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A Novel Peptide Mimotope Identified As a Potential Immunosuppressive Vaccine for Organ Transplantation

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We reported that anti-histone H1 autoantibody is one of the main immunosuppressive factors in serum that is induced after orthotopic liver transplantation in a rat tolerogenic model. We generated a novel anti-histone H1 IgM mAb produced by hybridoma 16G9 (16G9 mAb) that shows MLR-inhibitory activity. Identification of a functional epitope responsible for the immunosuppressive activity of 16G9 mAb may lead to the establishment of a novel therapeutic strategy. We used a combinatorial phage display peptide library to screen for peptides that bind to 16G9 mAb. Consequently, two peptides that bind to 16G9 mAb, SSV and LPQ, were selected from the library. The binding of 16G9 mAb to histone H1 was inhibited by SSV. SSV was recognized by rat tolerogenic post-orthotopic liver transplantation serum and the binding to SSV was inhibited by histone H1. Mice were immunized with keyhole limpet hemocyanin-conjugated SSV and LPQ. Abs induced by SSV immunization inhibited Con A-stimulated splenocyte proliferation, and the inhibition was neutralized by preincubation with SSV. Splenocytes stimulated by anti-CD3 Ab were inhibited by SSV-induced Abs using CFSE labeling. SSV immunization in rats before heterotopic heart transplantation resulted in significant prolonged allograft survival. These findings suggested that SSV is a functional histone H1-binding epitope for 16G9 mAb. SSV is capable of determining serum immunoreactivity against histone H1 as an index marker for tolerance. The inhibitory activity of SSV-induced Abs on blast cell proliferation and the prolonged graft survival that results from SSV immunization imply a potential for the development of an immunosuppressive vaccine. The Journal of Immunology, 2009, 182: 4282–4288.

Organ transplantation has become the therapeutic option for patients with irreversible organ diseases. Immunosuppressive regimens are usually required to prevent allograft rejection in patients who received organ transplants. When cyclosporine was introduced, it allowed for improvements in graft survival, and the incidence of acute rejection decreased. Cyclosporine and tacrolimus composes the class of immunosuppressive agents called calcineurin inhibitors. By binding calcineurin and preventing its translocation into the nucleus, these drugs prevent transcription and subsequent secretion of IL-2 (1). In addition to their immunosuppressive activity, the drugs have side effects such as nephrotoxicity, induction of cancer progression, and cardiotoxicity (2–4). These drugs have been proven to be effective in treating acute rejection, but not in preventing chronic rejection and extending long-term graft survival (5). Therefore, the development of more effective and less toxic immunosuppressive agents could improve the clinical outcomes of organ transplantation. Various new agents, including mAbs, are currently being investigated as potential immunosuppressants in the field of organ transplantation. Due to their target specificity, mAbs have become the most rapidly expanding class of therapeutic drugs for a wide variety of human diseases. There are several therapeutic mAbs approved for the prevention of rejection, including daclizumab and basiliximab.

In a rat tolerogenic model of orthotopic liver transplantation (OLT),3 PVG rats receiving a DA liver graft survived without immunosuppression and recipient post-OLT serum showed immunosuppressive activity (6–9). We have previously reported that anti-histone H1 autoantibody is transiently induced during the rejection phase after liver transplantation in a rat tolerogenic model and that anti-histone H1 polyclonal Ab is able to suppress MLR and to prevent allograft rejection (10). Furthermore, we have reported that anti-histone H1 polyclonal Ab reduces the cytotoxic effects of NK and LAK cells, induces CD4+CD25+ regulatory T cells, and drives dendritic cells toward a more tolerogenic state (11). These results suggested that anti-histone H1 polyclonal Ab could be used as a potential immunosuppressant. We have recently generated a novel anti-histone H1 IgM mAb produced by hybridoma strain 16G9 (16G9 mAb) that has MLR-inhibitory activity and may be potentially developed as a therapeutic drug. Due to its short half-life in the blood (12), IgM mAb may be a better candidate than IgG mAb in the development of therapeutic drugs.

In recent years, phage display has emerged as a powerful tool to identify small molecules that bind to receptor molecules and that mimic the interaction with natural ligands. A combinatorial phage display peptide library is likely to be useful in identifying molecules for therapeutic targets and in discovering pharmacologic

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3 Abbreviations used in this paper: OLT, orthotopic liver transplantation; HHT, heterotopic heart transplantation; KLH, keyhole limpet hemocyanin; MHCH, MHIC hapten; 7-AAD, 7-aminoactinomycin D.

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agents. Monoclonal and polyclonal Abs have been used to identify immunogenic mimic peptides (mitomotes) from random peptide libraries (13). A major goal of peptide library screening is to identify a peptide as an immunogenic mimic that will elicit in vivo Abs similar to the original Ab. Several reports have shown that peptide mitomotes identified by mAbs or serum Abs by phage display exhibit the potential to be developed into vaccines or diagnostic tools (14–17). Mimotopes are epitope-mimicking structures. Therefore, epitope specificity should be considered when designing a vaccine preparation aimed at inducing a humoral immune response (18). In the present study, we used a combinatorial phage display peptide library to screen for peptides that bind to 16G9 mAb. Identification of the functional epitope responsible for the immunosuppressive activity of 16G9 mAb may lead to the establishment of a novel diagnostic and therapeutic strategy.

Materials and Methods

16G9 mAb

The 16G9 mAb was generated, characterized, and purified as described previously (19).

Isolation and selection of 16G9 mAb-binding peptides

A linear 12-mer phage display peptide library (New England Biolabs) was screened with purified 16G9 mAb in a solid-phase support system. The biopanning procedure was performed according to the manufacturer’s instructions. Briefly, 16G9 mAb was immobilized and incubated with the peptide library. Unbound phages were washed away, and phages that were bound to 16G9 mAb were eluted. The eluted phages were amplified in Escherichia coli and were then put through more cycles of biopanning. After three rounds of panning, single clones that bound to 16G9 mAb were isolated. DNA from the isolated clones was extracted according to the manufacturer’s instructions.

Sequencing of isolated phage clones and peptide synthesis

The nucleotide sequence of peptides was determined with the 3100-Avant genetic analyzer (Applied Biosystems) using the 96-g primer (New England Biolabs). Peptides SSVLYGGPGSAA (SSV) and LPQNVLWHL GWHT (LPQ) were synthesized by the Peptide Institute. The peptides were then conjugated to a carrier protein, either keyhole limpet hemocyanin (KLH) or OVA, via the NH2 terminus.

Specificity of peptide-bound 16G9 mAb determined by ELISA

The reactivity and specificity of 16G9 mAb with SSV and LPQ were determined by ELISA. Briefly, 96-well microtiter plates (Nalgene Nunc International) were coated with KLH-conjugated SSV or LPQ, or with KLH (2 μg/ml) alone in 100 mM sodium bicarbonate buffer (pH 9.3). KLH was used as an internal control. The plates were washed with PBS-Tween 20 (0.05%) and blocked with 3% skim milk and 1% BSA in PBS for 1 h. Increasing amounts (0–0.5 μg/ml) of 16G9 mAb were added to the wells and incubated for 1 h. Bound 16G9 mAb was detected using biotin-conjugated anti-mouse IgM Ab (Bioscience) and incubated for 1 h. Peroxidase-conjugated streptavidin (Sigma-Aldrich) was added and incubated for 30 min. The streptavidin-biotinylated peroxidase complex was detected by ABTS substrate solution (Sigma-Aldrich). Multispan Ascent (Thermo Fisher Scientific) was used to determine absorbance at 405 nm.

Inhibition of 16G9 mAb binding to histone H1 by SSV

Microtiter plates (96-well) were coated with 25 ng per well histone H1 (Roche Diagnostics). 16G9 mAb (0.02 μg/ml) was preincubated with increasing amounts (0–160 μg/ml) of KLH-conjugated SSV or LPQ, a control peptide (A7), or KLH. A7 is KLH-conjugated peptide with a single amino acid sequence being replaced by alanine (SSVLYGAPP SAA). After 30 min, the mixture was added to the blocked wells coated with histone H1. Bound 16G9 mAb was detected by biotin-conjugated anti-mouse IgM Ab and streptavidin, and then followed by ABTS substrate solution for color development.

Reactivity of peptides with post-OLT serum

In OLT, the DA liver was implanted into DA (DA-DA, syngenic), PVG (DA-PVG, tolerogenic), or LEW (DA-LEW, acute rejection) rats recipients by the cuff method as described previously (20). Post-OLT serum was obtained from the recipients on days 7–83 after OLT. Rat post-OLT serum diluted 200-fold was added to the blocked wells coated with KLH-SSV, KLH-LPQ, or KLH (250 ng/well). Serum reactivity to SSV and LPQ was detected using peroxidase-conjugated anti-rat IgG Ab (Sigma-Aldrich) and then with the ABTS substrate solution. For competitive inhibition studies, DA-PVG post-OLT serum (200-fold dilution) was preincubated with increasing concentrations (0–20 μg/ml) of histone H1 or OVA. The mixture was added to the peptide-coated wells and detected by peroxidase-conjugated anti-rat IgG Ab (Sigma-Aldrich). Absorbance was determined at 405 nm.

Immunization of BALB/c mice with SSV and LPQ

Female BALB/c mice aged 6 wk were purchased from Charles River Laboratories and were housed under specific pathogen-free environments. All animal studies complied with the guidelines of the Experimental Ethics Committee at Josai International University. Three groups of mice (n = 4/group) were immunized by i.p. injection of 10 μg of KLH-conjugated SSV or LPQ mixed with Freund’s complete adjuvant (Sigma-Aldrich) at a ratio of 1:1. Mice immunized with KLH alone were used as negative controls. Two boosters mixed with Freund’s incomplete adjuvant (Sigma-Aldrich) were given. Blood was collected from the tail vein of the animals before vaccination and once weekly thereafter. For the detection of anti-peptide Abs, serum samples from immunized mice were evaluated using peptide-coated plates. Bound anti-SSV or anti-LPQ Ab was detected by incubation with peroxidase-conjugated anti-mouse IgG Ab (Sigma-Aldrich).

Evaluation of peptide-immunized serum by Con A stimulation assay

Female DA rats (MHC haplotype (MHCH): RT1a; age, 7–8 wk) were purchased from Japan SLC and were then maintained in specific pathogen-free environments. Splenocytes were obtained from DA spleens. Cells were suspended at 2 × 106 cells/ml in RPMI 1640 (Sigma-Aldrich) containing 10% PBS, 100 U/ml penicillin, and 100 μg/ml streptomycin (In-vitrogen) and were then stimulated with 2.5 μg/ml Con A (Sigma-Aldrich). Equal numbers of cells (2 × 105 cells/well) were cultured in 96-well round-bottom microplates. Peptide-immunized serum was added to Con A-stimulated splenocytes and the cells were then cocultured in a humidified atmosphere with 5% CO2 (37°C, 36 h). Splenocyte proliferation was detected using the BrdU labeling and detection kit III (Roche Diagnostics) with a Multispan Ascent (Thermo Fisher Scientific) with absorbance at 405 nm. For the neutralization experiment, SSV-immunized serum was incubated with KLH-SSV, a control peptide (A7), histone H1, or KLH for 30 min before being added to Con A-stimulated splenocytes and cocultured for 36 h.

CFSE labeling of responder cells

Splenocytes obtained from DA spleens were labeled with CFSE (Sigma-Aldrich) as described previously (21). The 5 μM stock solution of CFSE in DMEM (Sigma-Aldrich) was diluted to 5 μM in a volume of PBS equal to the one in which responder cells (1 × 106 cells/ml in PBS) were suspended, and the cells were then incubated at 37°C for 10 min. The labeling process was quenched by adding an equal volume of heat-inactivated FBS (Invitrogen) to the sample. After 1 min, CFSE-labeled cells were washed twice, recounted, and adjusted to a concentration of 5 × 105 cells/ml in the culture medium. Cultured cells from each well were harvested after 3 days and then preincubated with mouse anti-rat CD32 (FcγRII) receptor (BD Bio-sciences) to block the nonantigen-specific binding of immunoglobulins. CFSE-labeled cells were incubated at 4°C for 30 min with allopolyocytocin-conjugated anti-CD3 Ab. Cells were suspended in PBS/BSA with 0.01% NaN3 and 2 mg/ml 7-aminoactinomycin D (7-AAD; Invitrogen) to measure the population distributions of apoptotic/necrotic cell death. Three-color flow cytometry was performed on an EPICS ALTRA flow cytometer (Beckman Coulter) using EXPO32 software.

In vivo evaluation of SSV immunization in heterotopic heart transplantation

Male LEW (MHCH RT1a) rats, 4 wk of age, were obtained from the National Animal Breeding Center (Taipei, Taiwan). All animals were maintained in specific pathogen-free animal facilities with water and commercial rat food provided ad libitum. Our experimental design was reviewed and approved both by the Experimental Ethics Committee at Josai International University and the Institutional Animal Care and Use Committee at Chang Gung Memorial Hospital-Kaohsiung Medical Center. The LEW rats were immunized i.p. every 2 wk with KLH-SSV (100 μg), which had been preadsorbed with Freund’s complete adjuvant (1st immunization; Wako Biologicals) or Freund’s incomplete adjuvant (2nd immunization;
Wako Biochemicals). For a final boost, LEW rats were immunized with KLH-SSV (100 µg) without adjuvant. Control rats were immunized with KLH (Sigma-Aldrich) on the same dosage and schedule as KLH-SSV-immunized rats. For the detection of anti-SSV Abs, serum samples from immunized rats were evaluated using OVA-SSV-coated plates to eliminate the nonspecific binding of KLH from the serum. Bound anti-SSV Ab was detected by incubation with peroxidase-conjugated anti-rat IgG Ab (BioSource International).

After administering the final boost for 3 days, heterotopic heart transplantation (HHT) was performed as previously described (22). For HHT, hearts from DA rats (MHCH RT1a) were heterotopically transplanted into the neck of KLH-SSV- or KLH-immunized LEW rats. A sham control group was treated with saline. Actual heart allograft survival was calculated by the Kaplan-Meier product limit estimator. The log-rank test (Mantel-Cox) was used to test the equality of graft survival curves.

**FIGURE 1.** Specific binding of 16G9 mAb to KLH-conjugated SSV and LPQ by ELISA. A, 16G9 mAb bound to KLH-conjugated SSV and LPQ in a dose-dependent manner. KLH was used as a control to eliminate its nonspecific binding to 16G9 mAb. A significant difference ($p < 0.05$) was found between 16G9 mAb bindings to KLH-conjugated SSV and LPQ and to KLH. B, 16G9 mAb binding to histone H1 was dose-dependently inhibited by KLH-SSV, but not by KLH-LPQ. KLH was used as a control. A significant inhibition ($p < 0.05$) was observed in competition with KLH-SSV. C, A7, which is a KLH-conjugated SSV peptide with a single amino acid replaced by alanine, was used as a control in competition ELISA. * $p < 0.05$.

**FIGURE 2.** SSV (filled bar) was detected in rat (DA-PVG) tolerogenic post-OLT serum during the rejection phase (post-OLT days 14). A, SSV was not detected in syngenic (DA-DA) or acute rejection (DA-LEW) serum. KLH (open bar) was used as a control. B, SSV (filled bar) was detected transiently in rat tolerogenic post-OLT serum during rejection phase (post-OLT days 14 and 21). LPQ (open bar) and KLH (gray bar) did not react with OLT serum. **$p < 0.01$ on post-OLT day 14 between KLH-SSV and KLH-LPQ or KLH. C, Histone H1 inhibited binding between SSV and post-OLT day 14 serum dose-dependently. OVA was used as a control. A significant difference was found between histone H1 and OVA ($p < 0.05$).
Statistical analysis

Statistical analysis was performed using the Mann-Whitney U test and one-way ANOVA. Each sample was evaluated in triplicate, and three independent experiments were performed. Results are expressed as mean ± SD. A value of \( p < 0.05 \) was considered statistically significant. Statistical analysis was performed using the Excel statistical program file ystat 2002 (Igaku Tosho Shuppan, Tokyo, Japan).

Results

Identification and characterization of SSV and LPQ

In this study, a Ph.D.-12 phage display peptide library kit was used to screen for peptides that bind to 16G9 mAb. After three rounds of panning, two sequences were identified: SSVYGGPPSAA (SSV) and LPQWVLGHWHT (LPQ). SSV and LPQ were compared with mouse histone H1 amino acid sequence. Three residues (“GPP”) in SSV were found in mouse histone H1 sequence but not LPQ. Free and carrier protein-conjugated SSV and LPQ were synthesized. However, we had difficulty in detecting 16G9 mAb when LPQ-immunized serum did not neutralize the inhibitory effect of SSV-immunized serum. All figures are representatives of three independent experiments.

FIGURE 3. Immunization of mice with KLH-conjugated SSV or LPQ, or with KLH alone. Four female BALB/c mice were immunized with KLH-conjugated SSV or LPQ. Immunization with KLH alone was performed as a control. Serum samples from immunized mice were evaluated with OVA-conjugated SSV to eliminate the nonspecific binding of KLH from the serum. High anti-SSV Ab was obtained at 4 wk and 6 wk after immunization. * \( p < 0.05 \).

Elimination of KLH-conjugated SSV or LPQ

Results of Con A-stimulated splenocyte proliferation and cocultured with 2% mouse serum. All figures are representatives of three independent experiments.

Reactivity of SSV with rat post-OLT serum

Nakano et al. reported that anti-histone H1 Ab titers increased transiently on days 14–21 after OLT in a rat tolerogenic model (DA-PVG) (10). Our ELISA results suggested that SSV could be a histone H1-binding epitope. Therefore, we hypothesized that SSV should react with tolerogenic post-OLT serum with the same binding pattern as that of histone H1. Rat tolerogenic post-OLT serum was added to wells that were coated with KLH-conjugated SSV or LPQ, or with KLH alone, and was detected by peroxidase-conjugated anti-rat IgG Ab. Post-OLT serum from a tolerogenic, syngenic (DA-DA), or acute rejection (DA-LEW) model was examined. Anti-SSV Ab was detected on days 14–21 after OLT in the tolerogenic model (Fig. 2A). The binding pattern of OLT serum to SSV was similar to that of histone H1 (Fig. 2B) (10). OLT serum did not bind to LPQ and KLH (Fig. 2B), suggesting that the binding of SSV was specific. We then performed competitive ELISA to further investigate whether the binding of rat OLT serum to SSV is histone H1 related. A mixture of post-OLT day 14 serum, which showed the strongest reactivity to SSV (Fig. 2, A and B) and various concentrations of histone H1 were added to wells that were coated with KLH-conjugated SSV. The binding of post-OLT day 14 serum to SSV was inhibited by histone H1 dose-dependently (Fig. 2C). These results demonstrated the specificity of SSV and further confirmed that this peptide is a histone H1-binding epitope.
Peptide immunization

We speculated that Abs induced by SSV immunization should exhibit the same suppressive activity as anti-histone H1 Ab (10). Three groups of female BALB/c mice aged 6 wk (n = 10) were immunized with KLH-conjugated SSV or LPQ, or with KLH alone. Serum samples from immunized mice were examined using OVA-conjugated SSV or LPQ to eliminate the nonspecific binding from KLH present in serum. Anti-SSV Abs increased after three immunization of KLH-conjugated SSV (Fig. 3). Anti-LPQ Abs also increased after three injections of LPQ (data not shown); however, the increase was not as intense as with SSV. Serum from KLH-immunized mice did not react with these peptides (Fig. 3).

Suppression of splenocyte proliferation by anti-SSV serum

Serum samples from SSV- and LPQ-immunized mice were tested for suppressive activity in a Con A blast experiment. Splenocytes obtained from DA spleens were stimulated with Con A. Serum samples were added and cocultured with Con A-stimulated splenocytes for 36 h. Addition of SSV-immunized serum resulted in a significant suppression of Con A-stimulated splenocyte proliferation compared with LPQ-immunized, KLH-immunized, and normal mouse serum (Fig. 4A). Addition of different concentrations of SSV-immunized serum revealed a dose-dependent suppression (Fig. 4B). The inhibitory effect of SSV-immunized serum on proliferating cells was neutralized by preincubating the serum with KLH-SSV but not KLH or A7 (Fig. 4C), confirming that SSV is a functional epitope of 16G9 mAb and showed the immunosuppressive activity of Abs induced by SSV immunization in vitro.

Graft survival studies of rats immunized with SSV

The inhibitory effect of SSV-immunized serum on cell proliferation was shown in vitro. We next examined whether immunization of SSV has immunosuppressive effect in vivo. A rat HHT model was used to examine the effect of SSV immunization. LEW rats

FIGURE 5. A, Histogram plots show cell divisions associated with CFSE labeling of DA splenocytes. All plots were gated on CD3+ cells. SSV-immunized serum suppressed cell proliferation compared with LPQ-immunized serum, KLH-immunized serum, or with normal mouse serum. B, Cell proliferation percentage calculated from flow cytometric analysis: (A) SSV-immunized serum, (B) LPQ-immunized serum, (C) KLH-immunized serum, and (D) normal mouse serum. Cell proliferation was significantly suppressed only by SSV-immunized serum significantly (p < 0.05) compared with other sera. C, No difference is observed in gated or ungated 7-AAD(-) cell numbers between (A) SSV-immunized, (C) KLH-immunized, or (D) normal mouse serum.

FIGURE 6. SSV vaccination before HHT resulted in prolongation of graft survival. Graft survival in rats immunized with SSV (▲, n = 4) was significantly (p < 0.01) prolonged compared with rats immunized with KLH (■, n = 6) or sham control (saline) group (●, n = 10).
were immunized with KLH-SSV or KLH until we confirmed a higher titer of anti-SSV Ab (data not shown), and then HHT was performed. Graft survival was determined by the beating of the heart after transplantation. Graft survival in rats immunized with KLH-SSV was significantly prolonged ($p < 0.01$) more than 13 days ($n = 4$), whereas rats immunized with KLH rejected the heart within 7–11 days ($n = 6$). Rats treated with saline rejected the heart within 9 days (Fig. 6). The results demonstrated the immunosuppressive activity of SSV immunization in vivo, suggesting the potential of SSV to be used as a nontoxic therapeutic agent or immunosuppressive vaccine.

**Discussion**

In this study, we used a combinatorial phage display peptide library to isolate SSV and LPQ that bind to 16G9 mAb. 16G9 mAb was generated as a tool to elucidate the mechanism of immunological tolerance in liver transplantation (19) and was found to have MLR-inhibitory activity (data not shown). Here, we used phage display to identify peptides from a large library that bind to Ab-binding sites, thereby mimicking the conformational features of both linear and conformational epitopes. KLH- or OVA-conjugated SSV and LPQ were used in all ELISAs due to difficulties in working with their free forms. We could not detect the binding of 16G9 mAb and immobilized free peptides in an ELISA system. Our conjecture is that the particular peptide conformation that Abs will recognize is not predictable. When peptides are displayed on phages, they may adopt a more rigid conformation than free peptides, as they are anchored to the pIII protein at the NH$_2$ terminus in the phage display that was used in this study (23). Therefore, the conjugation of KLH and OVA may change their conformation to resemble that on the phages. In ELISAs, KLH-conjugated SSV and LPQ were used to examine their binding to 16G9 mAb; KLH alone was also used to eliminate its nonspecific binding to 16G9 mAb (Fig. 1). It was found that both SSV and LPQ bind to 16G9 mAb. However, the binding of 16G9 mAb to histone H1 was only inhibited by SSV (Fig. 1). Post-OLT serum at rejection phase in a rat tolerogenic OLT model (DA-PVG), which bound to histone H1 (10), bound to SSV only, and the binding was inhibited by histone H1 (Fig. 2). Therefore, SSV was selected as the more promising epitope for mimicking histone H1 and was studied in more depth.

Several reports have shown the effectiveness of vaccination with peptides screened with mAbs using a phage display peptide library in combating viral infection (24, 25). The development of a mimotope-based peptide vaccine with immunosuppressive activity would be a novel approach against rejection in organ transplantation. Our previous studies have suggested anti-histone H1 Ab as a potential immunosuppressant (10, 11). Histone H1 vaccination of transplant recipients led to the production of immunosuppressive factors and the modification of cytokine/cellular profiles. Histone H1 vaccination has great potential as a tolerance therapy for prospective transplantation (26). Recently, we evaluated the immunosuppressive state of histone H1-immunized rats using MLR (27). The results demonstrated that immunization with histone H1 induces allogeneic T cell unresponsiveness in vitro. Furthermore, in our unpublished data we show that anti-histone H1 autoantibody was transiently induced in the natural recovery stage from Con A-induced liver injury in rats. Our speculation is that the induction of anti-Histone H1 Ab may be a “weapon” to prevent a breakdown of the immune system (27). Our results have shown that SSV is a functional histone H1 epitope for 16G9 mAb. We speculate that SSV may work in the same way as histone H1 in tolerance induction. We hypothesized that immunization with SSV could induce an Ab similar to anti-histone H1 Ab that has immunosuppressive activity. One advantage of immunization with a peptide containing the minimal epitope is the acquisition of greater specificity than whole Ag immunization (28). Peptide immunization minimizes the risk of cross-reactivity between proteins with similar structures. Here we immunized mice with KLH-conjugated SSV or LPQ, or with KLH alone. Consequently, a good anti-SSV Ab was achieved after three immunizations (Fig. 3). Serum samples from SSV-immunized mice inhibited Con A- and anti-CD3 Ab-stimulated splenocyte proliferation significantly (Figs. 4 and 5) compared with those from LPQ- or KLH-immunized mice. The suppressive activity of SSV-immunized serum was neutralized by preincubation with SSV, confirming the immunosuppressive effect of anti-SSV Ab (Fig. 4C). The inhibition of the proliferation by anti-SSV Ab was not the result of cell death (Fig. 5C). SSV immunization in a rat HHT model resulted in a significant prolongation of graft survival (Fig. 6), suggesting that SSV is a functional epitope of 16G9 mAb and can be used as a nontoxic vaccine to induce tolerance in organ transplantation.

In summary, 16G9 mAb was generated as a tool to elucidate the mechanism involved in immunological tolerance and will be evaluated as a therapeutic agent in organ transplantation. Our results demonstrated that SSV recognizes 16G9 mAb and the binding of 16G9 mAb to histone H1 is inhibited by SSV. Rat tolerogenic post-OLT serum recognizes SSV during the rejection phase (post-OLT days 14–21), indicating the potential use of SSV as a marker for the withdrawal of immunosuppressive drugs in liver transplantation in the same way as histone H1 (29). In vitro and in vivo experiments have suggested that SSV can be used as an immunosuppressive vaccine. Furthermore, it has been reported that peptide mimotopes that can be identified for Abs can serve as Ag templates for Ab engineering (30). Therefore, SSV holds a promising aspect that it could be used as a mimotope-guided strategy for engineering Ab directed against histone H1 and could be used to establish humanized 16G9 mAb.

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**Disclosures**

The authors have no financial conflicts of interest.

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