

## **Prevention of Insulin-dependent Diabetes Mellitus in Nonobese Diabetic Mice by Immunogenic but Not by Tolerated Peptides**

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### **Summary**

In the nonobese diabetic (NOD) mouse, susceptibility to insulin-dependent diabetes mellitus is in part controlled by a single expressed class II major histocompatibility complex (MHC) molecule, I-A<sup>g7</sup>. This molecule probably exerts its control through the presentation of a self-peptide, derived from an unknown  $\beta$  cell antigen, leading to T cell activation and eventual islet destruction. In this paper, synthetic peptides have been used to compete for binding to the I-A<sup>g7</sup> molecule in an attempt to suppress the autoimmune response. The administration of an I-A<sup>g7</sup>-binding immunogenic peptide,  $\lambda$  repressor (cI) 12-26, in a water and oil emulsion (incomplete Freund's adjuvant) can prevent the transfer of IDDM into irradiated recipients by spleen cells from diabetic donors. Nonbinding, nonimmunogenic peptides have no effect in this situation. However, the immune response to the "blocking" peptide in these experiments was a complicating factor in interpreting the results. To establish that the effect was at the level of competition for MHC binding, two additional approaches were tried. First, tolerance was induced to the immunogenic peptide, cI 12-26, before using it to "block" disease. Tolerance abolished the effect on diabetes transfer. Second, an effort was made to identify peptides that were nonimmunogenic but that bound to I-A<sup>g7</sup>. Such a peptide, mouse prostatic secretory glycoprotein precursor 63-76, had no effect on the incidence of transferred disease. We conclude that the "blocking" effects seen in initial experiments in the NOD mouse were not caused by blockade of MHC presentation, but by other unknown effects related to the immunogenicity of the "blocking" peptide.

Class II MHC molecules bind peptide fragments of antigens for presentation to helper T cells. Peptides compete for binding to the class II molecule, and competition can be used to inhibit T cell activation in vivo (1). The competing or "blocking" peptide approach has been tested in several autoimmune disease models, including experimental autoimmune encephalomyelitis (2, 3), adjuvant arthritis (4), myosin-induced acute myocarditis (5), and graft versus host disease (6).

The nonobese diabetic (NOD) mouse develops a spontaneous autoimmune pancreatic insulinitis and has been used as a model for human insulin-dependent diabetes mellitus. In the NOD mouse, the islet cell antigen responsible for initiating the autoimmune response is unknown, although several candidates have been identified (7, 8). The I-A<sup>g7</sup> molecule has a critical role in NOD mouse diabetes, probably in the presentation of an autoantigenic peptide.

In this paper, synthetic peptides were used to compete for binding to the I-A<sup>g7</sup> molecule. Blocking peptide therapy was attempted in a transfer system, since diabetes develops rapidly in a high percentage of mice in this model (9). Similar work has recently been reported, but in spontaneous disease

in the NOD mouse (10). The mechanism by which peptide "blocking" occurs in vivo is still not clear.

### **Materials and Methods**

**Mice.** NOD/Mcd (McDevitt colony) mice and control strains were bred within the Stanford Department of Laboratory Animal Medicine facility under barrier conditions. The NOD/Mcd mice were originally derived from Dr. E. Leiter's colony (The Jackson Laboratory, Bar Harbor, ME). The incidence of spontaneous diabetes in the NOD/Mcd colony is ~70% in females and ~15% in males. Control strains used for immunization were B10.A(4R) (A<sup>d</sup>E<sup>b</sup>), BALB/c (A<sup>d</sup>E<sup>d</sup>), DBA/2J (A<sup>d</sup>E<sup>d</sup>), SJL/J (A<sup>s</sup>), PL/J (A<sup>u</sup>E<sup>u</sup>), B10.GD (A<sup>d</sup>E<sup>b</sup>).

**Peptides.** Peptides were synthesized by 9-fluorenylmethoxycarbonyl chemistry using a peptide synthesizer (model 431A; Applied Biosystems, Inc., Foster City, CA), and they were analyzed by reverse-phase HPLC, amino acid composition, and mass spectrometry. Peptides used in blocking experiments were HPLC purified.

**T Cell Proliferation Assays.** NOD mice and the appropriate control strain were immunized in the tail base or footpad with antigen emulsified in CFA. The antigen was dissolved in PBS at a concentration of 2 mg/ml and mixed 1:1 with CFA. Mice were

**Table 1.** Antigen Tested for T Cell Proliferation in the NOD Mouse

Antigen	Residues	Sequence	MHC restriction	Immune response	Binding to I-A <sup>g7</sup>
<b>OVA</b>					
Whole OVA				+	
OVA	323-339	ISQAVHAAHAEINEAGR	A <sup>d</sup>	+	+
<b>EMb</b>					
Whole EMb				+	
EMb	102-118	KYLEFISDAIHVLHSK	A <sup>d</sup>	-	-
	131-153	MTKALELFRNDIAAKYKELGFQG	A <sup>d</sup>	+	+
<b>Rat myelin basic protein (rMBP)</b>					
Whole rMBP				+	
rMBP	Ac1-11	AcASQKRPSQRHG	A <sup>u</sup>	-	
	35-47	TGILDSIGRFFSG	E <sup>u</sup>	-	
	89-101	VHFFKNI VTPRTP	A <sup>s</sup>	-	
<b>HEL</b>					
Whole HEL				+	
HEL	13-35	KRHGLDNYRGYSLGNWVCAAKFE	A <sup>d</sup>	+	+
	46-61	NTDGSTDYGI LQINSR	A <sup>k</sup>	-	-
	74-96	NLCNI PCSALLSSDI TASVNCAK	E <sup>k</sup> , A <sup>b</sup>	-	
<b>cI</b>					
Whole cI	1-102			+	
cI	12-26	LEDARRLKAIEYKKK	E <sup>d, k</sup>	+	+
	73-88	VEEFSPSIAREIYEMY	H-2 <sup>f, b</sup>	-	-
<b>Influenza hemagglutinin HA/PR8 (Flu HA)</b>					
Flu HA	111-120	FERFEIFPKE	A <sup>d</sup>	-	
	130-142	HNTNGVTAACSHE	A <sup>d</sup>	-	
<b>Staphylococcal nuclease (SNase)</b>					
SNase	61-79	FTKKMVENAKKIEVEFDKG	A <sup>d</sup>	+	+
<b>Tuberculosis 65-kD heat shock protein (TB)</b>					
TB	112-126	YEKIGAEVKEVAKK	H-2 <sup>b, d, k</sup>	-	
<b>Bovine cytochrome C (BCC)</b>					
BCC	13-25	KCAQCHTVEKGGK	A <sup>d</sup>	-	
<b>Reiterative peptides</b>					
ROIV		AHAHAHAHAHAHAHA	A <sup>d</sup>	-	
RLI		ARRLKARRLKARRLK	E <sup>d</sup>	-	
<b>Murine peptides</b>					
α-1 antitrypsin	148-163	LSQAVHKAVLTIDETG		+	+
Laminin b1 chain precursor	1594-1612	MVKEALEEAQVAAEKA		+	+
PSG-1	63-76	FENRKRIEPVLIRK		-	+
Mouse lysozyme	13-39	KRNGMAGYYGVSLADWV		-	-
Murine myoglobin	131-153	MSKALELFRNDIAAKYKELGFQG		+	+

Positive lymph node T cell proliferative responses are indicated. When tested, binding of peptides to I-A<sup>g7</sup> in hybridoma competition assays is shown. The sequence data for PSG, antitrypsin, and mouse lysozyme C are available under the SwissProt data base under accession numbers P09036, P07758, and P08905, respectively.

immunized with 0.1 ml of the mixture, equivalent to 100  $\mu\text{g}$  of antigen. Draining lymph nodes were removed 10–14 d later, and  $5 \times 10^5$  cells were cultured for 3 d in the presence of antigen in a total volume of 200  $\mu\text{l}$  RPMI 1640 supplemented with 1% mouse serum, 2 mM glutamine, 10 mM HEPES, 50  $\mu\text{M}$  2-ME, 100 U/ml penicillin, and 100  $\mu\text{g}/\text{ml}$  streptomycin. The cells were then pulsed with 0.5  $\mu\text{Ci}$  [ $^3\text{H}$ ]thymidine per well before being harvested for liquid scintillation counting  $\sim 18$  h later.

**Adoptive Transfer.** Spleens from diabetic females were disrupted in PBS, and red cells were lysed with Tris-buffered ammonium chloride. The cells were then washed and taken up in RPMI 1640. 8–12-wk-old male NOD mice were irradiated with 650 rads from a 250-kV x-ray source and were then injected via the tail vein with  $10^7$  spleen cells in a 0.2-ml vol. Mice were monitored from day 14 until day 28 with urinary glucose testing (Chemstrip uG; Boehringer Mannheim Biochemicals, Indianapolis, IN). Blood glucose was measured on day 28 (Accu-Chek IIm; Boehringer Mannheim Biochemicals, Indianapolis, IN).

**Peptide Treatment.** In the adoptive transfer experiments, peptides were dissolved in PBS and emulsified in IFA. IFA was used to provide a slow release of peptide during  $\sim 6$ –7 d. Peptides were administered intraperitoneally in a 0.2-ml vol.

**Peptide Tolerization.** Peptide was dissolved in PBS, sterile filtered, and spun twice at 13,000 rpm for 5 min to remove possible aggregates. Diabetic female donor mice were given two doses of either 600  $\mu\text{g}$  or 1.2 mg of cI 12-26 peptide intravenously in a 0.2-ml vol 10 and 5 d before the experiment. Diabetic female mice in this experiment were maintained on 5 U lente insulin (Lente Iletin 100 suspension; Lily) subcutaneously every 2 d. To prove that tolerization was effective, test mice were tolerized and then immunized with  $\lambda$  repressor (cI) 12-26 in CFA, and lymph node assays were set up as described. 50  $\mu\text{l}$  of supernatant was removed from each well after 24 h for IL-2 assay as described below, while the remaining culture was pulsed with [ $^3\text{H}$ ]thymidine after 48 h to measure proliferation.

**Profile Searching.** The programs PileUp and ProfileMake from the Sequence Analysis Software Package (Genetics Computer Group, Madison, WI) (11) were used to identify nonimmunogenic peptides. Profiles were made from OVA 323-339, cI 12-26, Staphylococcal nuclease 61-79, and hen egg lysozyme (HEL) 13-35. The profiles were run against the entire mouse protein database using ProfileSearch. The three best matches were synthesized and tested: prostatic secretory glycoprotein precursor (PSG)-1 63-76 (FEN-RKRIEPLIRK), mouse  $\alpha$ -1 antitrypsin (AT) 148-163 (LSQAVH-KAVLTIDETG), and mouse lysozyme C (ML) 13-29 (KRNGMAGYYGVSADWV). The peptides were tested for immunogenicity in lymph node proliferation assays and for binding to I-A $^b$ 7 by competition for presentation to a cI 12-26-specific T cell hybridoma.

**Hybridoma Competition Assays.** A cI 12-26-specific I-A $^b$ 7-restricted T cell hybridoma, 2.2E9, was produced by fusion of NOD lymph node cells with the BW5147 cell line. For competition assays, cI 12-26 was plated over a 0.625–5  $\mu\text{M}$  range in triplicate, and competitor peptide was then added at a constant concentration of 0.5 mg/ml ( $\sim 250$   $\mu\text{M}$ ). Next,  $10^5$  cells of an I-A $^b$ 7-expressing transfectant, M12.NOD (made by Dr. Steven Singer, Harvard School of Medicine, Boston, MA), and finally  $10^5$  2.2E9 cells were added to a total volume of 200  $\mu\text{l}$ . After 24 h, 50  $\mu\text{l}$  of supernatant was removed, frozen, and later added to  $10^4$  HT-2 cells. HT-2 cells were cultured for 24 h, with the addition of 1  $\mu\text{Ci}$  [ $^3\text{H}$ ]thymidine per well for the last 6 h of culture, before harvesting and counting.

## Results

**NOD Mouse T Cell Responses.** Lymph node T cell proliferative responses were used as an initial screen for peptides that bound to I-A $^b$ 7. Several whole antigens, and a panel of known T cell epitopes were tested (Table 1). Five T cell epitopes that are highly immunogenic in the NOD mouse (Table 2) have been identified. One of these peptides, cI 12-26, was then tested for its ability to block adoptively transferred diabetes in the NOD mouse. Whole OVA and two nonimmunogenic peptides were also tested initially.

**Prevention of Adoptive Transfer by Immunogenic Peptides.** Peptide blocking was tested in an adoptive transfer system, since diabetes develops in 80–100% of mice within 2–4 wk of transfer, compared with 3–6 mo for spontaneous disease. Peptides were administered intraperitoneally at a dose of 2 mg in IFA per mouse given at the time of transfer. Mice were monitored starting on day 14 until day 28 with urinary glucose testing and blood glucose measurement at the end of the experiment. Diabetes was defined as a blood glucose of  $\geq 200$  mg/dl.

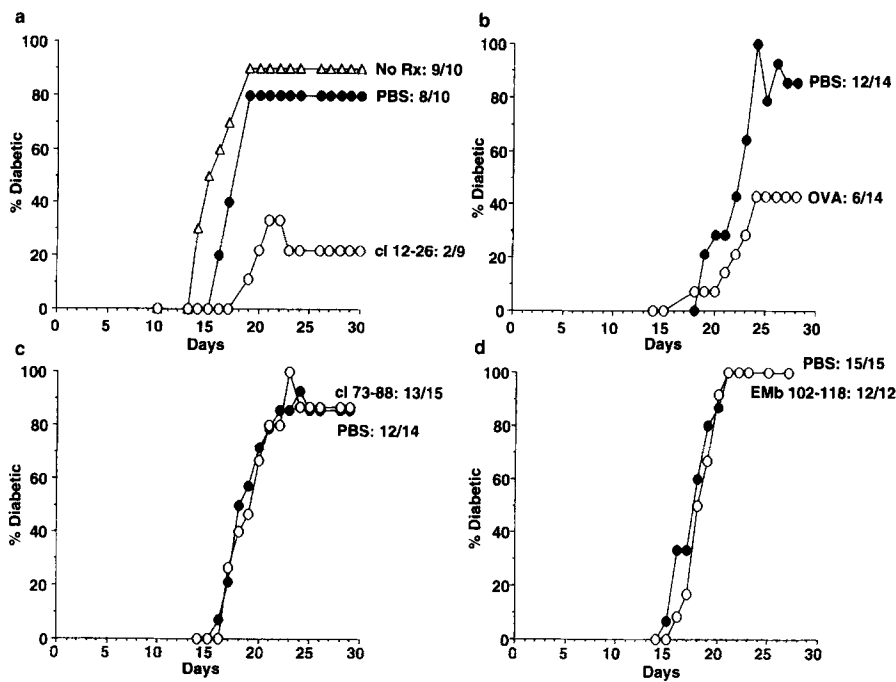
In initial experiments, a reduction in the incidence of adoptively transferred diabetes in the NOD mouse was seen after treatment with cI 12-26 (Fig. 1 *a*). Similar results were seen in a repeat experiment. This reduction in disease was also observed with whole OVA, another immunogen in the NOD mouse (Fig. 1 *b*). Treatment with the nonbinding peptides cI 73-88 or equine myoglobin (EMb) 102-118 had no effect on disease incidence (Fig. 1, *c* and *d*).

The immune response to the “blocking” peptide in these experiments, however, was a complicating factor in interpreting the results. To establish that the effect was at the level of competition for MHC binding, two further approaches were tried.

**Table 2.** Peptides Eliciting a Positive T Cell Response in the NOD Mouse

Peptide	Sequence	Known MHC restriction
OVA 323-339	I SQAVHAAHAE I NEAGR	A $^d$
cI 12-26	LEDARRLKA I YEKKK	A $^d$ , E $^d$ , k
Staphylococcal nuclease 61-79	FTKKMVENAKK I EVEFDK	A $^d$
EMb 131-153	MTKALELFRND I AAKYKELGFQG	E $^d$ , k
HEL 13-35	KRHGLDNYRGYSLGNWVCAAKFE	A $^d$

The peptides listed produced strong proliferative responses in lymph node T cell assays; murine peptides tested are not shown.



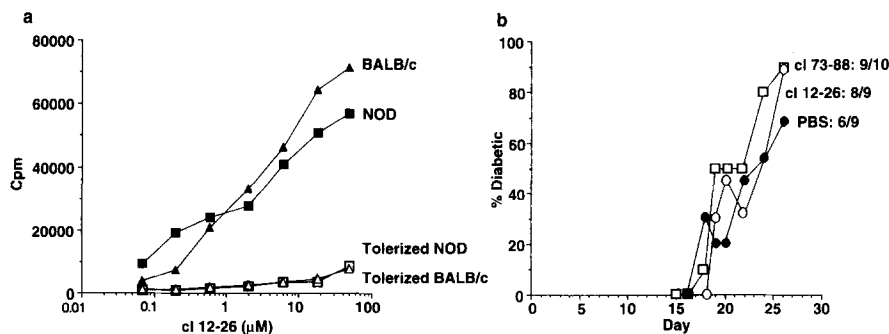
**Figure 1.** Adoptive transfer experiments with immunogenic and nonimmunogenic, nonbinding peptides in the NOD mouse. The number of diabetic mice over the total number of mice is shown to the right of each treatment. Disease inhibition (a) with cl 12-26 and (b) with whole OVA. Lack of disease inhibition with the nonimmunogenic, nonbinding peptides (c) cl 73-88 and (d) Emb 102-118.

**Peptide Treatment after Tolerization.** Tolerance to a peptide can be induced by giving it in soluble form intravenously in PBS before immunization. Two doses of at least 300  $\mu\text{g}$  given 10 and 5 d before immunization induce tolerance lasting up to 120 d (11a). cl 12-26 is strongly immunogenic in NOD and BALB/c mice. To prove that this tolerizing regimen was effective, NOD and BALB/c control mice received two doses of 600  $\mu\text{g}$  cl 12-26 intravenously in PBS before being immunized with 100  $\mu\text{g}$  of the same peptide in CFA. The proliferative response after tolerization was reduced by  $\sim 50\%$  (data not shown), and the IL-2 response was reduced by 80–90% (Fig. 2 a).

A transfer experiment was then done after tolerization to the blocking peptide cl 12-26. In this experiment, diabetic female donor mice were tolerized with 1.2 mg of peptide at days  $-10$  and  $-5$ . Recipient mice received two 3-mg doses

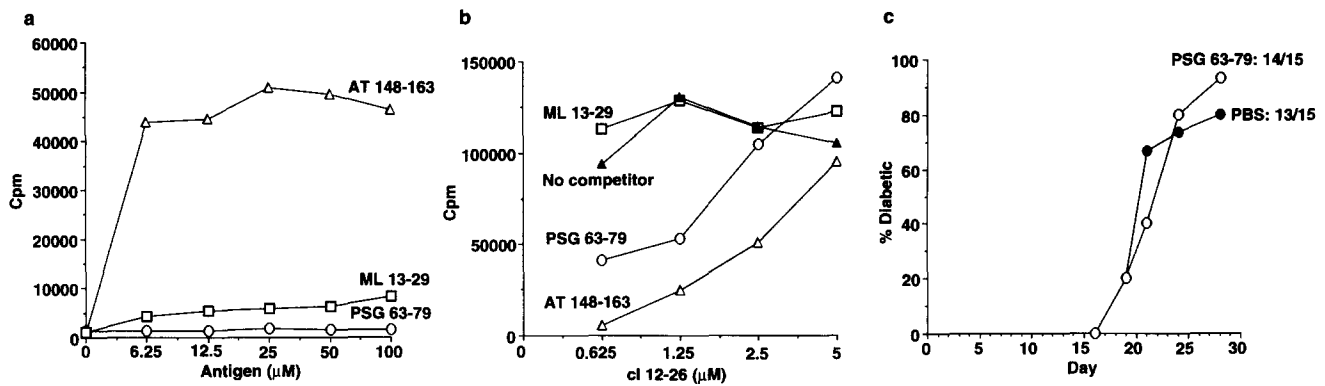
of cl 12-26 in IFA on the day of transfer and 7 d after transfer. The reduction in disease seen in the previous nontolerized experiments was abolished (Fig. 2 b). Similar results were found on repeating this experiment once.

**Peptide Treatment with a Nonimmunogenic Peptide.** Several peptides derived from murine protein sequences were also tested as potential blockers. These sequences were homologous to previously identified epitopes and were identified using ProfileMake and ProfileSearch from the GCG software package. Of the peptides tested, only PSG-1 63-79 was both nonimmunogenic when NOD mice were immunized with 100  $\mu\text{g}$  in CFA in footpads (Fig. 3 a) and bound to I-A<sup>s7</sup> (Fig. 3 b). PSG-1 63-79 was then used in a blocking experiment, with 2 mg being given in IFA at the time of transfer. This treatment had no effect on the incidence of transferred diabetes (Fig. 3 c).



**Figure 2.** (a) Peptide tolerization. Peptide was dissolved in PBS, sterile filtered, and spun twice at 13,000 rpm for 5 min to remove possible aggregates. In the experiment shown, mice were tolerized with two doses of 300  $\mu\text{g}$  cl 12-26 intravenously in PBS and then immunized with 100  $\mu\text{g}$  cl 12-26 in CFA in the footpads. Lymph node assays were set up as described. 50  $\mu\text{l}$  of supernatant was removed from each well after 24 h for IL-2 assay with HT-2 cells, while the remaining culture was pulsed with [<sup>3</sup>H]thymidine after 48 h to mea-

sure proliferation. IL-2 production is significantly reduced in T cell supernatants from NOD and BALB/c mice tolerized by the protocol described. (b) Adoptive transfer and treatment with cl 12-26 after previous tolerization to the peptide. In this experiment, diabetic female donor mice were given two 1.2-mg doses of cl 12-26 peptide intravenously in a 0.2-ml vol 10 and 5 d before the experiment. Spleen cells from these mice were then transferred into irradiated male recipients. The transferred mice were tested for glycosuria as described. No reduction in diabetes incidence was seen after previous tolerization with the blocking peptide.



**Figure 3.** (a) NOD mouse immunizations with murine self-peptides in CFA. (b) Binding of murine peptides to I-A<sup>g7</sup>, as measured by competition for presentation to the cl 12-26-specific hybridoma 2.2E9. For competition assays, cl 12-26 peptide was added first to a 96-well plate in triplicate over a 0.625–5  $\mu\text{M}$  concentration range. Next, competitor peptide was added at a constant concentration of 0.5 mg/ml ( $\sim 250 \mu\text{M}$ ) to all the wells.  $10^5$  cells of an I-A<sup>g7</sup>-expressing transfectant (M12.NOD) were then added, and finally  $10^5$  2.2E9 cells were added to a total volume of 200  $\mu\text{l}$ . After 24 h, 50  $\mu\text{l}$  of supernatant was removed, frozen, and later added to  $10^4$  HT-2 cells. HT-2 cells were cultured for 24 h with the addition of 1  $\mu\text{Ci}$  [<sup>3</sup>H]thymidine per well for the last 6 h of culture, before harvesting and counting. (c) Adoptive transfer and treatment with the nonimmunogenic but I-A<sup>g7</sup>-binding peptide PSG-1 63-76. In this experiment, irradiated, spleen cell recipient male NOD mice were each given 2 mg of PSG-1 63-76 dissolved in PBS and emulsified in IFA. The peptide was administered intraperitoneally in a 0.2-ml vol. Mice were tested for glycosuria as described.

## Discussion

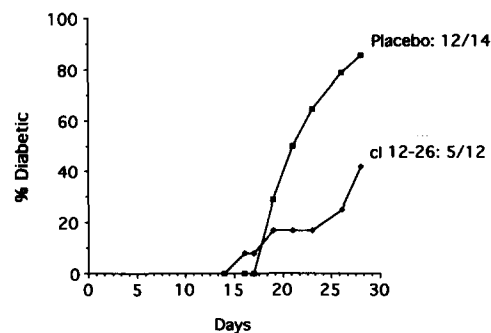
In summary, from these experiments, it now appears that “blocking” of diabetes transfer observed with cl 12-26 (or with whole OVA) was the result of an immune response directed against the peptide. A spleen cell proliferative response could be detected when peptide was given in this form, i.e., intraperitoneally in IFA or in slow-release pellet form (Innovative Research of America, Toledo, OH), where a blocking effect was also seen (Fig. 4).

When an immunogenic peptide (cl 12-26) was given after tolerization or when a nonimmunogenic non-binding peptide (cl 73-88, or Emb 102-118) was used, there was no effect on diabetes incidence. A nonimmunogenic but I-A<sup>g7</sup>-binding peptide (PSG-1 63-79), also had no effect on disease. The “blocking” effect on disease with cl 12-26 in the naive animal was therefore not caused by competitive binding to class II MHC, but was related to the immunogenicity of the blocking peptide. In an additional experiment, spleen cells were pulsed with 100  $\mu\text{M}$   $\lambda$  cl 12-26 for 6 h in culture before transfer into irradiated recipient mice. There was no effect on the incidence of diabetes between the group transferred with cells treated in this way and a control group, again arguing against a direct blocking effect (data not shown).

In the NOD mouse, any stimulation of the immune system appears to lower the incidence of disease. For example, the incidence of diabetes is higher in a “clean” mouse colony than in a “dirty” colony, where infections presumably stimulate the immune system. Immunization with CFA alone will prevent disease in the NOD mouse (12). The incidence of NOD mouse diabetes is also sensitive to a number of other factors, for example diet and local colony conditions (13). The generation of an additional alternative immune response may divert cells away from the pancreas, perhaps alter the balance between Th1 and Th2 cells and alter cytokine levels (14), or shift the balance between diabetogenic and suppressor T cells (15).

It has been reported that a peptide can block the spontaneous development of diabetes in the NOD mouse by interfering with antigen presentation (10). For the reasons discussed, caution is needed in accepting these results. It is clear that the “blocking” peptide was provoking a significant immune response in these experiments. In fact, the mice required coadministration of an antihistamine to prevent an anaphylactic reaction. The only peptide control provided was a group treated with a nonimmunogenic, nonbinding peptide. The experiments should perhaps also have included an MHC-binding, but nonimmunogenic, peptide.

Blocking peptides fall into two main categories, those that are related to the disease-initiating peptide and those that are unrelated. Related peptide analogues may be able to engage the TCR and cause differential signaling through it (16). The net result may depend on the affinity of this interaction with the TCR. Some analogues such as myelin basic protein



**Figure 4.** Transfer of insulin-dependent diabetes mellitus and treatment with cl 12-26 in slow-release pellet form. Irradiation and transfer was done as described in Materials and Methods. Transferred mice were treated with two pellets placed subcutaneously containing a total of 2 mg cl 12-26 peptide. Mice were tested for glycosuria as described.

1-11[4A] may be "partial agonists" (17) and perhaps induce anergy or cause T cell deletion. This type of peptide may be able to treat disease, as well as to prevent it (18).

The use of related peptides is clearly only possible in a disease model where the autoantigen is known. Experimental autoimmune encephalomyelitis has been prevented with unrelated peptides, but here, the competing peptide has been given at the same time as the disease-inducing peptide, thus preventing induction of disease. In the NOD mouse, T cells

were already primed against the autoantigen at the time of transfer and treatment. In NOD mouse diabetes, the affinity of the autoantigenic peptide(s) may be very high, making competition difficult. In a spontaneous disease model such as the NOD mouse, there may be several epitopes from more than one autoantigen being presented. Specific peptide immunotherapy in this model may be feasible once these autoantigenic peptides have been identified.

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