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A Synthetic CD4-CDR3 Peptide Analog Enhances Skin Allograft Survival Across a MHC Class II Barrier

Ute Koch, Swati Choksi, Lisa Marcucci, and Robert Korngold

The efficacy of a synthetic peptide analogue (rD-mPGPtide), mimicking the CDR3 region in the first domain of the CD4 surface molecule, was investigated in a murine model for CD4\(^+\) T cell-mediated skin allograft rejection. A single injection of rD-mPGPtide shortly before transplantation exhibited significantly prolonged graft survival in the B6 anti-B6.C-H\(^{2bm12}\) MHC class II-disparate strain combination. Long-term graft survival (>100 days) was achieved when thymectomized adult recipient mice were transplanted along with rD-mPGPtide treatment. The peptide also affected secondary rechallenge responses with MHC class II allografts. In addition, the inhibitory effect of the rD-mPGPtide in this transplantation model was directed against CD4\(^+\) T cells and was exclusively specific toward donor alloantigen. In vitro analysis of CD4\(^+\) T cells isolated from the draining lymph nodes of rD-mPGPtide-treated recipients indicated a 450-fold decrease in precursor frequency in response to donor allostimulation compared with the untreated control group. There was also significant down-regulation of the frequency of IL-2-, IFN-\(\gamma\), and IL-4-producing CD4\(^+\) T cells upon in vitro allogeneic restimulation of host cells 4 days posttransplantation. However, these same CD4\(^+\) T cells maintained the capacity to produce normal cytokine levels upon third-party allostimulation. Thus, these studies demonstrate that a CD4-CDR3 peptide analogue can specifically and effectively prolong skin graft survival across MHC class II barriers. The Journal of Immunology, 1998, 161: 421–429.

Clinical organ transplantation between genetically disparate individuals presently requires nonspecific immunosuppressive agents to prevent rejection. However, the use of such agents risks morbidity and mortality from a range of associated side effects and does not always guarantee success of the graft. Rejection of a solid organ transplant by a recipient is generally mediated by a strong alloreactive immune response that primarily involves CD4\(^+\) and CD8\(^+\) T cells, depending upon the specific associated antigenic differences (1). In an attempt to develop a more alloantigen-specific immunosuppressive therapy, CD4\(^+\) T cells are an obvious target, since they play such a pivotal role in alloreactivity (2, 3). In this regard, mAbs against the murine CD4 Ag have proven to be one of the most versatile means to prevent or delay the graft rejection process and to induce tolerance (4, 5). These mAb may inhibit the function of the mature alloreactive CD4\(^+\) T cells responsible for rejection of the graft by either blocking the molecular interactions of the CD4 molecule, modulating its expression, or causing the T cells to be anergized or deleted from the lymphoid compartment.

Recently, a synthetic peptide was developed to mimic the distinct molecular surface structure of the CDR3-like region in the first domain (D1) of the murine CD4 molecule, which is a member of the Ig superfamily (6). This analogue, designated rD-mPGPtide, exhibited significant inhibition of the clinical and pathologic symptoms of experimental allergic encephalomyelitis (EAE)\(^4\) in the SJL mouse model (6, 7). In addition, this peptide significantly inhibited the development of graft-vs-host disease in lethally irradiated recipients of a haploidentical bone marrow transplant (8). Likewise, rD-mPGPtide could enhance donor hematopoietic engraftment across either a full or class II MHC barrier and establish effective donor/host tolerance and subsequent immunocompetence (9).

In the present study, the prophylactic effect of the rD-mPGPtide in a murine model for CD4\(^+\) T cell-mediated skin allograft rejection was investigated. A single injection of the CD4-CDR3 peptide analogue 3 h before transplantation significantly delayed graft rejection across a MHC class II allogeneic barrier in the B6 anti-B6.C-H\(^{2bm12}\) strain combination and was capable of inducing long-term graft survival in adult thymectomized recipient mice. Furthermore, rD-mPGPtide treatment significantly delayed the rejection process in presensitized recipients and acted in a donor alloantigen-specific fashion in mice later challenged with an SJL third-party allograft. The rD-mPGPtide significantly decreased the precursor frequency of detectable donor-specific B6 CD4\(^+\) T cells by day 8 posttransplantation, and lowered both Th1 and Th2-like cytokine production. Overall, these results suggest that the CD4-CDR3 analogue can be an effective inhibitor of murine alloreactive CD4\(^+\) T cells involved in the rejection process of skin allografts.

Materials and Methods

Mice

Male C57BL/6J (B6; H\(2^b\)), B6-adult-thymectomized (B6-ATX; ATX was performed at 6 wk of age), B6.C-H\(^{2bm12}\) (bm12), and B6.C-H\(^{2bm1}\) (bm1) mice were purchased from The Jackson Laboratory, Bar Harbor, ME. Female SJL/J (H\(2^a\)) and BALB/c nu/nu mice were purchased from the National Cancer Institute, Bethesda, MD. Mice were 8 to 12 wk of age when

\(^4\) Abbreviations used in this paper: EAE, experimental allergic encephalomyelitis; B6, C57BL/6; CDR, complimentary determining region; LN, lymph node; MST, median survival time; ATX, adult thymectomized; PE, phycoerythrin; ELISPOT, enzyme-linked immunospot assay.
used in experiments. Mice were kept in a sterile environment in microisolators at all times and were provided with acidified water and autoclaved food ad libitum.

**Media**

PBS (BioWhittaker, Walkersville, MD) supplemented with 0.1% BSA (Sigma, St. Louis, MO) was used for all in vitro manipulations of the lymphocytes. RPMI 1640 medium (Mediatech, Herndon, VA) supplemented with 10% FCS (Atlanta Biologics, Norcross, GA), 2 mM l-glutamine, 50 IU/ml penicillin, 50 µg/ml streptomycin, and 0.05 mM 2-ME (Mediatech) was used for all in vitro assays (complete RPMI).

**CD4-CDR3 peptides**

The peptides were designed as previously described (6), synthesized on a 430A peptide synthesizer (Applied Biosystems, Foster City, CA) using standard F-moc chemistry, refolded to enrich for intramolecular disulfide bonding, and purified by HPLC on a system using a Waters 600E system controller and Waters 990E programmable multiwavelength detector (Milipore, Bedford, MA). The sequences of the peptides were as follows: rDmPGPId (CGPPEEKNELEG, all N-termino acids) and scrambled rDmPGPId (Scr-rDmPGPId, same amino acid composition, but scrambled sequence, CEKPKNLPEGEC, all N-termino acids). The rDmPGPId and the Scr-rDmPGPId were stored lyophilized at room temperature in a dehydrated chamber under house-vacuum pressure until use. For injection, the peptides were reconstituted in sterile PBS and injected i.v. at a concentration of 2.5 mg/ml in a volume of 0.2 ml.

**Monoclonal Abs**

Ascites fluid for anti-CD8 (3.168, rat IgM (10), 1:100 dilution in vitro), anti-CD8 (2.43, rat IgG2b, 1:50 dilution in vivo: American Type Culture Collection (ATCC), Rockville, MD) ATCC TIB210, and anti-CD4 (GK1.5, rat IgG2b, 1:100 dilution in vivo: ATCC TIB207) mAb were generated in our laboratory from in vivo passage of hybridoma cells in BALB/c mice. Injection of 0.2 ml of a 1:50 dilution of the 2.43 ascites fluid induced >98% depletion of CD8 T cells within both lymphoid organs. Goat anti-mouse IgG Ab was purchased from Cappel-Organon Teknika, West Chester, PA. Guinea pig serum, prepared in our laboratory, was used as a source of C for all Ab treatments. Supernatant containing anti-mouse FcRγII/III (2.4G2, rat IgG2b, ATCC HB917 (19)) mAb was generated in our laboratory. Surface phenotype was analyzed by immunofluorescent labeling and, as a negative control, rat IgG2a mAb were used to remove B cells. This treatment resulted in a cell population (>99% CD8 T cells) as quantitated by flow cytometry.

**Preparation of cells**

A panning procedure to deplete B cells was used to enrich T cells from pooled lymph node (LN) cell suspensions. LN cells were treated with ACK lysis buffer containing 0.15 M NH₄Cl, 1.0 mM KHCO₃, and 0.1 mM Na₂EDTA in H₂O (pH 7.2) for removal of RBC, and panned over a Falcon 150-mm plastic petri dish (Becton Dickinson, Lincoln Park, NJ) precoated with a 5 µg/ml solution of goat anti-mouse IgG (Cappel-Organon Teknika) for 1 h at 4°C to remove B cells. This treatment resulted in a cell population containing >97% TCRβ⁺ T cells, as detected by ELISPOT assay.

CD4 T cells analyzed in the ELISPOT assay were obtained from the pooled inguinal and mesenteric LN of graft recipients on day 4 posttransplantation or from naive B6 mice. LN cells were enriched for T cells as described above, and further isolation of CD4 T cells was performed by depletion of CD8 T cells with anti-CD8 mAb (3.168, 1:100 dilution) and C (1:50) for 60 min at 37°C, followed by filtration over nylon mesh and washing. These procedures resulted in highly enriched populations of CD4 T cells (>97%) with no detectable presence of CD8 T cells, as assessed by flow cytometry.

**Quantification of functional T cells by limiting dilution analysis**

CD4 T cells were isolated on day 8 posttransplantation from the pooled inguinal and mesenteric LN of eight graft recipients per treatment group. CD4 T cells were cultured at threefold dilutions ranging from 3 × 10⁵ to 1 × 10⁶ cells per well (24 wells per dilution) in a 96-well round-bottom microtiter plate (Costar, Cambridge, MA) in complete RPMI. Syngeneic B6, alloimmune (bm12), and third-party (SJL) T cell- and RBC-depleted and irradiated (20 Gy) spleen cells were used as stimulators (2.5 × 10⁴ cells per well, final volume of 200 µl) after a 48-h incubation at 37°C, 7% CO₂. Plates were irradiated at 20 Gy and 5 × 10⁷ cells of the IL-2-dependent CTLL-2 indicator cell line (ATCC TIB214) were added per well. All plates were incubated for an additional 24 h and 1 µCi [3H]TdR/well was added 6 h before harvesting onto glass fiber filters (Wallac Oy, Turku, Finland) with a Harvester 96 (TomTec, Orange, CT) and counted in a 1205 Beta-Plate reader (Wallac, Gaithersburg, MD). Estimates of the precursor frequency of CD4 T cells reactive to the stimulator cells were obtained by the maximum likelihood method based on the Poisson distribution and the number of responding cells and the log-rithm of the fraction of negative cultures (16). The cell dose that yielded 0.37 negative cultures was extrapolated for the frequency calculation.

**ELISPOT assay to detect single cytokine secreting CD4⁺ T cells**

The frequencies of IL-2-, IL-4-, and IFN-γ-secreting CD4 T cells in graft recipients were determined by the ELISPOT assay on day 4 posttransplantation and performed as previously described (14, 17). Briefly, CD4 T cells (1 × 10⁶ cells/5 ml) were purified from the draining LN, as described above, and incubated with or without (1:100 dilution) anti-CD4 mAb, followed by 4°C 30-min i.v. injection of rat anti-mouse IL-2 mAb (clone CTCL2/2 indicator cell line (ATCC TIB214) were added per well for 48 h at 37°C, 7% CO₂ in the presence or absence of human rIL-2 (50 U/ml) (Biologic Response Modifiers Program, NCI, Bethesda, MD). Ni-trocilulose-backed microtiter plates (Millipore) were coated with cytokine-specific primary capturing mAb, including either purified rat anti-mouse IL-2 mAb (1:100 dilution), or recombinant human rIL-2 (50 U/ml), or anti-IFN-γ mAb (50 U/ml), or IFN-γ-secreting cells were detected by the addition of 50 µl/well of biotin-labeled cytokine-specific secondary detecting mAb, including either biotin rat anti-mouse IL-2 (2 µg/ml), IL-4 (0.33 µg/ml), or IFN-γ (4 µg/ml)
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unpublished observations). To investigate how long rD-mPGP tide could have an inhibitory effect upon the development of CD4+ T cell responses in vivo, peptide was injected into B6 mice (0.5 mg i.v.) at varying time points (0, 3, 6, and 24 h) before skin grafting. The results indicated that the optimum time to administer the peptide was between 3 and 6 h before transplantation (MST of 49 days, p ≤ 0.001 and 31 days, p ≤ 0.012, respectively; Fig. 2). In contrast, peptide treatment 24 h before grafting showed no significant prolongation of graft survival compared with the saline-treated recipients (MST of 19 days, p ≥ 0.99), suggesting that within this period of time the peptide concentration in vivo dropped below effective levels. The injection of rD-mPGP tide immediately before surgery also had no significant effect (MST of 22 days; p ≥ 0.94; Fig. 2), but appeared to be due to technical problems related to the proximity of the injection site to the transplant sites on the tail, since injection of even saline at this time resulted in intensified tissue damage, increased bleeding, and faster rejection of allografts. Based on these collective findings, all i.v. treatments of peptide and PBS were administered 3 h before transplantation in all subsequent experiments.

Effect of rD-mPGP tide on allograft rejection in adult thymectomized (ATX) recipients

Although significantly delayed, the MHC class II–disparate strain combination still exhibited graft rejection in the peptide-treated recipient mice. It was hypothesized that, over time, newly emerging alloreactive CD4+ T cells from the thymus were not tolerized and could eventually cause rejection of the bm12 allografts, in the absence of rD-mPGP tide. To test this notion, B6-ATX mice were used as recipients of bm12 allografts, thereby having only a static population of peripheral T cells available to participate in the rejection process. Recipients were divided into four treatment groups receiving 3 h before transplantation either PBS (i.v.), rD-mPGP tide (0.5 mg; i.v.), anti-CD4 mAb (1:100 ascites dilution; i.p.), or Scr-mPGP tide (0.5 mg; i.v.) in a volume of 0.2 ml. Statistical analyses of MST in comparison to the PBS-treated control group (n = 9, injected at −3 h) were as follows: rD-mPGP tide at 0 h (n = 10), p ≥ 0.94; −3 h (n = 9), p ≤ 0.001; −6 h (n = 10), p ≤ 0.01; −24 h (n = 5), p ≥ 0.99. These data were pooled from two replicate experiments.

Efficacy and optimization of rD-mPGP tide treatment

To investigate the efficacy of the rD-mPGP tide analogue in a skin allograft model, a MHC class II–disparate strain combination (B6 anti-bm12) was selected to concentrate on the CD4+ T cell response involved in the rejection process. Three hours before transplantation of bm12 donor tail skin grafts, B6 mice received injections of either PBS, rD-mPGP tide (ranging 0.25 mg to 2 mg; i.v.), or anti-CD4 mAb (1:100 ascites dilution; i.p.) in a volume of 0.2 ml. The PBS-treated recipients fully rejected the bm12 allograft in 10 days (Fig. 3). In contrast, the peptide-treated mice showed significant prolongation of allograft survival (MST of 25–29 days; p ≤ 0.01), indicating a threshold effect of the peptide treatment. Lowering the dosage to 0.25 mg resulted in a MST of 14 days and loss of significant prolongation of graft survival compared with the PBS group (p > 0.90). Animals treated with anti-CD4 mAb had a MST of 36 days (p ≤ 0.001), whereas mice treated with 0.5 mg of a scrambled control peptide (Scr-mPGP tide) showed no significant graft prolongation (MST of 13 days; p > 0.50; data not shown). In all subsequent studies, 0.5 mg of rD-mPGP tide was used as the optimal prophylactic dose to ensure an effective inhibitory response.

The serum stability of rD-mPGP tide is an important issue to consider in regard to the optimum time for administration and the in vivo efficacy of this agent. Preliminary studies have indicated that the peptide appeared to be moderately resistant to protease degradation, with 60% intact retention after 40 h of in vitro incubation in 90% murine serum. In addition, the in vivo serum half-life of the rD-mPGP tide in mice upon i.v. injection is approximately 25 min, with most of the peptide recovered undergraded in the urine (R. Wiaderkiewicz, R. M. Townsend, and R. Korngold;
mAb, Statistical analyses of MST in comparison to the PBS-treated control group anti-CD4 mAb (1:100 ascites dilution; i.p.; \(n=5\), rD-mPGPtide (0.5 mg; i.v.; \(n=7\), Scr-mPGPtide (0.5 mg; i.v.; \(n=9\), or anti-CD4 mAb (1:100 ascites dilution; i.p.; \(n=4\)) in a volume of 0.2 ml. Statistical analyses of MST in comparison to the PBS-treated control group were for: rD-mPGPtide, \(p=0.001\); Scr-mPGPtide, \(p \geq 0.99\); and anti-CD4 mAb, \(p \geq 0.001\). These data were pooled from two replicate experiments.

(100% survival; MST of >100 days). This result supported the hypothesis that rD-mPGPtide treatment could cause peripheral long-term allograft survival, contingent upon exposure of mature CD4\(^+\) T cells to the peptide at time of activation.

**Effect of rD-mPGPtide on the secondary alloresponse**

Elimination of CD4\(^+\) T cells by treatment with mAb in vivo has long been used as a means of immunosuppression in transplantation. However, some studies have indicated that certain anti-CD4 mAb treatments had no inhibitory effect on secondary T cell responses (18, 19). To address this important issue in relation to rD-mPGPtide efficacy, B6 mice were presensitized by bm12 allograft transplantation; all grafts were rejected within 16 days and the scar tissue allowed to heal. At 30 days postprimary challenge, the mice were divided into four treatment groups 3 h before secondary bm12 skin allograft challenge, and given either PBS (i.v.), rD-mPGPtide (0.5 mg; i.v.), anti-CD4 mAb (1:100 ascites dilution; i.p.) or Scr-mPGPtide (0.5 mg; i.v.). As shown in Figure 4, the rD-mPGPtide-treated mice had a significantly prolonged allograft MST of 19 days (\(p \leq 0.019\)) compared with the accelerated rejection in the PBS- or Scr-mPGPtide-treated mice (MST of 8 and 10 days, respectively). Notably, significant survival prolongation was observed in the mice that received the rD-mPGPtide treatment, whereas there was little effect with the anti-CD4 mAb-treatment (11 days; \(p \geq 0.8\)). Thus, in this case treatment with the synthetic CD4-CDR3 peptide analogue proved to be more effective than the anti-CD4 mAb treatment in inhibiting the host secondary CD4\(^+\) T cell alloresponse.

**Specificity of rD-mPGPtide effect in vivo**

An important aspect to be considered was whether the peptide treatment targeted bm12 alloantigen-specific T cells, or whether the allograft prolongation was mediated by general immunosuppression. Recipient B6 mice were divided into the following six treatment groups: 1) PBS (i.v.); 2) rD-mPGPtide (0.5 mg; i.v.); 3) anti-CD4 mAb (1:100 ascites dilution; i.p.); 4) anti-CD8 mAb (1:50 ascites dilution, i.p.); 5) rD-mPGPtide plus anti-CD8 mAb; and 6) anti-CD4 mAb plus anti-CD8 mAb. All mice were transplanted 3 h later with syngeneic and bm12 skin allografts. On day 3 (by which time any peptide would be expected to have been cleared from the system), all of the mice received a SJL (H2\(^s\)) third-party tail skin graft. The PBS-treated control group rejected their donor allografts with a MST of 17 days (bm12, Fig. 5A) and 12 days (SJL, Fig. 5B), respectively. In contrast, the rD-mPGPtide- and the anti-CD4 mAb-treated mice challenged with bm12 allografts on day 0 exhibited significant prolongation of graft survival with a MST of 37 (\(p \leq 0.001\)) and 36 (\(p \leq 0.001\)) days, respectively. However, SJL allografts (Fig. 5B) were readily rejected by rD-mPGPtide-treated mice (MST of 11 days), whereas their survival was prolonged by the anti-CD4 mAb treatment (MST of 23 days; \(p \leq 0.001\)). The observed rejection process of SJL allografts in the peptide-treated group was not merely due to residual activity of CD8\(^+\) T cells, since the peptide recipient group that had also received anti-CD8 mAb treatment yielded a similar MST of 13 days (Fig. 5B). Treatment with anti-CD8 mAb alone had no significant effect upon either bm12 (MST of 14 days, \(p \geq 0.58\)) or SJL (MST of 14 days, \(p \geq 0.99\)) allograft rejection. This data suggested that the prophylactic effect of the peptide was specifically directed toward the primary bm12 antigenic challenge and that the rejection processes of both bm12 and SJL allografts were mediated predominately by CD4\(^+\) T cells.

**Effect of rD-mPGPtide in allograft rejection across a MHC class I barrier**

To further analyze the functional specificity of rD-mPGPtide, the peptide was tested in the MHC class I-disparate B6 anti-bml transplantation model. B6 mice were treated with either PBS, rD-mPGPtide, anti-CD4 mAb, or anti-CD8 mAb (as described earlier) 3 h before transplantation with syngeneic and bml allografts. The PBS-treated group rejected the allografts with a MST of 9 days, whereas anti-CD8 mAb treatment significantly prolonged the graft MST to 34 days (\(p \leq 0.001\); Fig. 6). In contrast, both rD-mPGPtide and anti-CD4 mAb treatments only had marginal effects upon allograft survival (both with MST of 17 days; \(p \geq 0.3\) and \(p \geq 0.5\), respectively). This effect could be due to interference with
the enhancing activity of CD4+ T cells by blocking their stimulation through the indirect processing pathway (20). The combined results suggested that the rD-mPGPtide had no direct effect on CD8+ T cells in vivo.

Effect of rD-mPGPtide on alloreactive precursor frequency in draining LN

To further investigate the mechanism of rD-mPGPtide inhibition of host alloresponses in vivo, alloreactive precursor frequencies of CD4+ T cells in the draining LN of B6 mice were determined 8 days after treatment with either PBS, rD-mPGPtide, or anti-CD4 mAb and transplantation with bm12 allografts. CD4+ T cells were isolated from the draining LN of rD-mPGPtide-treated allografted mice (day 4) and restimulated with either syngeneic (B6), donor-allogeneic (bm12), or third-party allogeneic (SJL) irradiated (20 cGy) splenocytes in the presence or absence of IL-2. After 425 h of bulk MLC, ELISPOT analysis was performed to quantitate the number of cells capable of producing IL-2, IFN-γ, or IL-4. The frequencies shown in Table I were achieved in the presence of IL-2 in the bulk culture, and the levels for naive B6 mice and the PBS-treated control group were three- to fivefold higher than without IL-2 (data not shown). However, no significant difference was observed in the frequency levels of peptide-treated or anti-CD4 mAb-treated mice cultured with or without IL-2 (data not shown). The CD4+ T cells from
CD4 PEPTIDE EFFECT ON SKIN ALLOGRAFTS

Discussion

The effort to develop approaches to achieve long-term tolerance of allografts has been one of the central goals of transplantation immunology. Various immunosuppression protocols can successfully retard graft rejection processes, but at the same time they often result in generalized immunoincompetency. More selective and specific means to inhibit alloreactivity are needed, and toward this end, the recent development of the CD4-CDR3 peptide analogue has created intriguing possibilities (6–9). The current study demonstrates the efficacy of the rD-mPGPtide in a murine skin allograft model and supports the hypothesis that antidonor alloreactive CD4+ T cells are specifically targeted for deletion or induction of long-term nonresponsiveness.

The use of specific mAb directed toward either homing, co-stimulatory, cytokine receptor, or T cell-specific molecules is a widely considered approach to inhibit T cell-mediated graft rejection in various model systems (25, 26). However, several factors have limited the usefulness of these mAb approaches, particularly their inherent xenogeneic immunogenicity (27, 28), necessitating attempts to humanize the molecules (29, 30), and the fact that they cannot be administered by the oral route. In contrast, small m.w. cyclized peptides tend not to be immunogenic and, if not readily orally active, can often lend themselves to further peptidomimetic modifications that can make them available by this route of administration. In the case of anti-CD4 mAb, there are also concerns about pan-CD4 suppression (31, 32) and the fact that several laboratories have reported the ineffectiveness of anti-CD4 mAb treatments upon memory T cell responses (18, 19, 33). The memory effector population has even been shown to have enhanced responsiveness following anti-CD4 mAb treatment. This potential effect could certainly have important implications in cases of chronic graft rejection. In this regard, it is interesting to note that in the current study, the CD4-CDR3 peptide also exhibited some inhibitory activity in a secondary allograft rechallenge situation. This

Table I. Frequency of cytokine-producing cells after peptide treatment in vivo

<table>
<thead>
<tr>
<th>In Vivo Treatment (day 0)</th>
<th>Stimulating Cells in MLR</th>
<th>IL-2-Producing Cells Reciprocal Frequency (× 10^3)</th>
<th>IFN-γ-Producing Cells Reciprocal Frequency (× 10^3)</th>
<th>IL-4-Producing Cells Reciprocal Frequency (× 10^3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>Syngeneic</td>
<td>21.4</td>
<td>200</td>
<td>75</td>
</tr>
<tr>
<td>None</td>
<td>bm12</td>
<td>0.64</td>
<td>4.8</td>
<td>6.6</td>
</tr>
<tr>
<td>None</td>
<td>SJL</td>
<td>1.3</td>
<td>6.2</td>
<td>3.1</td>
</tr>
<tr>
<td>bm12 skin + PBS (i.v.)</td>
<td>Syngeneic</td>
<td>16.6</td>
<td>200</td>
<td>28</td>
</tr>
<tr>
<td>bm12 skin + PBS (i.v.)</td>
<td>bm12</td>
<td>0.62</td>
<td>10.5</td>
<td>2.6</td>
</tr>
<tr>
<td>bm12 skin + PBS (i.v.)</td>
<td>SJL</td>
<td>1.3</td>
<td>6.2</td>
<td>3.2</td>
</tr>
<tr>
<td>bm12 skin + rD-mPGPtide (i.v.)</td>
<td>Syngeneic</td>
<td>9.4</td>
<td>200</td>
<td>100</td>
</tr>
<tr>
<td>bm12 skin + rD-mPGPtide (i.v.)</td>
<td>bm12</td>
<td>12.5b</td>
<td>150b</td>
<td>69b</td>
</tr>
<tr>
<td>bm12 skin + rD-mPGPtide (i.v.)</td>
<td>SJL</td>
<td>1.1</td>
<td>8.6</td>
<td>2.7</td>
</tr>
</tbody>
</table>

* B6 CD4+ T cells were isolated on day 4 from the draining LN of naive or bm12-allografted recipient mice treated with either saline solution or rD-mPGPtide as described in Materials and Methods. Bulk MLR cultures were prepared and incubated for 24 h in the presence of IL-2 (50 U/ml), and ELISPOT analysis for cytokine production was performed.

b Indicates a significant decrease in frequency determined by Students t test; p = 0.001 using bm12 stimulators in the rD-mPGPtide-treated group. There were no significant differences between the PBS-treated allograft recipients and the naive B6 mice.
efficacy was also evident in an autoimmune model, in which rD-mPGptide blocked memory responses to Ags responsible for induction of EAE (7).

A variety of other therapeutic approaches have tried to inhibit T cell activation by interfering with the formation of the TCR-Ag-MHC complex, including mAb to either TCR (34) or CD3 (35) components, and blockade of MHC class II or depletion of APCs (36, 37). Other approaches target adhesion and lymphocyte homing molecules (38) or costimulatory molecules mediating secondary signals (39). Blockade of CD28 with CTLA4-Ig has been successful in preventing rejection of cardiac allografts (40) and xenogeneic islet grafts (41) in mice. In addition, nondepleting mAb to LFA-1, ICAM-1, and VLA-4 can inhibit graft rejection and may induce transplantation tolerance (42, 43). However, these mAb methods also share some of the same concerns with anti-CD4 mAb treatment, including general immunosuppressive effects and the inherent immunogenicity of the mAb.

Although the anti-CD4 mAb treatment effectively prolonged skin graft survival in the primary challenges in naive (Figs. 1 and 2) and ATX (Fig. 3) mice, it clearly also inhibited responses against third-party alloantigens (Figs. 5 and 6), as would be expected with a pan-deletional effect. In contrast, the CD4-CDR3 peptide specifically inhibited the host alloreactivity to a transplant given 3 h later, but did not impair responses to another allograft 3 days later. The findings based on rejection times, overall alloreactive precursor frequency, and the frequency of cytokine-producing cells in response to donor or third-party alloantigens all support the specificity of the peptide effect. Similar findings of Ag specificity were obtained in use of the CD4-CDR3 peptide in the EAE SJL model (7). It has also been demonstrated here that although the half-life retention of the peptide in serum of mice is approximately 25 min, host alloreactivity was significantly inhibited for up to 6 h after peptide administration, but there was no effect on allograft rejection if the peptide was given 24 h before transplantation (Fig. 2). An equivalent short window of peptide effectiveness was found in earlier studies with keyhole limpet hemocyanin primary responses in vivo (7). Thus, it would appear that the peptide can inhibit CD4+ T cell responses initiated in its presence. The advantage of this approach is that the peptide can be effective in any MHC class II-restricted Ag-specific situation, whether it be alloreactive or autoimmune. In addition, the short-term range of activity allows for manipulation of responses without establishing a general state of immunosuppression. Obviously, caution must be taken to avoid the co-inhibition of antipathogenic responses that might be underway at the time of peptide administration, but this concern would apply to any immunosuppressive approach. The CD4-CDR3 peptide also offers another advantage over several other therapeutic approaches in that it does not seem to be immunogenic and has failed to generate Ab responses upon repeated injections into mice, as analyzed by ELISA (our unpublished observations).

It is not yet clear how the CD4-CDR3 peptide inhibits T cell activation and/or expansion on a molecular level. Earlier in vitro studies demonstrated that the inhibitory activity of similar CD4-CDR3 peptide analogues could be localized to effects on the Th cell itself (44). It is currently hypothesized that these analogues act by uncoupling a CD4-CD4 homodimer or oligomer that is critical to the generation of an appropriate immune response (45). Based on recent investigations, the CD3 region of CD4-D1 is suspected of playing a critical role in CD4 homodimerization (46). Dimerization/oligomerization of CD4 may be required for stable interaction with the nonpolymorphic sites in the α2 and β2 domains of MHC class II molecules presenting Ag to the TCR of the CD4+ T cell (47–49). This stable interaction then allows for CD4-mediated signal transduction via the noncovalently associated protein tyrosine kinase p56lck (50, 51). Disruption of dimerization/oligomerization by the peptide would thus interfere with the proper signal cascade of the T cell, initiated upon TCR engagement by MHC-presented Ag. The consequence of this disruption would hypothetically lead either to programmed cell death (52) and deletion of the alloreactive cell population, anergy of the Ag-specific T cells (53, 54), possibly a switch in cytokine production (55, 56), or merely a lack of any response.

It has always been difficult to discern the mechanism of alloreactive tolerance and lack of expansion at the population level between clonal deletion and some form of anergic state. Clues to the mechanism of action of the peptide could be derived from the analysis of the precursor frequency (Fig. 7), as well as the cytokines produced at the single cell level (Table I). Limiting dilution analysis performed with LN CD4+ T cells from peptide-treated allograft recipients exhibited an almost 450-fold reduction (~8.6 × 10−6) in alloreactive precursor frequency compared with the PBS-treated recipients, which could be due to either clonal deletion or anergy. Since the precursor frequency of bml2-specific naive B6 LN T cells (~2.4 × 10−4, data not shown) is considerably higher than that seen from the peptide-treated mice, the data would suggest that the mechanism of action involves more than a merely temporary lack of responsiveness in the presence of peptide. The ELISPOT single cell cytokine analysis indicated that peptide-treated allograft recipients by day 4 already had a significant decrease in the absolute number of alloreactive cells producing either IL-2, IFN-γ, or IL-4. However, normal levels of cytokine-producing cells were detected when the mice were restimulated with the third-party SJL alloantigens. Previously, it has been shown that IL-2 is capable, under certain conditions, to reactivate anergized cells and induce the transition from G0 to G1 in the cell cycle (22). Therefore, the inability of IL-2 to reverse the lack of alloreactivity of CD4+ T cells from peptide-treated mice would favor a clonal deletion mechanism. However, since not all forms of anergy are reversible with IL-2 (57), further investigation of this issue will be undertaken with cells from TCRαβ transgenic mice to clearly follow the fate of Ag-specific cells after peptide exposure.

In regard to the possibility of a potential cytokine switch, polarization of CD4+ T cell responses from Th1- to Th2-type cytokine production has altered the pathology in experimental graft-vs-host disease (58, 59), EAE (60), and diabetes (61). Nevertheless, it is apparent from the current results that the peptide-treated group exhibited a significant down-regulation of both Th1- and Th2-related cytokines, suggesting that cytokine shifts are not involved in the establishment of peptide-mediated allograft tolerance. On the other hand, it is interesting that the potent effects of combined CTLA4-Ig and CD40 ligand mAb treatment, which results in uniform heart allograft tolerance, are also accompanied by a silencing of both the Th1 and Th2 pathways in the early posttransplant period (62). The CD4-CDR3 peptide may be operating through a similar, as yet unidentified, mechanism of tolerance induction.

The ability of the CD4-CDR3 peptide to affect secondary CD4+ T cell responses (Fig. 4) is of high interest because of its implication upon the application of the approach to both accelerated and chronic graft rejection situations. Several important issues need to be further investigated to understand how the peptide might affect memory T cells. It is not clear whether memory T cells that become selected for higher affinity TCR-Ag interactions will still require CD4 coreceptor activity. However, even in this situation it is still possible that the peptide might induce or allow a negative or inappropriate signal through the CD4 molecule that could effect
activation. Future experiments with memory T cell populations from TCRβ transgenic mice will also help address some of these questions.

It is clear from the result of the B6-ATX anti-bm12 study that a single treatment with the CD4-CDR33 peptide could inhibit peripheral T cell alloreactivity and induce long-term survival of the skin allograft (Fig. 3). This experiment strongly suggests that in normal recipients, de novo-derived T cells in the absence of peptide can still be stimulated by donor-derived APC, presumably dendritic cells that would find their way into the draining LN. It may therefore be possible to induce long-term graft survival in normal mice by administering peptide intermittently (e.g., every 5 days) over a period of perhaps 30 days, until the donor APC have significantly decreased in number. Studies to address the induction of long-term graft survival are in progress.

In summary, the CD4-CDR33 peptide appears to be an effective prophylactic agent for inhibition of skin allograft rejection across a MHC class II barrier. Of most importance, the peptide treatment was specific for the host alloreactive CD4+ MHC class II barrier. Of most importance, the peptide treatment decreased in number. Studies to address the induction of long-term period of perhaps 30 days, until the donor APC have significantly decreased in number. Studies to address the induction of long-term graft survival are in progress.

References

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