Prevention of Graft-Versus-Host Disease by Peptides Binding to Class II Major Histocompatibility Complex Molecules

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Graft-versus-host disease across minor histocompatibility barriers was induced in two different models by transplanting allogeneic bone marrow and spleen cells into irradiated H-2-compatible recipient mice. In this report, we show that administration of peptides with high binding affinity for the respective class II major histocompatibility complex molecules after transplantation is capable of preventing the development of graft-versus-host disease in two different murine models. The peptides used were myelin basic protein residues 1 through 11 with alanine at position 4 (Ac 1-11[4A]) for I-A" (Aa"Aa") and the antigenic core sequence 323 through 339 of ovalbumin with lysine and methionine extension (KM core) for I-A" (Aa"Aa"). In both systems, the mechanism of prevention was found to be major histocompatibility complex-associated, because nonbinding control peptides did not have any effect. Engraftment of allogeneic bone marrow cells was shown by polymerase chain reaction analysis of DNA polymorphisms in a microsatellite region within the murine interleukin-5 gene.

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MATERIALS AND METHODS

Animals and transplantation. Female PL/J, B10.PL (both H-2") and SJL mice were purchased from The Jackson Laboratories (Bar Harbor, ME). B10.S (H-2") female mice were bred in our own facility. SJL and B10.S mice express class II A" (Aa"Aa") only; I-Eα is deleted.25 GVHD was induced by transplanting 10 × 10^6 allogeneic BM and 50 × 10^6 allogeneic spleen cells into sublethally irradiated (560 R) H-2 identical recipient mice.29 Recipient mice were 12 weeks old at the time of transplantation. The experimental group design is outlined in Table 1. Engraftment was shown by polymerase chain reaction (PCR) analysis of a polymorphic microsatellite region.
**PREVENTION OF GVHD BY PEPTIDES**

### Assessment of GVHD
Skin biopsies were examined by two independent observers for histologic evidence of GVHD. Epidermal dyskeratotic cells (EDC; individual cell death as defined by nuclear pyknosis and cytoplasmic hypereosinophilia) and vacuolated cells (basal epithelial cells with vacuolar degeneration of the cytoplasm) were quantitated as previously described. Given 95% confidence intervals for groups B and D, GVHD prevention in any particular mouse was defined as histologic changes identical to syngeneic controls.

### PCR analysis
Engraftment of donor BM was documented by PCR amplification of a polymorphic microsatellite region within the interleukin-5 (IL-5) gene. Primer sequences are as follows: 5'-CCCTTCCTGGAAGTGATTAGG-3' and 5'-AGACCATCTG-CATATTCACG-3'. These sequences are available from EMBL/GenBank/DBJ (Bethesda, MD) under the accession numbers X75904 and X75905. Oligonucleotides were synthesized on a 391/PE-Genie synthesizer (Perkin Elmer, Emeryville, CA). Amplification was performed for 30 cycles (1 minute of denaturation at 94°C, 1 minute of annealing at 55°C, and 1 minute of elongation at 72°C).

### Peptides
Peptides were synthesized by standard FMOC chemistry as previously described. All peptides were 95% to 99% pure, as determined by high-performance liquid chromatography, and were checked by amino-acid analysis and mass spectroscopy. Sequences are given in single letter codes: Ac 1-11[4A], AcASQARPSQVRHG; MBP 35-47, TGILDISGIRFSGS; KM-core extension peptide KMKMVHAHKM; HEL 46-61, NTGDSTDYGLINLSR. MBP 89-101, VHHFKNVTRPTP, was synthesized by t-butoxycarbonyl chemistry.

### Peptide treatment
Transplanted mice were treated with Ac 1-11[4A] and MBP 35-47 (B10.PL) or KM core (B10.S; see Table 1). Control mice received either phosphate-buffered saline (PBS) or a nonbinding control peptide (MBP 89-101 for I-A^d^ and I-E^b^; HEL 46-61 for I-A^b^). Syngeneic control mice received PBS. For the first 3 weeks after transplantation, peptides were injected in PBS intraperitoneally (IP) daily, based on published data of the half-life of peptide-class II complexes being between 5 and 38 hours in live antigen-presenting cells. The frequency of injections was tapered to 3 times per week for the following 2 weeks and to 2 times per week for another 2 weeks. Once a week, peptides were administered with incomplete Freund’s adjuvant (IFA) as a depot dose. Treatment was discontinued after 7 weeks. Each injection contained 100 μg (60 to 80 nmol) of each peptide, except for control peptide MBP 89-101, which contained 200 μg (Table 1). The total dose of peptide per mouse was 6.2 mg for the B10.PL recipients or 3.1 mg for the B10.S recipients.

### RESULTS

**Ac 1-11[4A] and MBP 35-47 prevent GVHD in the PL/J → B10.PL (H-2b) system.** Mice were followed up for 100 days after BMT for evidence of GVHD. Skin biopsy specimens were taken 30 days after transplantation during peptide therapy and on day 70 after transplantation, 21 days after peptide therapy had been stopped. Figures 1A and B show GVHD-specific skin changes on day 30 and day 70 in the PL/J → B10.PL system. Data points represent individual mice and their values of dyskeratotic cells (EDC) per millimeter of skin and vacuolated cells per millimeter of skin in the different experimental groups. Data pooled from two identical experiments showed that the incidence of histologic GVHD on day 30 was 16 of 16 in control groups B (PBS) and C (nonbinding peptide) and was 0 of 14 in group D (syngeneic controls). Given 95% confidence intervals for groups B and D, GVHD prevention in any particular mouse was defined as histologic changes identical to syngeneic controls and is represented graphically as a dashed box (Fig 1A and B). No prevention was found with the nonbinding peptide MBP 89-101 (n = 16). However, GVHD was prevented...
Fig 1. GVHD-specific skin changes on day 30 (A) and day 70 (B) in the PL/J → B10.PL model. Data points represent individual mice and their values of EDC per millimeter of skin and epidermal vacuolated cells per millimeter of skin. Data are pooled from two identical experiments. The group design is outlined in Table 1. Given 95% confidence intervals for groups B and D, a cutoff for GVHD prevention was selected as described in Materials and Methods and is represented graphically as a dashed box. Significance levels determined by \( \chi^2 \) distribution are as follows: ** \( P < .001 \), * \( P < .05 \), incidence of GVHD in group A compared with group B or C.
in 10 of 18 mice (55.6%) from group A that received the binding peptides Ac 1-11[4A] and MBP 35-47 (P < .001; see Fig 1A). Figure 2 shows representative skin biopsy specimens from animals of the experimental groups A through D. Skin biopsy specimens from control-transplanted mice of groups B and C that were treated with PBS or MBP 89-101 show prominent EDC and vacuolated cells in the epidermis with diffuse basal cell layer hyperplasia and infiltrates of superficial dermal mononuclear cells. Figure 2A depicts a biopsy specimen from a mouse that received Ac 1-11[4A] and MBP 35-47 and did not develop GVHD, a finding identical to the histology of a syngeneic control mouse (Fig 2D). The effect of the binding peptides was transient in some mice and permanent in others. After discontinuation of treatment GVHD recurred in 4 of 18 mice of group A, when they were tested 70 days posttransplant (Fig 1B); 6 of 18 mice remained without evidence of GVHD (P < .05).

Documentation of engraftment. PCR analysis was performed 100 days posttransplant to document long-term engraftment of allogeneic BM cells. DNA polymorphism based on length variation in tandem repeat sequences of a microsatellite in the murine IL-5 gene32 was used as marker to differentiate between donor-derived (PLJ) and recipient (B10.PL) cells. Figure 3 depicts one representative analysis on an ethidium bromide-stained 2% agarose gel. Long-term engraftment of donor-derived cells was shown in all allogeneic mice by PCR analysis.

KM core extension peptide prevents GVHD in the SJL → B10.S (H-2s) system. To determine whether the above results were a unique property of the peptides Ac 1-11[4A] and MBP 35-47 or whether a similar mechanism might operate in other H-2-identical donor-recipient strain combinations as well, an additional strain combination was chosen. KM core extension peptide was selected for its well-characterized high-binding affinity for I-A2 and for its resistance to serum proteases.11 Moreover this peptide is structurally unrelated to the MBP. Data from two similar experiments showed that the incidence of GVHD on day 30 and day 70 was 14 of 14 in mice treated with PBS and 13 of 13 in mice treated with the nonbinding control peptide HEL 46-61. However, in mice that received KM-core, the incidence of GVHD was 10 of 16 (62.5%) on day 30 and 11 of 16 (68.8%) on day 70 posttransplant.

Ac 1-11[4A] is presented by peritoneal macrophages. To further elucidate the mechanism(s) of GVHD prevention, we showed in vivo presentation of Ac 1-11[4A] by peritoneal macrophages after IP injection of the peptide. The IL-2 production of the Ac 1-11[4A]-specific 1934.4 T-cell hybridoma8 provided an assay for the presence of the peptide on the surface of peritoneal macrophages from transplanted B10.PL mice of group A. Macrophages from this group elicited a response from the hybridoma, whereas macrophages from transplanted B10.PL mice that had been injected with PBS (group B) or control peptide MBP 89-101 (group C) failed to stimulate 1934.4 (Fig 4A). Moreover, the IL-2 response in groups B and C could be completely restored by exogenous addition of Ac 1-11[4A] to the assay. Ac 1-11[4A] could be detected on peritoneal macrophages with this assay as long as 2 to 4 hours after IP injection (data not shown).

Immunogenicity of the competitor peptides. As another important step in our understanding of the mechanism of in vivo prevention, we have tested whether the soluble peptides in IFA are able to induce proliferative T-cell responses against themselves. For these assays, nonirradiated mice from donor strains were chosen, because donor T cells are responsible for inducing GVHD in transplanted animals. A total of 150 nmol of Ac 1-11[4A] or MBP 35-47 emulsified in IFA was injected IP into nonirradiated PLJ mice. Similarly, 150 nmol of KM core in IFA was injected into nonirradiated SJL mice. T-cell proliferation assays showed no proliferative response to Ac 1-11[4A] or MBP 35-47 (Fig 4B). In contrast, however, KM core in IFA was found to be highly immunogenic (Fig 4B). The implication of these findings on differential mechanisms of prevention in the H-2s and H-2q systems are discussed below. It was then asked whether daily administration of Ac 1-11[4A] and MBP 35-47 according to the treatment schedule would induce a proliferative T-cell response in transplanted B10.PL mice. In two similar experiments, no proliferative T-cell response to either peptide could be detected (Fig 4C).

Selective in vitro inhibition of secondary mixed lymphocyte reaction (MLR). We then determined whether T-cell-mediated proliferative responses against mH antigens could also be inhibited in vitro. In secondary MLR, Ac 1-11[4A], which binds to I-A2, or MBP 35-47, which binds to I-E2, inhibited the proliferative response of responder spleen cells from primed B10.PL mice to irradiated PLJ stimulator cells by 60% or 43%, respectively (Fig 5A). Inhibition by class II-binding peptides was MHC-associated, because the control peptides KM core and MBP 89-101, which bind with high affinity to a different allele (I-A2), failed to block the response.

MHC-specific inhibition of T-cell priming to whole OVA. After the observation of inhibition of T-cell responses to mH antigens in vitro, we investigated whether in vivo T-cell responses could be inhibited in a similar fashion. We decided to test whether priming to a native structurally nonrelated, nonself protein (OVA) in PL/J mice could be inhibited by coinmunization of OVA with Ac 1-11[4A] or MBP 35-47 at a molar excess of competitor peptide over OVA of 1:1, 25:1, and 100:1. As for coinmunization with Ac 1-11[4A], maximum inhibition (77.6% to 96%) was already found at the ratio of 1:1 (Fig 5B). For MBP 35-47, no inhibition was found at a ratio of 25:1 (data not shown); at a ratio of 100:1, maximum inhibition of OVA-specific T-cell proliferation was observed.
ranged from 45.9% to 51.4%. Specificity controls included the nonbinding control peptide 89-101, which did not block T-cell responses at any of the molar ratios tested.

**DISCUSSION**

The molecular basis of antigen recognition by T cells has been intensely studied in recent years, and the various pathways of T-cell activation and functional specialization have been defined. Consequently, it is now possible to delineate various strategies for intervention with allorecognition in the context of BMT, namely, interference with TCR-peptide-MHC recognition, blocking of T-cell costimulation, differential activation of CD4+ T cells by antigen-presenting cells, and interference with recruitment of T cells mediated through homing receptors. Previous studies have shown that both CD4+ and CD8+ T-cell subsets can be responsible for the induction of GVHD across mH barriers. Although one purified subset of T cells may be sufficient to initiate GVHD, a combination of CD8+ and CD4+ T cells induces a much more severe form of GVHD in a variety of experimental strain combinations, a conclusion that is consistent with the classical paradigm that the cellular response to alloantigens involves both class I-restricted and class II-restricted T cells. Furthermore, isolation of T cells from the skin of mice with GVHD and clonal analysis of murine GVHD on days 14 and 50 after transplant have shown the importance of CD4+ T cells in the early and later phases of GVHD.

In this series of studies, we focused on the interaction of T cells with antigens presented by class II MHC molecules. Results of four independent experiments in two distinct murine models of GVHD across mH barriers showed that class II-binding competitor peptides can be used to prevent GVHD in vivo. The mechanism of prevention was MHC-associated. Binding of the competitor peptide to the class II molecule was a prerequisite for prevention in both systems. Nonbinding control peptides did not prevent the induction of GVHD. Thus, the mechanism of prevention in both model systems is mediated by interference with T-cell recognition of host peptide-MHC complexes. Furthermore, PCR analysis of transplanted mice showed that prevention of GVHD was not an artefact related to regrowth of autologous BM. All allogeneic mice showed long-term engraftment of donor-derived cells.

GVHD across mH barriers is caused by donor T cells recognizing mH antigens of the host as "nonself." Over 40 murine MHC loci have been defined, and almost every chromosome, including the mitochondrial genome, carries at least one. Minor antigens share unique features. Whereas MHC antigens are recognized by both T and B cells, responses to mH antigens are strictly T-cell-mediated, and their recognition is MHC-restricted. Detectable T-cell re-
responses to mH antigens require in vivo immunization (priming) before in vitro restimulation. Many of the mH antigens display a restricted tissue distribution. In some instances, latent viral sequences may encode for some mH antigens or control their expression. Our results of selectively preventing GVHD by class II-binding peptides support the recently proposed concept that mH antigens are genetically polymorphic peptides derived from a variety of cellular proteins that are subsequently presented by MHC molecules.

To further elucidate the mechanism(s) of prevention, we demonstrated (partial) occupancy of class II molecules by Ac 1-11[4A] on live antigen-presenting cells from transplanted animals using the IL-2 response of the 1934.4 hybridoma as a read-out system. The latest positive signals up to 4 hours after IP injection (data not shown) are in accordance with previously published data showing the in vitro half-life of peptide-class II complexes in live antigen-presenting cells to range from 5 to 38 hours and illustrate the need for daily injections of peptide during the first weeks after transplantation. The dose rationale for the peptide treatment in the experiments described was based on the calculation that 100 nmol of competitor peptide provides a 10^3 molar excess of competitor peptide over constitutively expressed class II-binding sites modified by a factor of 10^2 to 10^3 for the upregulation of class II molecules on professional antigen-presenting cells and for the induction of class II molecules on cells that do not constitutively express MHC class II after BMT. Consequently, 100 nmol of competitor peptide is estimated to result in approximately 10^2 to 10^3 molar excess of competitor peptide over class II-binding sites. In the con-

Fig 4. (A) Presentation of injected Ac 1-11[4A] by peritoneal macrophages after IP injection. The peritoneal cavity of transplanted B10.PL mice was flushed with 3 mL of PBS before and at different intervals (1, 2, and 15 minutes) after IP injection of Ac 1-11[4A] (■), PBS (○) or MBP 89-101 (▲). 1934.4 Hybridoma cells (5 × 10^5), which recognize Ac 1-11[4A] in the context of I-A*, were incubated with 5 × 10^5 peritoneal macrophages. IL-2 production was assayed by measuring the [3H]thymidine uptake of the IL-2–dependent cell line HT-2 in the presence of supernatants from the 1934.4 cells. The results are the mean values of triplicate measurements. T-cell proliferation assays from (B) nonirradiated PL/J SJL mice in response to Ac 1-11[4A] (■), MBP 35-47 (□) or KM core (△) and from (C) transplanted B10.PL mice after 21 days of daily peptide treatment in response to Ac 1-11[4A] (■) or MBP 35-47 (□). (C) A total of 150 nmol of Ac 1-11[4A] or MBP 35-47 emulsified in IFA was injected IP into nonirradiated PL/J mice; 150 nmol of KM core in IFA was injected IP into nonirradiated SJL mice. Ten days after injection spleen cells (draining site for IP injections) were incubated in triplicate at 5 × 10^5 cell/well with the relevant peptides (0.1 to 100 μmol/L) or controls as stimulators. After 72 hours of incubation, cultures were pulsed with 1 μCi [3H] thymidine for an additional 12 hours. Controls included medium as background (2,124 cpm/3,343 cpm/3,791 cpm), purified protein derivative (PPD; 25,449 cpm/25,566 cpm/19,519 cpm), and nonbinding control peptide (2,336 cpm/3,399 cpm/3,940 cpm). Data are representative of two identical experiments. Similarly, retroperitoneal and mesenteric lymph node cells from nonirradiated PL/J injected with 150 nmol of Ac 1-11[4A] or MBP 35-47 in IFA showed no proliferative T-cell response on in vitro rechallenge with the peptides (data not shown). (C) After 21 days of daily peptide treatment and after administration of the peptides with IFA once a week, spleen cells (primary draining site after IP injection) from transplanted B10.PL mice were incubated in triplicate at 5 × 10^5 cell/well with relevant peptides (0.1 to 100 μmol/L) or controls as stimulators. Controls included medium as background (4,304 cpm), Con A (8,178 cpm), and nonbinding control peptide (4,066 cpm). Note that positive controls (Con A) are considerably lower after irradiation and transplantation. Data are representative of two identical experiments. Culture medium was RPMI 1640 supplemented with 1% fresh normal mouse serum, 2 nmol/L glutamine, 50 μmol/L 2-mercaptoethanol, 100 U/mL penicillin, and 100 μg/mL streptomycin.
tides may reduce presentation of endogenous peptides so tides are presented by class I1 MHC molecules, some of which will bind to as little as 0.001% of class II molecules. On the basis of our data, it is feasible that partial occupancy of class II-binding sites by the competitor peptides may reduce presentation of endogenous peptides so that T-cell activation and consequently induction of GVHD is inhibited. The difficulty of achieving a constant level of saturation of class II molecules over extended periods of time and the lack of available class I-binding inhibitor peptides may account for the fact that GVHD was not prevented in all animals. In the EAE system, it has been shown that coimmunization of the disease-inducing peptide with the structurally nonrelated competitor peptide OVA 323-339 at a molar excess of 25:1 is able to prevent disease induction. Moreover, we were able to show that T-cell proliferative responses to minor antigens in vitro and T-cell priming to an unrelated whole protein in vivo could be inhibited by MHC class II-binding peptides. Both peptides, Ac 1-11[4A] (binding to class II I-A") and MBP 35-47 (binding to class II I-E") were found to inhibit T-cell responses in vitro and in vivo.

Experimental models of autoimmune diseases in which class II-binding peptides have been used can be assigned to two categories, based on the presence or absence of structural homology between the inhibiting peptide and the disease-inducing antigens/peptides. In systems where the inhibitor peptide is closely related to the antigenic peptide, antigen-specific mechanisms in addition to interference with TCR-peptide-MHC recognition must be considered. These additional regulatory mechanisms include induction of cross-reactive T-cell tolerance, induction of regulatory suppressor T cells, alteration in the signalling pathway through TCR occupancy leading to anergy, or a shift in the lymphokine profile. However, in both murine models of GVHD described in this manuscript, MHC-specific mechanisms rather than antigen-specific mechanisms must be considered, because competitor peptides and multiple endogenous self peptides are nonhomologous. Competitive blockade of class II molecules in vivo and immunodominance have been invoked as MHC-specific mechanisms of inhibiting T-cell activation. Using a panel of T-cell hybridomas generated from 52M/- knock-out mice, Guéry et al. have recently presented evidence that exogenous MHC antagonists can inhibit constitutive presentation of an endogenous, naturally processed self epitope. Our results show that partial occupancy of class II-binding sites was sufficient to inhibit the development of GVHD. Furthermore, it was shown that Ac...
1-11[4A] and MBP 35-47 were nonimmunogenic in both nontransplanted PL/J donor mice and transplanted B10.PL recipients after 21 days of daily peptide treatment. In contrast, a profound T-cell response to KM core was found in SJL mice. These in vitro data suggest different mechanisms by which the above peptides prevent GVHD, namely, interference with TCR-peptide-MHC recognition without T-cell activation and immunodominance (MHC interference with a marked T-cell response to the competitor peptide resulting in clonal dominance of competitor-specific over antigen-specific T-cells).\(^{1,11,12,26}\) As for Ac 1-11[4A] and MBP 35-47, we propose interference for MHC class II binding, as evidenced by the fact that both peptides are nonimmunogenic by virtue of their binding capacity and by the observation that nonbinding control peptides do not block GVHD. As for KM core, immunodominance will have to be considered as a contributing mechanism. Gammon and Sercarz\(^{47}\) have shown that such a mechanism of immunodominance or determinant hierarchy could inhibit the ability of less dominant epitopes to elicit effective T-cell responses in vivo in the presence of a dominant epitope.

In conclusion, our data have shown for the first time that peptides with high binding affinity for class II MHC molecules are able to prevent GVHD across mH barriers in two different murine systems. The results support the recently proposed concept that mH antigens are polymorphic peptides, derived from a variety of cellular proteins, that are subsequently presented by MHC molecules. The feasibility of this new approach of competitively inhibiting MHC-peptide interaction opens an area of intense investigation to define competitor peptides that will bind with high affinity to human class II molecules and that may be potentially useful for the prevention of GVHD in allogeneic BMT patients.

ACKNOWLEDGMENT

We would like to thank C. O. Jacob (University of Southern California, Los Angeles, CA), H. Jürgens (University of Münster, Germany), and D. Niethammer (University of Tübingen, Germany) for stimulating discussions; and Karl G. Blume (Stanford University, Stanford, CA) for his critical review and support throughout the course of these studies.

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