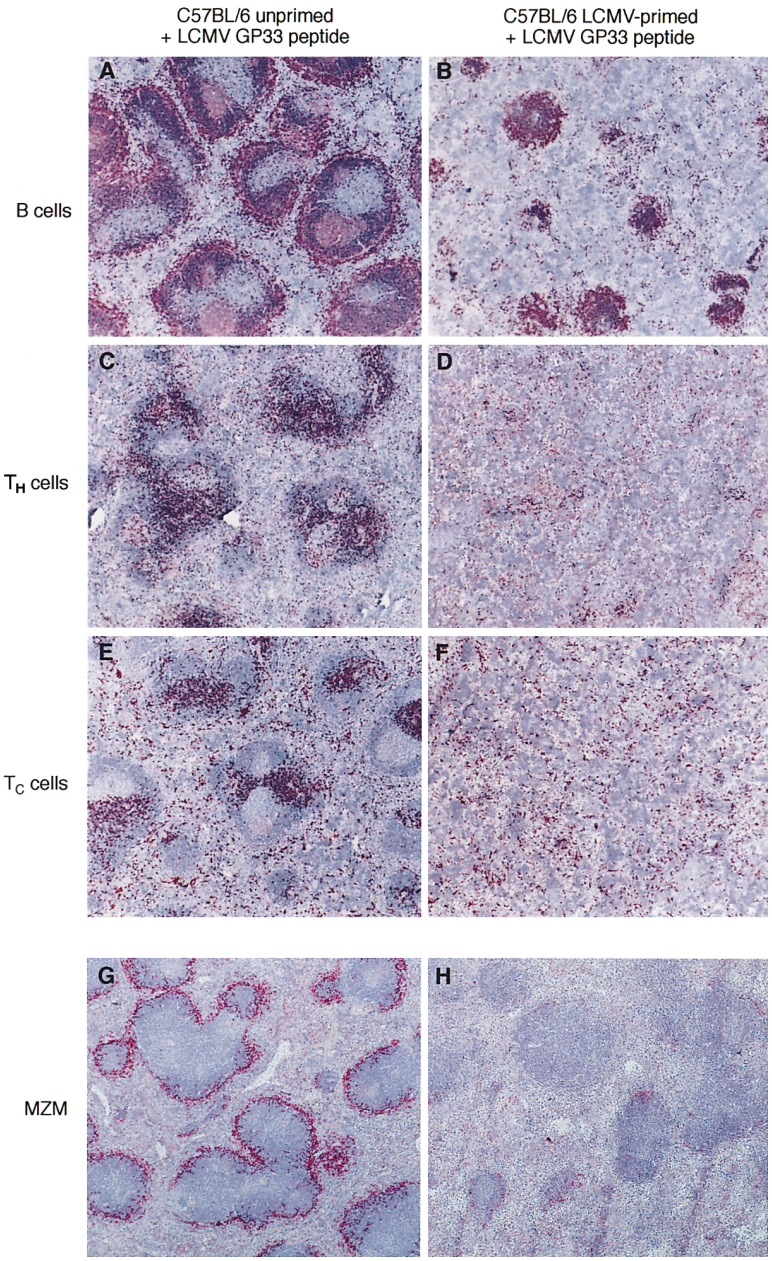

Figure 3. Immunohistological Analysis of Spleen Sections from Naive and LCMV-Primed Mice Treated with LCMV GP33 Peptide

Naive and LCMV-primed C57BL/6 mice were treated intraperitoneally three times at 3-day intervals with 500 μg of GP33 peptide in IFA. Three days after the last peptide treatment, spleens were removed, snap-frozen in liquid nitrogen, and cut into 5 μm sections. Spleen sections from GP33 peptide–treated naive (A, C, and E) and LCMV-primed mice (B, D, and F) were stained with monoclonal antibodies specific for B220 (A and B), CD4 (C and D), and CD8 (E and F). Metallophilic marginal zone macrophages were already stained with monoclonal antibody MOMA-1 after the first application of GP33 peptide (G and H).

when mice primed with both VSV and LCMV were treated with LCMV GP33 peptide, the VSV-specific CTL response was completely abrogated (Figure 2B, left), while control IFA-treated VSV/LCMV memory mice had normal VSV-specific CTL activities (Figure 2B, right). Thus, only in the presence of LCMV-specific memory CTL did the application of LCMV GP33 peptide exhibit a nonspecific immunosuppressive effect on unrelated third-party antigen-specific CTL responses.

To test whether this phenomenon was unique for the LCMV GP33 peptide treatment, mice primed with both VSV and LCMV were treated intraperitoneally three times with the VSV NP52 peptide, and LCMV-specific CTL activities were examined. Mice primed with VSV and LCMV failed to generate LCMV-specific CTL activities when treated with VSV NP52 peptide (Figure 2D, left), while IFA treatment alone did not affect the LCMV-specific CTL response (Figure 2D, right). In contrast, mice primed with LCMV only, when treated with VSV NP52 peptide, mounted a normal LCMV-specific CTL response against target cells loaded with LCMV GP33 peptide (Figure 2C, left). These findings confirm that only in the presence of the specific memory CTL population did administration of the corresponding peptide antigen lead to nonspecific suppression of unrelated CTL responses in vivo.

LCMV GP33 Peptide Treatment of LCMV-Primed Mice Induced Severe Immunopathology in the Spleen

To investigate the possible mechanisms underlying the observed immunosuppression, frozen sections of spleens

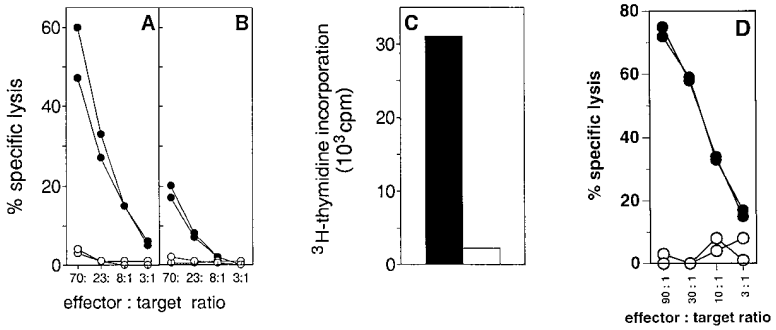

Figure 4. Activation of GP33-Specific Memory CTL after a Single Peptide Injection and In Vivo Loading of Spleen Cells and Peritoneal Macrophages with Peptide

LCMV-primed mice (30–50 days after infection) were treated intravenously with 500 μg of GP33 peptide in HBSS (A) or left untreated (B). Twenty-four hours later the ex vivo CTL activity of splenocytes was tested on GP33-loaded (closed circles) or unloaded (open circles) EL-4 target cells at the indicated effector- to- target ratios in a 5 hr standard ^{51}Cr release assay.

(C) Splenocytes of GP33 peptide–treated (closed bar) or untreated (open bar) LCMV-primed mice were irradiated (3000 rad) and used as stimulators for GP33-specific responder CTL in a proliferation assay.

(D) Peritoneal macrophages of C57BL/6 mice treated intraperitoneally either with 500 μg of GP33 peptide in IFA (closed circles) or with IFA alone (open circles) were removed 24 hr later and used as target cells in a ^{51}Cr release assay. In vivo sensitization of peritoneal macrophages with peptide was assessed with splenocytes of acutely LCMV-infected mice as effector cells.

from naive and LCMV-primed mice that had been treated three times with GP33 peptide intraperitoneally according to the standard protocol were stained with monoclonal antibodies for B220, CD4, and CD8 (Figure 3). Unprimed mice treated with GP33 peptide exhibited normal splenic architecture: the white pulp contained distinct primary B cell follicles, germinal centers, marginal zone B lymphocytes, and T cell–rich periarteriolar lymphoid sheaths (Figures 3A, 3C, and 3E). In striking contrast, evaluation of spleen sections from LCMV-primed mice treated with GP33 peptide revealed extensive destruction of lymphoid follicle structures. The size and number of B cell follicles was partially reduced (Figure 3B), and the T cell–rich region of the periarteriolar lymphoid sheaths was undefinable. The remaining $CD4^+$ and $CD8^+$ T lymphocytes were randomly distributed throughout the whole organ (Figures 3D and 3F). Histological analysis of different splenic antigen–presenting cells revealed that the metallophilic marginal zone macrophages (stained with monoclonal antibody MOMA-1) were absent in LCMV-primed mice after GP33 peptide treatment, whereas the dendritic cell populations (stained with monoclonal antibody N418) were only slightly reduced, when compared with GP33 peptide–treated naive mice (data not shown). Metallophilic marginal zone macrophages had already disappeared in some cases after the first peptide application (Figures 3G and 3H). Thus, peptide treatment of specific memory CTL induced severe immunopathological damage to the spleen. This finding may well explain the observed suppressive effect on unrelated, third-party antigen-specific CTL responses.

GP33-Specific Memory CTL Are Activated after a Single Injection of Peptide

To provide further evidence that the peptide-induced immunopathology was caused by GP33-specific memory CTL, LCMV-primed mice were treated once with GP33 peptide (Figure 4A) or were left untreated (Figure 4B). After 24 hr, the GP33-specific ex vivo CTL activities of splenocytes were assayed in a 5 hr standard ^{51}Cr release assay on GP33-loaded (closed symbols) or unloaded (open symbols) EL-4 target cells. Spleen cells from untreated LCMV-primed mice exhibited a low level of GP33 peptide–specific CTL activity ex vivo (Figure 4B), whereas LCMV-primed mice treated with GP33 peptide generated strong GP33-specific CTL responses (Figure 4A) when assayed within 24 hr after peptide treatment.

To evaluate the efficiency of peptide loading in vivo after peptide injection, the same spleen cells from peptide-treated LCMV-primed mice were used as stimulator

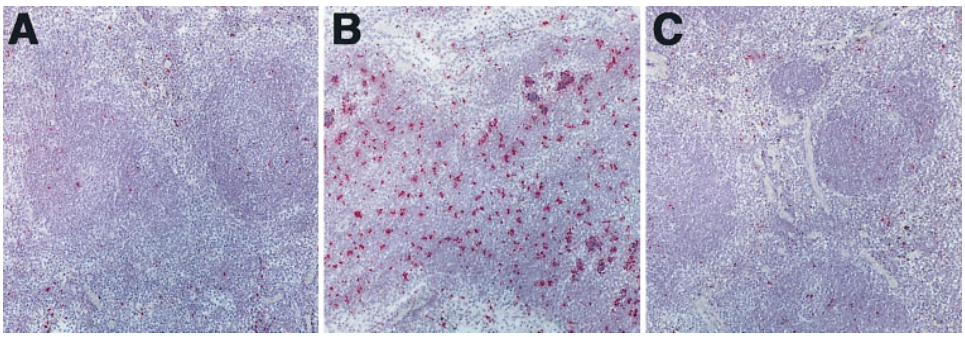

Figure 5. Detection of Apoptotic Cells in Spleens from Peptide-Treated LCMV-Primed Mice

Naive (A) and LCMV-primed (B) C57BL/6 mice were treated once with 500 μg of GP33 peptide and killed after 14 hr, and the spleens were snap frozen in liquid nitrogen. Five-micron spleen sections were stained for apoptotic cells with the TUNEL assay as described in Experimental Procedures. Spleen sections from untreated LCMV-primed mice were used as negative controls (C).

cells to provoke proliferation of specific T cells in an in vitro assay (Figure 4C). Responder CTL from transgenic mice expressing a GP33-specific T cell receptor (TCR) were strongly stimulated by irradiated spleen cells from mice injected 24 hr previously with GP33 peptide (Figure 4C, closed bar), but not by spleen cells from untreated controls (Figure 4C, open bar). To further address the question of whether peptide administration is sufficient to sensitize cells in vivo for CTL-mediated lysis, peritoneal macrophages from peptide treated or untreated mice were used as target cells in ^{51}Cr release assays. As shown in Figure 4D, peritoneal macrophages from GP33 peptide–treated mice were efficiently lysed by LCMV-specific effector cells, whereas the corresponding macrophage population from untreated mice was not susceptible to killing.

These results indicate that GP33 peptide treatment of LCMV-primed mice rapidly activated GP33-specific memory CTL and simultaneously loaded spleen cells in vivo with the GP33 peptide. It is tempting to speculate that these peptide-loaded spleen cells may serve as target cells in vivo for the GP33 peptide–activated memory CTL, which may explain the observed immunopathology after GP33 peptide administration in LCMV-primed mice. To test this hypothesis, frozen sections of spleens from GP33 peptide–treated, naive, or LCMV-primed mice were examined for apoptotic cells using the terminal deoxynucleotidyl transferase (TdT)–mediated dUTP-biotin nick end labeling (TUNEL) assay. Immunohistological analysis of spleen sections from LCMV-primed mice revealed a high number of apoptotic cells 12–24 hr after the GP33 peptide administration, mainly localized in the lymphoid follicles of the white pulp (Figure 5B). In contrast, almost no apoptotic cells could be detected in spleen sections from GP33 peptide–treated naive mice (Figure 5A), demonstrating that the peptide treatment itself is not toxic to spleen cells. Furthermore, only a few apoptotic cells could be found in untreated LCMV-primed control mice (Figure 5C). Thus, the high number of apoptotic cells in the spleens of LCMV-primed mice may be a direct consequence of the peptide application, which readily activated the specific memory CTL and in parallel loaded the spleen cells with GP33 peptide, thereby sensitizing them for CTL-mediated lysis.

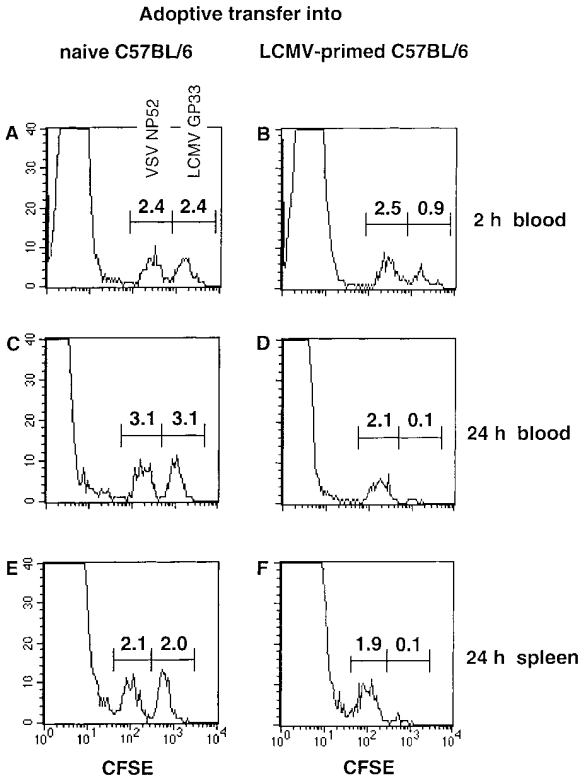

Figure 6. Antigen-Specific and Contact-Dependent Elimination of an Indicator Cell Population in LCMV-Primed Mice

C57BL/6 mice were treated intraperitoneally either with 500 μg LCMV GP33 peptide or VSV NP52 peptide in IFA for peptide antigen loading of spleen cells in vivo. Fifteen hours after the peptide application, spleens were removed. Splenocytes from LCMV GP33 peptide–treated mice were labeled with CFSE at a high concentration, and spleen cells from VSV NP52 peptide–treated mice were labeled with a low CFSE concentration. This resulted in two cell populations of different CFSE fluorescence intensities. The two cell populations were mixed 1:1, and 6×10^7 cells were transferred intravenously as indicator cells into naive or LCMV-primed mice that had been pretreated intraperitoneally with 500 μg of GP33 peptide in IFA 5 hr before. Lymphocytes from naive (A, C, and E) and LCMV-primed mice (B, D, and F) were taken 2 and 24 hr after transfer and analyzed by flow cytometry.

Peptide-Mediated Immunopathology in LCMV-Primed Mice Is an Antigen-Specific and Contact-Dependent Process

To evaluate further the mechanism of peptide-induced immunopathology in LCMV-primed mice, the following experiment was performed. C57BL/6 mice were treated intraperitoneally either with LCMV GP33 peptide or with VSV NP52 peptide to load splenocytes in vivo with peptide. Fifteen hours later, spleens were removed and splenocytes were labeled in vitro with the vital dye 5- (and 6-) carboxyfluorescein diacetate succinimidyl ester (CFSE). Splenocytes from LCMV GP33 peptide–treated mice were labeled with a high intensity of CFSE fluorescence, whereas splenocytes from VSV NP52 peptide were labeled with a low intensity of CFSE fluorescence, to allow distinction between the two populations. The two spleen cell preparations were mixed 1:1 and then transferred intravenously as indicator cells into naive or LCMV-primed mice that had been pretreated with GP33 peptide 5 hr before transfer. Blood samples of the recipient mice were taken 2 and 24 hr after adoptive transfer to follow the fate of peptide-loaded spleen cells in vivo. Fluorescence-activated cell sorter analysis of blood samples from naive mice 2 hr after transfer revealed two CFSE-labeled indicator cell populations of comparable size: VSV NP52 peptide–loaded cells with a low fluorescence intensity ($CFSE^{lo}$) and LCMV GP33 peptide–loaded cells with a high fluorescence intensity ($CFSE^{hi}$) (Figure 6A). The two differentially labeled cell populations were also detectable in blood samples of LCMV-primed mice 2 hr after transfer; however, the LCMV GP33 peptide–loaded, $CFSE^{hi}$ cell population was already reduced in numbers, when compared to the VSV NP52 peptide–loaded, $CFSE^{lo}$ cell population (Figure

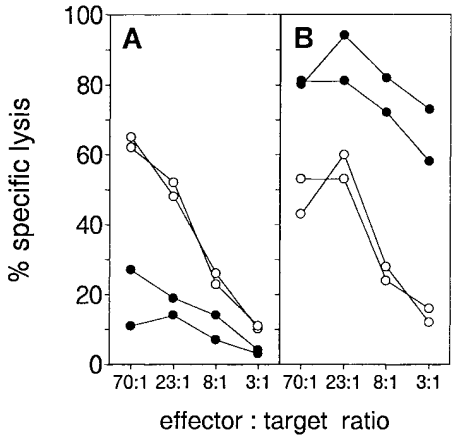

Figure 7. Peptide-Specific Tolerization of Naive CTL in TCR Transgenic Mice

TCR transgenic mice were treated intraperitoneally three times at 3-day intervals with 500 μg of GP33 peptide in IFA (A) or with IFA alone (B). Five days after the last peptide treatment the mice were infected intravenously with 2×10^4 pfu LCMV-WE, and ex vivo CTL activities of splenocytes were assayed 8 days later in a ^{51}Cr release assay. EL-4 target cells were either loaded with GP33 peptide (closed circles) or with NP394 peptide (open circles).

6B). This reduction was even more pronounced 24 hr after transfer of labeled cells into LCMV-primed mice. At this time the LCMV GP33-loaded, $CFSE^{hi}$ cell population was completely absent, while the VSV NP52 peptide–loaded, $CFSE^{lo}$ cell population was virtually unchanged (Figure 6D). In contrast, blood samples from naive mice 24 hr after transfer exhibited both peptide-loaded cell populations of comparable sizes (Figure 6C). The same result was obtained when splenocytes were analyzed 24 hr after transfer (Figures 6E and 6F). These results demonstrate that GP33 peptide–induced immunopathology and immunosuppression in LCMV-primed mice is an antigen-specific, contact-dependent mechanism and therefore most likely not mediated by cytokines.

Lack of GP33 Peptide–Induced Immune Suppression in Naive Transgenic Mice Expressing a GP33-Specific TCR

Virus-primed memory mice have a 100- to 1000-fold higher CTL precursor frequency when compared to naive mice. This may explain the GP33 peptide–induced immunosuppression in LCMV-primed mice. To determine whether this quantitative aspect accounted for the different outcome of peptide treatment in unprimed and LCMV-primed mice, transgenic mice expressing a GP33 peptide–specific TCR on 50% of all $CD8^+$ T cells were used. TCR transgenic mice were treated three times at 3-day intervals intraperitoneally with 500 μg of GP33 peptide in IFA and then were infected with LCMV. Splenocytes of these mice were assayed for CTL activities 8 days later. IFA-treated TCR transgenic control mice exhibited potent CTL activities against both GP33 peptide– (Figure 7B, closed circles) and NP394 peptide–loaded target cells (Figure 7B, open circles). In contrast, TCR transgenic mice treated with GP33 peptide failed to generate GP33-specific CTL activities (Figure 7A, closed circles), while the NP394 peptide–specific CTL responses were unimpaired (Figure 7A, open circles). Thus, despite the extremely high GP33 peptide–specific CTL precursor frequency in TCR transgenic mice, the GP33 peptide treatment tolerized the naive, GP33-specific CTL in a highly specific manner without inducing general immunosuppression. These data indicate that yet undefined qualitative differences between naive and memory CTL, rather than the quantitative differences in precursor frequencies alone, account for the observed immunopathology in LCMV-primed mice following LCMV GP33 peptide treatment.

Discussion

In the current study, we compared virus-specific naive and memory CTL for their susceptibility to peptide-induced tolerance in vivo. GP33 peptide administered intraperitoneally three times at 3-day intervals tolerized naive CTL in a highly antigen-specific manner. In contrast, the same peptide treatment in the presence of GP33-specific memory CTL in LCMV-primed mice induced severe immunopathological destruction in the spleen, accompanied by a nonspecific immunosuppression rather than antigen-specific tolerance.

Several mechanisms may account for the specific tolerance induction of naive $CD8^+$ T lymphocytes after repetitive and systemic peptide application. CTL may be tolerized because of the lack of specific TH cells (Guerder and Matzinger, 1992) or because they are triggered mainly by nonprofessional antigen-presenting cells and therefore will not receive the appropriate costimulation required for clonal expansion (Schwartz, 1990; Gimmi et al., 1991; Linsley et al., 1991; Matzinger, 1994) and for the activation of the cytolytic machinery (Azuma et al., 1992; Guerder et al., 1995; Lanier et al., 1995). This view correlates well with the observation that T cells are transiently activated during tolerance induction but never gain full effector function (Kyburz et al., 1993; Mamalaki et al., 1993; Kearney et al., 1994; Vidard et al., 1994). Furthermore, systemic application of high doses of peptide may trigger virtually all potentially available specific CTL, rather than only a fraction as is usually the case after a local initiation of an infection. These CTL become terminally differentiated and will die within a few days, as has been demonstrated for superantigens and rapidly replicating viruses (Webb et al., 1990; Moskophidis et al., 1993). In addition, CTL may undergo activation-induced cell death upon reencountering antigen during the cell cycle (Lenardo, 1991; Critchfield et al., 1994; Zheng et al., 1995).

Are memory CTL more resistant to these antigen-driven tolerance mechanisms? To answer this question, quantitative and qualitative aspects of CTL responses have to be compared in unprimed and virus-primed mice. Virus-primed mice exhibit a 100- to 1000-fold higher virus antigen-specific CTL precursor frequency (Moskophidis et al., 1987) when compared to unprimed mice. However, the increased precursor frequency alone cannot account for the observed immunosuppression in LCMV-primed mice, since GP33 peptide treatment of TCR-transgenic mice expressing a GP33-specific TCR on more than 50% of the $CD8^+$ T cells tolerized

the naive GP33-specific CTL completely, without affecting the LCMV NP394 peptide–specific CTL response. Thus, qualitative differences between naive and memory CTL appeared to be more crucial for explaining the different outcomes of the peptide treatment. These results fit well with the finding that male B lymphocytes transferred into female hosts efficiently tolerized H-Y–specific naive but not memory CTL (Fuchs and Matzinger, 1992). Furthermore, our data indicate that memory CTL, in contrast to naive CTL, gain full cytolytic effector function within 24 hr after peptide treatment, measurable directly in a standard ^{51}Cr release assay. At this time, peptide antigen still persists in the spleen and sensitizes virtually all spleen cells as targets for the activated memory CTL. Several reports have demonstrated that peptide-loaded T cells can serve as targets for activated CTL in vitro and in vivo (Pemberton et al., 1990; Walden and Eisen, 1990; Moss et al., 1991; Dutz et al., 1992; Kyburz et al., 1993). Such a mechanism may explain the extensive destruction of lymphoid architecture in the spleen of peptide-treated memory mice, which is likely to be the cause of the observed immunosuppression. Although we changed several parameters of our peptide application protocol by varying the dose of antigen, the use of adjuvants, and the time intervals of application, we were unable to avoid this peptide-induced immunopathological process (data not shown). Therefore the question of whether memory CTL are finally tolerized in our model remains open since the absence of a GP33-specific CTL response after GP33 peptide treatment of LCMV-primed mice could be due to tolerance induction or to immunosuppression or to both.

Taken together, the data presented suggest that peptide treatment of memory CTL in vivo will activate the cytolytic machinery before an antigen-specific tolerance status can be achieved. Such a difference in the in vivo kinetics of CTL activation versus CTL tolerization after peptide application may well explain the immunopathology and immunosuppression observed. This is confirmed by a recent study showing that high doses of peptides can induce apoptotic death of $CD8^+$ effector T lymphocytes in vitro within 40–48 hr. During this period, cloned CTL exposed to peptide were completely inhibited in their proliferative response but not in their cytolytic effector function (Alexander-Miller et al., 1996). Similarly, signaling via the TCR alone in the absence of interleukin-2 will inhibit proliferation of an antigen-experienced CTL clone in vitro but not block the cytolytic effector function (Otten and Germain, 1991).

The immunosuppression described in our model is different from the bystander suppression of $CD4^+$ T lymphocytes observed in certain experimental systems after oral application of antigens. In these cases the suppressive effect of antigen application on unrelated third-party antigen-specific $CD4^+$ T lymphocytes is most probably mediated by so-called suppressor cells, by the release of transforming growth factor β, or possibly by a T_{H1}–T_{H2} shift in the cytokine production (Khoury et al., 1990; Miller et al., 1992; Miller et al., 1993; Al-Sabbagh et al., 1994; Chen et al., 1995). Immunopathological damage after antigen application as demonstrated in our model have not been described in these systems. Experiments using differentially CFSE-labeled spleen cells, loaded in vivo with either LCMV GP33 peptide or with VSV NP52 peptide as indicator cell populations, revealed that after adoptive transfer into LCMV-primed mice only the GP33 peptide–loaded cell population was eliminated by the GP33-specific memory CTL, whereas the VSV NP52 peptide–loaded population was not destroyed. Therefore the immunosuppression and immunopathology, demonstrated in LCMV-primed mice after treatment with LCMV GP33 peptide, is mediated by an antigen-specific and contact-dependent mechanism rather than by the release of cytokines. This view is further supported by the finding that splenocytes of LCMV-primed mice treated once with GP33 peptide exhibited the same cytokine pattern as LCMV-primed mice challenged with LCMV. Both spleen cell populations produced interferon-γ, whereas interleukin-4 and tumor necrosis factor α were not detectable (data not shown).

Experimental adoptive transfer of LCMV-primed spleen cells into congenital virus carriers expressing antigen over the whole body has revealed peripheral deletion of memory CTL (Moskophidis et al., 1993). However, the virus carrier model is different from the peptide system studied here, since most lymphocytes are not infected by LCMV and therefore do not serve as targets for activated memory CTL (Ahmed et al., 1987). This may explain why no immunosuppression was observed in this experimental approach.

So far, peptide-specific immunotherapy of ongoing diseases and down-regulation of activated $CD4^+$ T cells has been successfully demonstrated only in the experimental autoimmune encephalomyelitis model (Smilek et al., 1991; Gaur et al., 1992). The tolerization of memory $CD4^+$ T cells may be achieved without the complications demonstrated in this study since $CD4^+$ T cells normally do not exhibit cytolytic activities and therefore are less likely to mediate acute immunopathological damage. Furthermore, since murine T cells do not express MHC class II molecules, they would not be loaded with class II-binding peptides to become a direct target for activated T helper cells.

In conclusion, this study shows that peptide treatment of antigen-primed hosts may have deleterious and undesirable effects, since specific memory CTL are rapidly activated and cause severe immunopathology in the spleen, leading to immunosuppression. For therapeutic intervention in autoimmune diseases, such a protocol would constitute a risk, because the potentially autoreactive memory CTL are activated and gain full effector function before the death of these cells is induced. Therefore, other strategies must be developed to overcome these problems and to render peptide-specific tolerization of memory CTL feasible for antigen-specific intervention in autoimmune diseases and graft rejections.

Experimental Procedures

Animals

C57BL/6 (H-2^b) mice were obtained from the breeding colony of the Institut für Zuchthygiene (Tierspital Zürich, Switzerland). The LCMV TCR transgenic mouse line 318 has been described previously (Kyburz et al., 1993). Animals were kept under conventional conditions and used in the experiments 14–24 weeks after birth.

Cell Lines

MC57G (H-2^b), a methylcholanthrene-induced fibroblast cell line, and EL-4 (H-2^b), a thymoma cell line, were used as target cells. The N1 cell line is a clone from EL-4 cells transfected with the *VSV-IND* nucleoprotein gene under the control of the Rous sarcoma virus long terminal repeat and constitutively expresses the VSV nucleoprotein (Puddington et al., 1986).

Viruses

The LCMV-WE strain was originally obtained from Dr. F. Lehmann-Grube (Hamburg, Germany) and was propagated in our laboratory on L929 fibroblast cells (Lehmann-Grube, 1971). Virus stocks were diluted in minimal essential medium supplemented with 2% fetal calf serum (FCS). Mice were infected intravenously with 200 plaque-forming units (pfu) of LCMV-WE and used in the experiments 30–60 days later. VSV-IND (VSV Indiana, Mudd-Summer isolate) seeds, originally obtained from Dr. D. Kolakofsky (Geneva, Switzerland), were grown on BHK 21 cells and plagued on Vero cells (McCaren et al., 1959; Charan and Zinkernagel, 1986). Mice were infected intravenously with 2×10^6 pfu of VSV-IND and used 30–60 days later. To generate memory responses against both viruses, mice were first injected with LCMV-WE and 30 days later with VSV-IND at the doses described above.

Peptide Treatment

LCMV glycoprotein peptide GP33–41 (KAVYNFATM) (Pircher et al., 1990), LCMV nucleoprotein peptide NP394–408 (AIFQPQNGQFI HFYR) (Schulz et al., 1989), and VSV nucleoprotein NP52–59 (RGYV YQGL) (van Bleek and Nathenson, 1990) were synthesized by the solid-phase method and purchased from Neosystem Laboratoire (Strasbourg, France). For technical reasons (to prevent dimer formation), the original cysteine at the anchor position 41 in the LCMV glycoprotein peptide GP33–41 was replaced by methionine. For injections, peptides were dissolved in Hanks' balanced salt solution (HBSS), emulsified 1:1 (vol/vol) in IFA (Difco Laboratories, Detroit, MI), and injected intraperitoneally at 3-day intervals at the indicated doses in a total volume of 200 μl.

Restimulation of Primed Antiviral CTL In Vitro

Spleen cells (4×10^6/well) from LCMV-primed mice were restimulated in 24-well tissue culture plates with 2×10^5 irradiated (3000 rad), LCMV-infected macrophages in Iscove's modified Dulbecco's medium supplemented with 10% FCS penicillin/streptomycin and 0.001 M 2-mercaptoethanol. Correspondingly, spleen cells (4×10^6/well) from VSV-primed animals were cultivated together with 4×10^5 irradiated (3000 rad) VSV-NP–transfected N1 cells under the same conditions. After 5 days, restimulated spleen effector cells from two wells were pooled and resuspended in 1 ml of complete minimal essential medium. Serial 3-fold dilutions were made (indicated as dilution of culture) and tested in a ^{51}Cr release assay.

Cytotoxicity Assay

The cytolytic activity of in vitro restimulated virus-specific spleen cells was determined in a ^{51}Cr release assay as described (Zinkernagel et al., 1985). MC57G or EL-4 cells were coated with LCMV peptides GP33 or NP394 or with VSV peptide NP52 at a concentration of 10^{-6} M and were labeled with 250 μCi ^{51}Cr for 2 hr at 37°C on a rocking platform. Target cells were washed three times and counted. Next, 10^4 target cells were incubated in 96-well round-bottom plates with a serial 3-fold dilution of restimulated spleen effector cells in a final volume of 200 μl. After a 5 hr incubation at 37°C, 70 μl supernatants were harvested and assayed. Spontaneous release was always less than 20%. Peritoneal macrophages were washed twice in balanced salt solution and resuspended in 100 μl of Iscove's modified Dulbecco's medium supplemented with 10% FCS for ^{51}Cr labeling. Spontaneous release of peritoneal macrophages was between 28% and 33%.

Proliferation Assay

Spleen cells from TCR transgenic mice (line 318), specific for the LCMV peptide GP33 + D^b, were used as responder cells (5×10^5 cells/well) and were incubated in 96-well plates with irradiated (3000 rad) spleen cells (5×10^5 cells/well) as stimulators from GP33 peptide–treated or untreated LCMV memory mice. Forty-eight hours later cultures were pulsed with 1 μCi/well of [^{3}H]thymidine for 12–15 hr and then harvested onto filter papers. Results are displayed as counts per minute determined in a scintillation counter.

Immunohistochemistry

Spleens were immersed in HBSS and snap-frozen in liquid nitrogen, and 5 μm–thick cryostat sections were cut and fixed in acetone for 10 min. These sections were incubated with the primary antibodies YTS.169.4.2 (anti-CD8; 1:6000 diluted) (Cobbold et al., 1984), YTS191.1 (anti-CD4; 1:10,000 diluted) (Cobbold et al., 1984), anti-B220 (1:50 diluted), MOMA-1 (1:50 diluted), or N418 (1:50 diluted) for 60 min followed by a two-step indirect immunoenzymatic staining with alkaline phosphatase-labeled goat anti-rat immunoglobulin (TAGO, Burlingame, CA) for 30 min. After washing, an alkaline phosphatase-labeled donkey anti-goat immunoglobulin (Jackson Immunoresearch, PA) was used for an additional 30 min. Antibody dilutions were prepared in 0.1 M Tris-HCl (pH 7.4) containing 5% normal mouse serum. Alkaline phosphatase was detected by a red color reaction by incubating with naphthol A-BI phosphate and with New Fuchsin for 15 min. Sections were counterstained with Mayer's hemalum for 5 min.

For the TUNEL assay, frozen sections were dried for 48 hr at 4°C and fixed with 2% paraformaldehyde for 15 min at room temperature. After washing with 50 mM Tris buffer (pH 7.5), the sections were first incubated for 15 min with Tris buffer containing 0.05 M glycine and then for 2 min with a solution of 0.1% sodium citrate and 0.1% Triton X-100 at 4°C. The sections were washed with Tris buffer, immersed in TdT buffer (Promega), and then incubated for 1 hr with the reaction mixture containing digoxigenin-dUTP and TdT according to the manufacturer's instructions. Sections were washed with Tris buffer, blocked for 15 min with Tris buffer containing 2% FCS, incubated for 1 hr with a alkaline phosphatase–labeled anti-digoxigenin immunglobulin (1:800 diluted in Tris buffer; Boehringer Mannheim, Germany), washed with Tris buffer, and developed for 7 min with naphthol A-BI phosphate and with New Fuchsin. Sections were counterstained with Mayer's hemalum for 5 min.

CFSE Labeling of Splenocytes

CFSE was purchased from Molecular Probes (Eugene, OR). C57BL/6 mice were treated intraperitoneally with 500 μg of either LCMV GP33 peptide or VSV NP52 peptide in IFA. Fifteen hours later, spleens were removed and a single cell suspension was made. Erythrocytes were depleted from spleen cell suspension by water lysis. The cells were washed with ice-cold phosphate-buffered saline and resuspended at a concentration of 2×10^7 cells/ml. CFSE was kept as a 0.5 mM stock in DMSO and stored at −20°C. Splenocytes from LCMV GP33 peptide–treated mice were labeled with CFSE at a final concentration of 0.5 μM, and splenocytes of VSV NP52 peptide–treated mice were labeled at a 10-fold lower concentration (0.05 μM), by incubating them for 10 min at 37°C. After labeling, FCS was added to a final concentration of 5% and cells were washed twice with ice-cold phosphate-buffered saline. The two splenocyte populations of different CFSE intensities were mixed at a 1:1 ratio, and 6×10^7 cells were adoptively transferred into recipient mice.

Acknowledgments

Correspondence should be addressed to P. A. This work was supported by Swiss National Foundation grants. We thank Lenka Vlk for excellent histology and Norbert Wey and Hannes Nef for photography.

Received November 8, 1996; revised February 14, 1997.

References

Ahmed, R., King, C., and Oldstone, M. (1987). Virus-lymphocyte interaction: T cells of the helper subset are infected with lymphocytic choriomeningitis virus during persistent infection in vivo. J. Virol. *61*, 1571–1576.

Aichele, P., Kyburz, D., Ohashi, P.S., Zinkernagel, R.M., Hengartner, H., and Pircher, H. (1994). Peptide induced T cell tolerance to prevent autoimmune diabetes in a transgenic mouse model. Proc. Natl. Acad. Sci. USA *91*, 444–448.

Aichele, P., Brduscha-Riem, K., Zinkernagel, R.M., Hengartner, H., and Pircher, H. (1995). T cell priming versus T cell tolerance induced by synthetic peptides. J. Exp. Med. *182*, 261–266.

Aichele, P., Bachmann, M.F., Hengartner, H., and Zinkernagel, R.M. (1996). Immunopathology or organ-specific autoimmunity as a consequence of virus infection. Immunol. Rev. *152*, 21–45.

Alexander-Miller, M.A., Leggatt, G.R., Sarin, A., and Berzofsky, J.A. (1996). Role of antigen, CD8, and cytotoxic T lymphocyte (CTL) avidity in high dose antigen induction of apoptosis of effector CTL. J. Exp. Med. *184*, 485–492.

Al-Sabbagh, A., Miller, A., Santos, L., and Weiner, H.L. (1994). Antigen-driven tissue-specific suppression following oral tolerance: orally administered myelin basic protein suppresses proteolipid protein-induced experimental autoimmune encephalomyelitis in the SJL mouse. Eur. J. Immunol. *24*, 2104–2109.

Azuma, M., Cayabyab, M., Buck, D., Phillips, J.H., and Lanier, L.L. (1992). CD28 interaction with B7 costimulates primary allogeneic proliferative responses and cytotoxicity mediated by small, resting T lymphocytes. J. Exp. Med. *175*, 353–360.

Blackman, M.A., Gerhard, B.H., Woodland, D.L., Palmer, E., Kappler, J.W., and Marrack, P. (1990). A role for clonal inactivation in T cell tolerance to Mls-1a. Nature *345*, 540–542.

Burkly, L.C., Lo, D., Kanagawa, O., Brinster, R.L., and Flavell, R.A. (1989). T-cell tolerance by clonal anergy in transgenic mice with nonlymphoid expression of MHC class II I-E. Nature *342*, 564–566.

Burstein, H.J., and Abbas, A.K. (1993). In vivo role of interleukin 4 in T cell tolerance induced by aqueous protein antigen. J. Exp. Med. *177*, 457–463.

Charan, S., and Zinkernagel, R.M. (1986). Antibody mediated suppression of secondary IgM response in nude mice against vesicular stomatitis virus. J. Immunol. *136*, 3057–3061.

Chen, Y., Inobe, J., Marks, R., Gonella, P., Kuchroo, V., and Weiner, H. (1995). Peripheral deletion of antigen-reactive T-cells in oral tolerance. Nature *376*, 177–180.

Chen, Y., Inobe, J., and Weiner, H.L. (1995). Induction of oral tolerance to myelin basic protein in CD8-depleted mice: both CD4+ and CD8+ cells mediate active suppression. J. Immunol. *155*, 910–916.

Cobbold, S.P., Jayasuriya, A., Nash, A., Prospero, T.D., and Waldmann, H. (1984). Therapy with monoclonal antibodies by elimination of T cell subsets in vivo. Nature *312*, 548–551.

Critchfield, J.M., Racke, M.K., Zuniga, P.J., Cannella, B., Raine, C.S., Goverman, J., and Lenardo, M.J. (1994). T cell deletion in high antigen dose therapy of autoimmune encephalomyelitis. Science *263*, 1139–1143.

Dresser, D.W. (1962). Specific inhibition of antibody production: II. Paralysis induced in adult mice by small quantities of protein antigen. Immunology *5*, 378–388.

Dutz, J.P., Walden, P.R., and Eisen, H.N. (1992). Effects of cognate peptides on cytolytic and proliferative activities of cloned cytotoxic T lymphocytes. Int Immunol *4*, 571–580.

Ferber, I., Schönrich, G., Schenkel, J., Mellor, A.L., Hämmerling, G.J., and Arnold, B. (1994). Levels of peripheral T cell tolerance induced by different doses of tolerogen. Science *263*, 674–676.

Fuchs, E.J., and Matzinger, P. (1992). B cells turn off virgin but not memory T cells. Science *258*, 1156–1159.

Gaur, A., Wiers, B., Liu, A., Rothbard, J., and Fathman, C.G. (1992). Amelioration of autoimmune encephalomyelitis by myelin basic protein synthetic peptide-induced anergy. Science *258*, 1491–1494.

Gimmi, C.D., Freeman, G.J., Gribben, J.G., Sugita, K., Freedman, A.S., Morimoto, C., and Nadler, L.M. (1991). B-cell surface antigen B7 provides a costimulatory signal that induces T cells to proliferate and secrete interleukin 2. Proc Natl Acad Sci Usa *88*, 6575–6579.

Guerder, S., and Matzinger, P. (1992). A fail-safe mechanism for maintaining self-tolerance. J. Exp. Med. *176*, 553–564.

Guerder, S., Carding, S.R., and Flavell, R.A. (1995). B7 costimulation is necessary for the activation of the lytic function in cytotoxic T lymphocyte precursors. J. Immunol. *155*, 5167–5174.

Hämmerling, G.J., Schönrich, G., Ferber, I., and Arnold, B. (1993). Peripheral tolerance as a multi-step mechanism. Immunol. Rev. *133*, 93–104.

Hoyne, G.F., O'Hehir, R.E., Wraith, D.C., Thomas, W.R., and Lamb, J.R. (1993). Inhibition of T cell and antibody responses to house dust mite allergen by inhalation of the dominant T cell epitope in naive and sensitized mice. J. Exp. Med. *178*, 1783–1788.

Jardetzky, T.S., Lane, W.S., Robinson, R.A., Madden, D.R., and Wiley, D.C. (1991). Identification of self peptides bound to purified HLA-B27. Nature *353*, 326–329.

Kappler, J.W., Roehm, N., and Marrack, P. (1987). T cell tolerance by clonal elimination in the thymus. Cell *49*, 273–280.

Kappler, J.W., Staerz, U.D., White, J., and Marrack, P. (1988). Self tolerance eliminates T cells specific for Mls-modified products of the major histocompatibility complex. Nature *332*, 35–40.

Kaufman, D.L., Clare, S.M., Tian, J., Forsthuber, T., Ting, G., Robinson, P., Atkinson, M.A., Sercarz, E.E., Tobin, A.J., and Lehmann, P.V. (1993). Spontaneous loss of T-cell tolerance to glutamic acid decarboxylase in murine insulin-dependent diabetes. Nature *366*, 69–72.

Kearney, E.R., Pape, K.A., Loh, D.Y., and Jenkins, M.K. (1994). Visualization of peptide-specific T cell immunity and peripheral tolerance induction in vivo. Immunity *1*, 327–339.

Khoury, S.S., Lider, O., Al, S.A., and Weiner, H.L. (1990). Suppression of experimental autoimmune encephalomyelitis by oral administration of myelin basic protein. Cell. Immunol. *131*, 302–310.

Kisielow, P., Blüthmann, H., Staerz, U.D., Steinmetz, M., and von Boehmer, H. (1988). Tolerance in T cell receptor transgenic mice involves deletion of nonmature CD4+8+ thymocytes. Nature *333*, 742–746.

Kyburz, D., Aichele, P., Speiser, D.E., Hengartner, H., Zinkernagel, R., and Pircher, H. (1993). T cell immunity after a viral infection versus T cell tolerance induced by soluble viral peptides. Eur. J. Immunol. *23*, 1956–1962.

Kyburz, D., Speiser, D.E., Aebischer, T., Hengartner, H., and Zinkernagel, R.M. (1993). Virus specific cytotoxic T-cell mediated lysis of lymphocytes in vitro and in vivo. J. Immunol. *150*, 5051–5058.

Lanier, L.L., O'Fallon, S., Somoza, C., Phillips, J.H., Linsley, P.S., Okumura, K., Ito, D., and Azuma, M. (1995). CD80 (B7) and CD86 (B70) provide similar costimulatory signals for T cell proliferation, cytokine production, and generation of CTL. J. Immunol. *154*, 95–105.

Lehmann-Grube, F. (1971). Lymphocytic choriomeningitis virus. Virol. Monogr. *10*, 1–173.

Lenardo, M.J. (1991). Interleukin-2 programs mouse alpha beta T lymphocytes for apoptosis. Nature *353*, 858–861.

Linsley, P.S., Brady, W., Grosmaire, L., Aruffo, A., Damle, N.K., and Ledbetter, J.A. (1991). Binding of the B cell activation antigen B7 to CD28 costimulates T cell proliferation and interleukin 2 mRNA accumulation. J. Exp. Med. *173*, 721–730.

Londei, M., Bottazzo, G.F., and Feldmann (1985). Human T cell clones from autoimmune thyroid glands: specific recognition of autologous thyroid cells. Science *228*, 85–89.

MacDonald, H.R., Schneider, R., Lees, R.K., Howe, R.C., Acha-Orbea, H., Festenstein, H., Zinkernagel, R.M., and Hengartner, H. (1988). T cell receptor Vb use predicts reactivity and tolerance to Mlsa - encoded antigens. Nature *332*, 40–45.

Mamalaki, C., Tanaka, Y., Corbella, P., Chandler, P., Simpson, E., and Kioussis, D. (1993). T cell deletion follows chronic antigen specific T cell activation in vivo. Int. Immunol. *5*, 1285–1292.

Matzinger, P. (1994). Tolerance, danger, and the extended family. Annu. Rev. Immunol. *12*, 991–1045.

McCaren, L.C., Holland, J.J., and Syverton, J.T. (1959). The mammalien cell-virus relationship: I. attachment of poliovirus to cultivated cells of primate and non-primate origin. J. Exp. Med. *109*, 475–485.

Metzler, B., and Wraith, D.C. (1993). Inhibition of experimental autoimmune encephalomyelitis by inhalation but not oral administration

of the encephalitogenic peptide: influence of MHC binding affinity. Int. Immunol. *5*, 1159–1165.

Miller, A., Hafler, D.A., and Weiner, H.L. (1991). Immunotherapy in autoimmune diseases. Curr. Opin. Immunol. *3*, 939–940.

Miller, A., Lider, O., Roberts, A.B., Sporn, M.B., and Weiner, H.L. (1992). Suppressor T cells generated by oral tolerization to myelin basic protein suppress both in vitro and in vivo immune responses by the release of transforming growth factor beta after antigen-specific triggering. Proc. Natl. Acad. Sci. USA *89*, 421–425.

Miller, A., Al-Sabbagh, A., Santos, L.M., Das, M.P., and Weiner, H.L. (1993). Epitopes of myelin basic protein that trigger TGF-beta release after oral tolerization are distinct from encephalitogenic epitopes and mediate epitope-driven bystander suppression. J. Immunol. *151*, 7307–7315.

Miller, J., and Morahan, G. (1992). Peripheral T cell tolerance. Annu. Rev. Immunol. *10*, 51–69.

Mitchison, N.A. (1964). Induction of immunological paralysis in two zones of dosage. R. Soc. Proc. *161*, 275–292.

Moskophidis, D., Assmann, W.U., Simon, M.M., and Lehmann, G.F. (1987). The immune response of the mouse to lymphocytic choriomeningitis virus. V. High numbers of cytolytic T lymphocytes are generated in the spleen during acute infection. Eur. J. Immunol. *17*, 937–942.

Moskophidis, D., Laine, E., and Zinkernagel, R.M. (1993). Peripheral clonal deletion of antiviral memory CD8+ T cells. Eur. J. Immunol. *23*, 3306–3311.

Moskophidis, D., Lechner, F., Pircher, H.P., and Zinkernagel, R.M. (1993). Virus persistence in acutely infected immunocompetent mice by exhaustion of antiviral cytotoxic effector T cells. Nature *362*, 758–761.

Moss, D.J., Burrows, S.R., Baxter, G.D., and Lavin, M.F. (1991). T cell-T cell killing is induced by specific epitopes: Evidence for an apoptotic mechanism. J. Exp. Med. *173*, 681–686.

Oehen, S.U., Ohashi, P.S., Bürki, K., Hengartner, H., Zinkernagel, R.M., and Aichele, P. (1994). Escape of thymocytes and mature T cells from clonal deletion due to limiting tolerogen expression levels. Cell. Immunol. *158*, 342–352.

Ohashi, P.S., Oehen, S., Bürki, K., Pircher, H.P., Ohashi, C.T., Odermatt, B., Malissen, B., Zinkernagel, R., and Hengartner, H. (1991). Ablation of "tolerance" and induction of diabetes by virus infection in viral antigen transgenic mice. Cell *65*, 305–317.

Oki, A., and Sercarz, E. (1985). T cell tolerance studied at the level of antigenic determinants. I. Latent reactivity to lysozyme peptides that lack suppressogenic epitopes can be revealed in lysozyme-tolerant mice. J. Exp. Med. *161*, 897–911.

Oldstone, M., Nerenberg, M., Southern, P., Price, J., and Lewicki, H. (1991). Virus infection triggers insulin-dependent diabtes mellitus in a transgenic model: Role of anti-self (virus) immune response. Cell *65*, 319–331.

Otten, G.R., and Germain, R.N. (1991). Split anergy in a CD8+ T cell: Receptor-dependent cytolysis in the absence of interleukin 2 production. Science *251*, 1228–1231.

Pemberton, R.M., Wraith, D.C., and Askonas, B.A. (1990). Influenza peptide-induced self-lysis and down-regulation of cloned cytotoxic T cells. Immunology *70*, 223–229.

Pette, M., Fujita, K., Wilkinson, D., Altmann, D.M., Trowsdale, J., Hinkkanen, A., Epplen, J.T., Kappos, L., and Wekerle, H. (1990). Myelin autoreactivity in multiple sclerosis: recognition of myelin basic protein in the context of HLA-DR2 products by T lymphocytes of multiple-sclerosis patients and healthy donors. Proc. Natl. Acad. Sci. USA *87*, 7968–7972.

Pircher, H.P., Mak, T.W., Lang, R., Ballhausen, W., Ruedi, E., Hengartner, H., Zinkernagel, R.M., and Bürki, K. (1989). T cell tolerance to Mlsa encoded antigens in T cell receptor b-chain transgenic mice. EMBO J. *8*, 719–727.

Pircher, H.P., Moskophidis, D., Rohrer, U., Bürki, K., Hengartner, H., and Zinkernagel, R.M. (1990). Viral escape by selection of cytotoxic T cell-resistant virus variants in vivo. Nature *346*, 629–633.

Puddington, L., Bevan, M.J., and Lefrancois, L. (1986). N protein is the predominant antigen recognized by vesicular stomatitis virus-specific cytotoxic T cells. J. Virol. *60*, 708–717.

Rajewsky, K., and Brenig, C. (1974). Tolerance to serum albumins in T and B lymphocytes in mice. Dose dependence, specificity and kinetics of escape. Eur. J. Immunol. *4*, 120–125.

Rammensee, H.G., Kroschewski, R., and Frangoulis, B. (1989). Clonal anergy induced in mature Vb6+ T lymphocytes on immunizing Mls-1 mice with Mls-1 expressing cells. Nature *339*, 541–544.

Rammensee, H.-G., Falk, K., and Rötschke, O. (1993a). Peptides naturally presented by MHC class I molecules. Annu. Rev. Immunol. *11*, 213–244.

Rammensee, H.G., Falk, K., and Rötzschke, O. (1993b). MHC molecules as peptide receptors. Curr. Biol. *5*, 35–44.

Ramsdell, F., Lantz, T., and Fowlkes, B.J. (1989). A nondeletional mechanism of thymic self tolerance. Science *246*, 1038–1041.

Ria, F., Chan, B., Scherer, M.T., Smith, J.A., and Gefter, M.L. (1990). Immunological activity of covalently linked T-cell epitopes. Nature *343*, 381–383.

Roberts, J.L., Sharrow, S.O., and Singer, A. (1990). Clonal deletion and clonal anergy in the thymus induced by different cellular elements. J. Exp. Med. *171*, 935–940.

Rocha, B., and von Boehmer, H. (1991). Peripheral selection of the cell repertoire. Science *251*, 1225–1228.

Romball, C.J., and Weigle, W.O. (1993). In vivo induction of tolerance in murine CD4+ cell subsets. J. Exp. Med. *178*, 1637–1644.

Rötzschke, O., Falk, K., Deres, K., Schild, H., Norda, M., Metzger, J., Jung, G., and Rammensee, H.G. (1990). Isolation and analysis of naturally processed viral peptides as recognized by cytotoxic T cells. Nature *348*, 252–254.

Rudensky, A.Y., Preston, H.P., Hong, S.C., Barlow, A., and Janeway, C.A. (1991). Sequence analysis of peptides bound to MHC class II molecules. Nature *353*, 622–627.

Schild, H.J., Rötzschke, O., Kalbacher, H., and Rammensee, H.G. (1990). Limit of T cell tolerance to self proteins by peptide presentation. Science *247*, 1587–1589.

Schluesener, H.J., and Wekerle, H. (1985). Autoaggressive T lymphocyte lines recognizing the encephalitogenic region of myelin basic protein: in vitro selection from unprimed rat T lymphocyte populations. J. Immunol. *135*, 3128–3133.

Schulz, M., Aichele, P., Vollenweider, M., Bobe, F.W., Cardinaux, F., Hengartner, H., and Zinkernagel, R.M. (1989). MHC dependent T cell epitopes of LCMV nucleoprotein and their protective capacity against viral disease. Eur. J. Immunol. *19*, 1657–1667.

Schwartz, R.H. (1990). A cell culture model for T lymphocyte clonal anergy. Science *248*, 1349–1356.

Sha, W.C., Nelson, C.A., Newberry, R.D., Kranz, D.M., Russell, J.H., and Loh, D.Y. (1988). Positive and negative selection of an antigen receptor on T cells in transgenic mice. Nature *336*, 73–76.

Sinha, A.A., Lopez, M., and McDevitt, H.O. (1990). Autoimmune diseases: the failure of self tolerance. Science *248*, 1380–1388.

Smilek, D.E., Wraith, D.C., Hodgkinson, S., Dwivedy, S., Steinman, L., and McDevitt, H.O. (1991). A single amino acid change in a myelin basic protein peptide confers the capacity to prevent rather than induce experimental autoimmune encephalomyelitis. Proc. Natl. Acad. Sci. USA *88*, 9633–9637.

Tisch, R., Yang, X.D., Singer, S.M., Liblau, R.S., Fugger, L., and McDevitt, H.O. (1993). Immune response to glutamic acid decarboxylase correlates with insulitis in non-obese diabetic mice. Nature *366*, 72–75.

van Bleek, G.M., and Nathenson, S.G. (1990). Isolation of an endogenously processed immunodominant viral peptide from the class I H-2Kb molecule. Nature *348*, 213–216.

Vidard, L., Colarusso, L.J., and Benacerraf, B. (1994). Specific T-cell tolerance may be preceded by a primary response. Proc. Natl. Acad. Sci. USA *91*, 5627–5631.

Walden, P.R., and Eisen, H.N. (1990). Cognate peptides induce self-destruction of CD8+ cytolytic T lymphocytes. Proc. Natl. Acad. Sci. USA *87*, 9015–9019.

Webb, S., Morris, C., and Sprent, J. (1990). Extrathymic tolerance of mature T cells: Clonal elimination as a consequence of immunity. Cell *63*, 1249–1256.

Weiner, H.L., Friedman, A., Miller, A., Khoury, S.J., Al-Sabbagh, A., Santos, L., Sayegh, M., Nussenblatt, R.B., Trentham, D.E., and Hafler, D.A. (1994). Oral tolerance: immunologic mechanisms and treatment of animal and human organ-specific autoimmune diseases by oral administration of autoantigens. Annu. Rev. Immunol. *12*, 809–837.

Wraith, D.C., Smilek, D.E., Mitchell, D.J., Steinman, L., and McDevitt, H.O. (1989). Antigen recognition in autoimmune encephalomyelitis and the potential for peptide-mediated immunotherapy. Cell *59*, 247–255.

Wucherpfennig, K.W., and Strominger, J.L. (1995). Molecular mimicry in T cell-mediated autoimmunity: viral peptides activate human T cell clones specific for myelin basic protein. Cell *80*, 695–705.

Zheng, L., Fisher, G., Miller, R.E., Peschon, J., Lynch, D.H., and Lenardo, M.J. (1995). Induction of apoptosis in mature T cells by tumour necrosis factor. Nature *377*, 348–351.

Zinkernagel, R.M., Leist, T.P., Hengartner, H., and Althage, A. (1985). Susceptibility to lymphocytic choriomeningitis virus isolates correlates directly with early and high cytotoxic T cell activity, as well as with footpad swelling reaction, and all three are regulated by H-2D. J. Exp. Med. *162*, 2125–2141.