2003

Regulation of type 1 diabetes by a self-MHC class II peptide: role of transforming growth factor beta (TGF-beta).

Bhagirath Singh

R Mukherjee

P Chaturvedi
University of Western Ontario

Babita Agrawal
University of Alberta

Cellular and Molecular Biology™ **49** (2), 159-169
Printed in France

ISSN 0145-5680/03
2003 Cell. Mol. Biol.™

REGULATION OF TYPE 1 DIABETES BY A SELF-MHC CLASS II PEPTIDE: ROLE OF TRANSFORMING GROWTH FACTOR BETA (TGF-β)

R. MUKHERJEE*[1], P. CHATURVEDI*[1], B. AGRAWAL[3] and B. SINGH[1,2,4✉]

[1] Department of Microbiology and Immunology, University of Western Ontario, John P. Robarts Research Institute, London, Ontario, Canada
[2] Institute of Infection and Immunity, Canadian Institutes of Health Research, London, Ontario, Canada
[3] Department of Surgery, University of Alberta, Edmonton, Alberta, Canada
[4✉] Department of Microbiology and Immunology, University of Western Ontario, London, Ontario N6A 5C1 Canada
Fax: +1 519 661 4226; E-mail: bsingh@uwo.ca
* Both authors contributed equally to this work.

Received March 18, 2003; Accepted April 25, 2003

Abstract - The present study was undertaken to analyze the regulatory T cells generated in response to MHC class II derived self-I-Aβ^{g7} (54–76) peptide. It was observed that T cells from young unprimed type 1 diabetes (T1D) prone NOD mice did not respond to self-I-Aβ^{g7} (54–76) peptide although T cells from primed young NOD mice showed a strong response. T cells from young unprimed BALB/c mice responded to self-I-Aβ^{d} (62–78) peptide. However, a breakdown of tolerance to these peptides was observed with age in both the strains. Culture supernatant from I-Aβ^{g7} (54–76) peptide-primed cells secreted large amounts of TGF-β and inhibited T cell responses in allogeneic-MLR. Further, I-Aβ^{g7} (54–76) peptide specific T cell lines from young (I-A.Y) and diabetic (I-A.D) NOD mice were established. I-A.Y secreted IL-4, TGF-β and IL-10 while I-A.D T cell line secreted IL-10 and IFN-γ. We found that I-A.D T cell line induced diabetes when transferred in NOD/SCID mice but I-A.Y T cell line did not induce disease. These results show that immunization of NOD mice with I-Aβ^{g7} (54–76) peptide at a younger age induces a regulatory T cell response suggesting that correcting the defects in immunoregulatory mechanisms using self-MHC peptides may be one of the approaches to prevent autoimmune diseases like T1D.

Key words: Self-MHC-class II, tolerance, type 1 diabetes, peptides, TGF-β, T cell lines

INTRODUCTION

The repertoire of T cells is determined in the thymus during the process of selection. As T cells develop in the thymus they express antigen-specific receptors, those with high-affinity to self-antigens are deleted. The process of binding of the receptors by weak ligands positively selects the T cell receptor (TCR) repertoire. These ligands originate from self-proteins, generating self-peptides that, in turn, bind to self-major histocompatibility (MHC) class II. Low-affinity auto-reactive T cells escape deletion and join the peripheral T cell pool. This process in the thymus, and secondarily in the periphery, results in tolerance of the immune system toward components of self (33).

The T lymphocytes that are positively selected from the thymus do not exhibit strong reactivity toward self-MHC molecules. In the periphery, these thymically selected weakly autoreactive T cells are subsequently exposed to self-MHC peptides or some cross reactive peptide derived from microbial agents that can trigger the activation of these T cells. Any cross reactivity between the triggering peptide and some tissue specific peptides may lead to T cell mediated autoimmune diseases, T1D is one such classical example of T cell mediated autoimmune disease. T1D is characterized by the destruction of insulin producing β cells in the pancreatic islets of Langerhans (5,56,60). Both genetic and environmental factors contribute to the development of T1D. There is a strong association between susceptibility to T1D and MHC class II molecules, HLA-DR and HLA-DQ in humans and -DQ homologue I-A^{g7} in non-obese diabetic (NOD) mice (23,25,55,58,61). Several unique properties of the MHC class II molecules I-A^{g7} and

Abbreviations: **APC**: antigen presenting cells; **MHC**: major histocompatibility complex; **MLR**: mixed lymphocyte reaction; **NOD**: non-obese diabetic mice; **TCR**: T cell receptor; **T1D**: type 1 diabetes mellitus; **TGF-β**: transforming growth factor-beta

HLA-DQ8 probably contribute to diabetes pathogenesis. I-A^{g7} and HLA-DQ8 positively select for high-affinity T cells, because they appear to bind peptides with low affinity. Results of several groups suggest that unstable I-A^{g7}-peptide complexes produce an effectively decreased density of MHC-peptide complexes on the cell surface (40). NOD mouse T cells show unusual self-reactivity, attributed to the poor negative selecting ability of the I-A^{g7} allele in the thymus and the periphery, presumably arising from poor peptide binding (41). This reduced expression of MHC class II would bias positive selection towards a T cell repertoire with a predisposition towards high-affinity T cells and a lack of regulatory T cells in NOD mice. However, this suggested role for MHC molecules in diabetogenesis is, probably, just one of multiple possible roles. A recent review of the HLA associations in human type 1 diabetes points out clearly that susceptibility to disease is conferred by particular combinations of the highly polymorphic genes encoding HLA-DQ and HLA-DR, rather then the mere presence or absence of certain variants (59).

The animal model of T1D, the NOD mouse has proven to be very important in understanding the pathogenesis of the disease. A number of studies have reported the association of structural abnormalities in the thymus of NOD mice and inadequate central tolerance (35,65). A number of aberrations like poorly defined demarcation between the cortex and medulla, increased number of mature T and B cells and deficiency of regulatory T cells in addition to defective negative selection have been attributed to the NOD thymus (3,17,26,45,46). It has also been shown that grafting of thymic epithelium into newborn C57BL/6 mice resulted in development of insulitis (54). This suggests a possible role of thymic epithelium in the development of diabetes by way of positive selection of auto-reactive T cells. A number of the peptides presented to T cells in the thymus may derive from self-MHC molecules. A number of studies have examined the response of peripheral T cells to self-MHC derived peptides (1,6,16).

A number of cytokines play a major role in down regulating the immune response in autoimmune diseases. Several approaches have been applied for correcting T1D including deletion of beta cell autoreactive Th1 cells and cytokines (IFN-γ and IL-2), or increasing regulatory Th2 or Th3/Tr1 cells and their cytokines (IL-4, IL-10 and TGF-β) reviewed in (52). A role of TGF-β has been implicated in a wide variety of *in vivo* models including NOD mice making this cytokine an important candidate for T cell mediated immunoregulation. TGF-β is a multifunctional cytokine with both positive and negative effects on the immune system. TGF-β has been shown to suppress inflammation, and is important for the induction of tolerance induced by oral antigen (31). A number of studies have reported the secretion of TGF-β by self-MHC-reactive regulatory T cells that mediate oral and peripheral tolerance and prevent the development of autoimmune diseases (22,27).

The peptide I-Aβ^{g7} (54–76) represents an immunodominant region on the I-A^{g7} molecule. This region has also been implicated in contacting the TCR in the recognition of MHC-peptide complex (8). Previously, using self-MHC class II β chain derived peptides, we found that tolerance to a peptide corresponding to 54–76 region is broken with age suggesting a role for peptide specific T cells in pathogenesis of diabetes in NOD mice (11). However, immunization of NOD mice at a younger age prevented the development of diabetes by induction of peptide specific antibodies and Th2 type cytokines (11). These results suggested that autoimmunity could be regulated using self-MHC peptides. The present study was undertaken to study breakdown of tolerance to self-MHC peptides in different strains of mice and to further characterize the regulatory T cells generated in response to self- I-Aβ^{g7} (54–76) peptide and understand the mechanism involved in the prevention of diabetes.

MATERIALS AND METHODS

Mice

Female NOD/Lt and NOD/SCID mice were bred and housed in the animal facilities at the John P. Robarts Research Institute and the University of Western Ontario, respectively (London, Ontario, Canada). Diabetic NOD mice were pooled and used within the week following disease onset. Female BALB/c mice were purchased from The Jackson Laboratory (Bar Harbor, ME).

Antigens

Peptides used in this study were synthesized in this laboratory, as previously described (1), using the Merrifield solid-phase peptide synthesis technique on an ABI 431A peptide synthesizer (Applied Biosystems, Mississauga, Ontario, Canada). The crude peptides were purified by reverse-phase HPLC on a semi-preparative synchropak RP-P C18 (250 x 10 mm ID) column using a linear gradient from 0.1% trifluoroacetic acid in water to 0.1% trifluoroacetic acid in acetonitrile (1% of the second solvent/min). Peptide purity and composition were confirmed by Mass spectral analysis. For functional assays, peptides were dissolved in saline by adjusting the pH with 0.1 M NaOH and were sterilized by filtration through a 0.22 μm filter. The sequences of the peptides used in this study are I-Aβ^{g7} (1–14), GDSERHFVHQFKGE, I-Aβ^{g7} (48–60), RAVTELGRHSAEY, I-Aβ^{g7} (54–76), GRHSAEYYN KQYLERTRAELDTA, I-Aβ^{g7} (82–95), EETEVPTSLRRLEQ, OVA (323–339), ISQAVHAAHAEINEAGR and I-Aβ^{d} (62–78), NSQPEIL ERTRAEVDTA. Concanavalin A (ConA) used as a positive control was obtained from Sigma Chem. (St. Louis, MO).

Purification of T cells

T cells were purified using nylon wool columns. Briefly, 1 x 10^8 spleen cells or lymph node cells were suspended in 1 ml RPMI (Life Technologies, Grand Island, NY) supplemented with 5 x 10^{-5} M 2-ME, 10 mM HEPES, 2 mM glutamine, 5 IU/ml penicillin-streptomycin and 10% heat-inactivated FCS (HyClone Laboratories, Logan, UT). The cells were then loaded onto a 10 ml column containing 0.6 g of nylon wool (Robbins Scientific, Sunnyvale, CA). After 45 min incubation at 37°C in the presence of 5% CO_2, the columns were washed with warm RPMI and

the effluent containing T cells was collected. The purity of T cells obtained was >90%.

Immunization and T cell proliferation assays

For primed T cell proliferation assay, 8 wk old mice were immunized subcutaneously in each hind footpad with 50 μg of peptide in saline mixed with an equal volume of incomplete freund's adjuvant (IFA) (Sigma Chem.). After 10 days, draining popliteal lymph nodes were collected and a single cell suspension was prepared. T cells were purified using nylon wool columns as described above. T cells (2×10^5) were then cultured in 96-well flat-bottom plates (Becton Dickinson, Bedford, MA) with I-A β-chain peptides (1-100 μg/ml) or ConA (5 μg/ml) in 200 μl of 10% RPMI (Life Technologies). Irradiated (3000 rad) spleen cells (10^6 cells/well) from normal syngeneic mice (8-9 wk old) were used as a source of APCs. After 3 days, cultures were pulsed with 1 μCi/well of ^{3}H-thymidine (NEN-DuPont, Boston, MA) for 16-20 hr. Incorporation of ^{3}H-thymidine was measured using a liquid scintillation counter (LKB Instruments, Gaithersburg, MD).

Proliferation assays using T cells from unprimed NOD mice

For experiments with unprimed mice, spleens were harvested and single cell suspensions were prepared. Purified T cells (5×10^5) were then cultured in 96-well flat-bottom plates (Becton Dickinson) with I-Aβ-chain peptides (1-100 μg/ml) or ConA (5 μg/ml) in 200 μl of medium. Irradiated (3000 rad) spleen cells (10^6 cells/well) from normal syngeneic mice (8-9 wk old) were used as a source of APCs. After 4 days, cultures were pulsed with 1 μCi/well of ^{3}H-thymidine (NEN-DuPont) for 16-20 hr. Incorporation of ^{3}H-thymidine was measured using a liquid scintillation counter (LKB Instruments).

Mixed lymphocyte reaction (MLR)

Allogeneic MLRs were performed using purified T cells from NOD as responders and irradiated (3000 rads) spleen cells from BALB/c mice as stimulators. Cells collected from lymph nodes of NOD mice (4-6 wk) primed with I-Aβ^{g7} (54–76) peptide were cultured in the presence of I-Aβ^{g7} (54–76) peptide and supernatants were collected after 24 and 72 hr. These culture supernatants were then added to the MLR cultures containing NOD T cells (Responders, 2×10^5) and irradiated BALB/c spleen cells (Stimulators, 4×10^5) and incubated for 3 days at 37°C. Untreated MLR cultures containing responders and stimulators (1:2) were used as positive controls. For one experiment, various I-Aβ^{g7} peptides (50 μg/ml) were added to the MLR cultures. After 72 hr, cultures were pulsed with 1 μCi/well of ^{3}H-thymidine (NEN-DuPont) for 16-20 hr. Incorporation of ^{3}H-thymidine was measured using a liquid scintillation counter (LKB Instruments).

Generation of I-Aβ^{g7}(54–76) peptide-specific T cell lines

Female NOD mice (4-6 wk old) were immunized with I-Aβ^{g7} (54–76) peptide (50 μg/footpad) emulsified in IFA. After 10 days, popliteal lymph nodes were harvested and T cells were purified using nylon wool as described above. T cells (4×10^6) were cultured for 4 days with I-Aβ^{g7} (54–76) peptide (50 μg/ml) in the presence of irradiated (3000 rads) syngeneic spleen cells from normal NOD (8 wk) as APCs (1×10^6) in a final volume of 4 ml. Cells were washed and incubated in medium alone at 37°C for 7 days. This process was repeated twice. Cells were collected and dead cells were removed using lympholyte M (Cedarlane, Hornby, Ontario, Canada). T cells (2×10^6) were cultured with the peptide (50 μg/ml) in the presence of irradiated APCs. Two days later, cells were further diluted in 10 ml medium and expanded in the presence of 15 U/ml IL-2 (Becton Dickinson). Two days later cells were diluted again in 100 ml media and rested. After 10 days, I-Aβ^{g7} (54–76) peptide-specific T cell lines from young NOD mice (I-A.Y) were used for the experiments or restimulated to maintain the cell line. Similarly an I-Aβ^{g7} (54–76) peptide-specific T cell line was generated from unprimed diabetic NOD mice (I-A.D) and used in the experiments.

Cytokine ELISA

Female NOD mice (4-6 wk old) were immunized with I-Aβ^{g7} (54–76) peptide (50 μg/footpad) emulsified in IFA. After 10 days, popliteal lymph nodes were harvested and cells (2×10^6) were cultured in the presence of I-Aβ^{g7} (54–76) peptide (50 μg/ml) or control peptides (50 μg/ml) in 24 -well plates (Becton Dickinson). Culture supernatants were collected after 24 hr and 72 hr and secretion of TGF-β was measured. Samples were acidified by addition of HCl for 10 min, neutralized by NaOH and the TGF-β content was measured using a TGF-β DuoSet Elisa Development Kit (R&D Systems, Minneapolis, MN). Similarly, I-Aβ^{g7} (54–76) peptide specific cell lines (2×10^6) were cultured in the presence of I-Aβ^{g7} (54–76) peptide (50 μg/ml) and syngeneic irradiated (3000 rads) spleen cells from NOD mice (8 wk) as APCs in 24 -well plates (Becton Dickinson). Culture supernatants were collected after 24 hr and IL-4, IL-10 and IFN-γ secretion was assayed using cytokine specific BD OptEIA™ ELISA Sets (BD Pharmingen, Mississauga, Ontario, Canada). TGF-β secretion was measured as described above. Optical densities were measured at 450 nm using a micro plate ELISA reader (Bio-Rad, Richmond, CA).

Adoptive transfer of diabetes by T cell lines

NOD/SCID (6-8 wk-old) mice were injected intravenously with either I-A.Y or I-A.D T cell line (1×10^7/ mouse). As a positive control NOD/SCID mice were injected with splenocytes (1×10^7/ mouse) from diabetic NOD mice alone. Recipient (n= 10/group) mice were then monitored twice per week for glycosuria and regarded as overtly diabetic based on two consecutive positive (>11.5 mmol) glycosuria tests.

Statistical analysis

Results were analyzed using Student's *t* test, in which p values of 0.05 or less were considered significant.

RESULTS

Response of T cells from NOD and BALB/c mice to self I-Aβ 54-76 peptide

Previously, using self-MHC class II β chain derived peptides, we found that young NOD mice are tolerant to a self-MHC class II peptide corresponding to region 54-76 (I-Aβ^{g7} 54–76 peptide). However, there is a breakdown of tolerance to this peptide in old diabetic NOD mice suggesting a role for peptide specific T cells in the pathogenesis of diabetes in NOD mice (11). This experiment was done to find out if a similar breakdown of tolerance occurs in non-autoimmune mouse strains as well. We determined the response of primed or unprimed T cells from BALB/c and NOD mice towards self I-Aβ^d (62–78) and I-Aβ^{g7} (54–76) peptide, respectively. There was no proliferative response observed in T cells isolated from young (8 wk old) unprimed NOD mice towards self I-Aβ^{g7} (54–76) peptide suggesting that young NOD mice are tolerant to this peptide (Fig. 1A). However, T cells from BALB/c mice showed moderate proliferation to I-Aβ^d (62–78) peptide. There was a dose dependent increase in the response of T cells from BALB/c mice. The response was abrogated at 100 μg/ml dose probably due to activation induced cell death.

To examine primed T cell responses, young mice were immunized subcutaneously in the hind footpads with

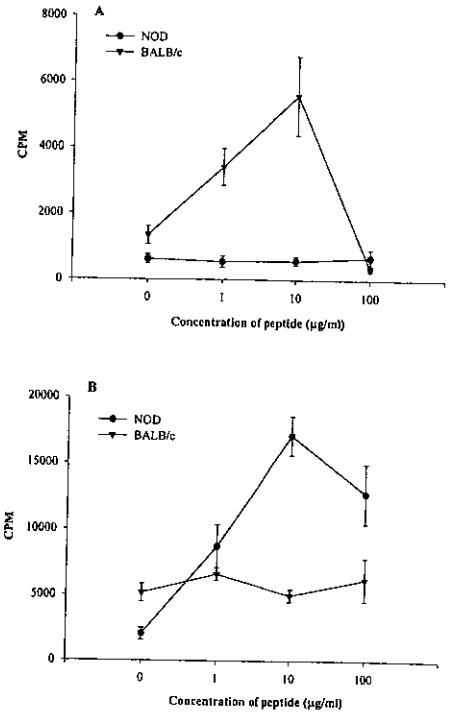

Fig. 1 Response of T cells from NOD and BALB/c mice to self I-Aβ chain peptides. **A)** Splenic T cells from unprimed young NOD and BALB/c (8 wk) mice were cultured with I-Aβ^{g7} 54–76 or I-Aβ^{d} (62–78) peptide (1-100 μg/ml) respectively along with irradiated (3000 rads) syngeneic spleen cells as APCs. **B)** For primed T cell responses, young NOD and BALB/c (8 wks) mice were immunized with I-Aβ^{g7} (54–76) or I-Aβ^{d} (62–78) peptide (50 μg/foot pad), respectively. After ten days, lymph nodes were harvested and T cells were cultured with irradiated (3000 rads) syngeneic spleen cells as APCs and respective I-Aβ peptides at various concentrations. T cell proliferation was measured by ^{3}H-thymidine incorporation as described in Materials and Methods. Results represent triplicate culture ± SD.

peptide emulsified in IFA. After ten days, draining lymph nodes were harvested and T cells were purified. T cells were cultured with irradiated spleen cells as APCs and I-Aβ^{d} (62–78) or I-Aβ^{g7} (54–76) peptide at various concentrations. T cell proliferation was measured as described in Materials and Methods. ConA served as a positive control to demonstrate that the T cells were competent (data not shown). The data presented in Fig. 1B show that T cells from primed NOD mice proliferate in response to I-Aβ^{g7} (54–76) peptide and the proliferation was dose dependent. However, response in primed T cells from young BALB/c mice towards self I-Aβ^{d} (62–78) peptide was similar to that obtained in young unprimed mice. These results suggest that although young NOD mice are tolerant to self I-Aβ^{g7} (54–76) peptide this tolerance can be broken by immunization but non-autoimmune mice remain tolerant even after immunization.

Breakdown of tolerance to self I-Aβ peptide with age in diabetes prone and non-diabetes prone mice

To further determine if tolerance/unresponsiveness to self I-Aβ peptide is maintained throughout life, we examined the response of T cells from old BALB/c mice (6 months old) and diabetic NOD mice towards the I-Aβ^{d} (62–78) or I-Aβ^{g7} (54–76) peptide, respectively. T cells were separated from spleen cells of unprimed old BALB/c and diabetic NOD mice and cultured with irradiated spleen cells (from 8 wk old syngeneic mice) and I-Aβ^{d} (62–78) or I-Aβ^{g7} (54–76) peptide, respectively at various concentrations and proliferation was assayed as described in Materials and Methods. Surprisingly, the T cell proliferative response to these self I-Aβ peptides was considerably greater than that of T cells from young mice in both BALB/c and NOD mice but the overall magnitude of response was higher in autoimmune diabetic NOD mice (Fig. 2). After an initial dose dependent increase the response decreased at highest peptide concentration possibly due to activation induced cell death. These results suggest that T cell tolerance to a self-peptide corresponding to an immunodominant region on I-A is broken at an older age in both autoimmune and non-autoimmune strains of mice. However, this breakdown of tolerance in NOD mice suggests a role for these cells in the development of diabetes.

Inhibition of NOD T cell response to BALB/c spleen cells by I-Aβ^{g7} (54–76) peptide specific culture supernatants in MLR

We have previously shown that immunization of young NOD mice with I-Aβ^{g7} (54–76) peptide induces a Th2 type response and cells from I-Aβ^{g7} (54–76) peptide-immunized mice significantly delays the transfer of

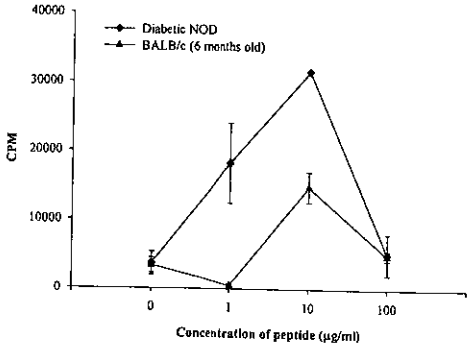

Fig. 2 Breakdown of tolerance to self I-Aβ peptide in diabetes prone and non-diabetes prone mice with age. T cells from unprimed old BALB/c mice (6 months old) and diabetic NOD mice were cultured with irradiated (3000 rads) syngeneic spleen cells and respective self I-Aβ peptide at various concentrations. Cultures were harvested and proliferation was assayed by ^{3}H-thymidine uptake as described in Materials and Methods. Results represent triplicate culture ± SD.

diabetes in an adoptive transfer model. Also, anti-peptide antibodies inhibited antigen-specific T cell responses in NOD mice (11). Therefore, experiments were conducted to see if cytokines secreted by peptide-specific T cells have any effect on T cell responses. An allogeneic-MLR system was used with NOD T cells (responder) and irradiated BALB/c spleen cells (stimulator) as described in Materials and Methods. Supernatants from I-Aβ^{g7} (54–76) peptide primed T cells collected after 24 and 72 hr were added to the MLR cultures. The data presented in Fig. 3 show that the I-Aβ^{g7} (54–76) peptide-specific culture supernatants (24 and 72 hr) significantly inhibited the response of NOD T cells to BALB/c spleen cells. The supernatants collected from control I-Aβ^{g7} (1–14) peptide primed cells did not have any effect on the T cell response in MLR cultures. These results suggest that the I-Aβ^{g7} (54–76) peptide-specific cells secrete cytokines that can inhibit T cell response in allogeneic-MLR.

Effect of self-MHC peptides on the NOD T cell response to BALB/c spleen cells in MLR

The results from the previous experiment showed that culture supernatants from I-Aβ^{g7} (54–76) peptide-primed cells inhibit T cell responses. Further experiments were done to find out if the inhibition observed was due to cytokines or the presence of I-Aβ^{g7} (54–76) peptide in the culture supernatant. Various peptides from I-Aβ^{g7} corresponding to regions 1–14, 54–76, 84–91 and OVA (223–239) peptide were added to the MLR cultures containing NOD T cells and BALB/c spleen cells. The data presented in Fig. 4 show that various I-Aβ^{g7} peptides including the I-Aβ^{g7} (54–76) peptide did not inhibit the response of NOD T cells to BALB/c spleen cells. These results suggest that inhibition of the T cell response is due to the cytokines secreted by I-Aβ^{g7} (54–76) peptide-primed cells and not because of the presence of peptide in the supernatant.

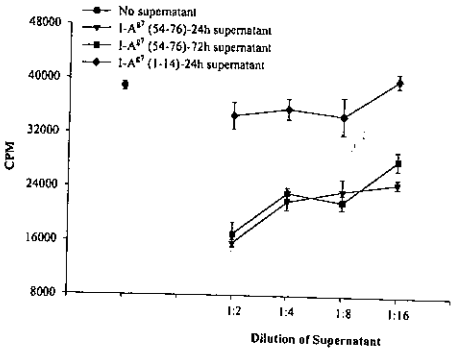

Fig. 3 Culture supernatants from I-Aβ^{g7} (54–76) peptide-primed cells inhibit allogeneic-MLR. Culture supernatants from I-Aβ^{g7} (54–76) peptide-primed cells collected after 24 and 72 hr were added to the allogeneic-MLR cultures using purified T cells from NOD as responders and irradiated (3000 rads) spleen cells from BALB/c mice as stimulators (1:2). Untreated MLR cultures containing responders and stimulators were used as positive controls. I-Aβ^{g7} (1–14) peptide-primed T cell supernatant was used as a control. Supernatants were used at a dilution of 1:2, 1:4, 1:8 and 1:16. After 3 days, cultures were harvested and ^{3}H-thymidine uptake was measured as described in Materials and Methods. Statistical analysis was performed using Student's *t* test. P values for cultures with supernatant (24 hr and 72 hr) from I-Aβ^{g7} (54–76) peptide-primed cells compared with untreated responders and stimulators was <0.01 and for I-Aβ^{g7} (1–14) peptide-primed supernatant p= 0.35. Difference between I-Aβ^{g7} (54–76) peptide-primed cell supernatant and I-Aβ^{g7} (1–14) peptide-primed supernatant was significant with p<0.001.

Effect of TGF-β on inhibition of MLR of NOD responders to BALB/c stimulators

We have previously shown that I-Aβ^{g7} (54–76) peptide-primed cells secrete IL-4 but no IL-2 or IFN-γ (11). In order to find out the cytokine responsible for inhibiting the T cell response, additional experiments were done to determine if the I-Aβ^{g7} (54–76) peptide-specific cells secreted TGF-β. Culture supernatants from I-Aβ^{g7} (54–76) peptide primed cells were tested for the presence of TGF-β as described in Materials and Methods. It was observed that the I-Aβ^{g7} (54–76) peptide-primed cells secreted large amounts of TGF-β when cultured with I-Aβ^{g7} (54–76) peptide *in vitro* for 24 and 72 hr (Fig. 5). These cells also secreted some IL-10 (data not shown). Culture supernatants from control I-Aβ^{g7} (1–14) peptide treated cells contained significantly lower levels of TGF-β.

Further, to find out if TGF-β was responsible for inhibiting the response of NOD T cells to BALB/c spleen cells in MLR, recombinant TGF-β1 (final concentration of 10 ng/well) was added to the MLR cultures. Proliferation was assayed as described in Materials and Methods. It was

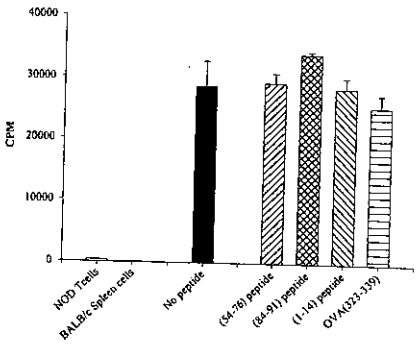

Fig. 4 Self-MHC peptides do not inhibit allogeneic-MLR. Various I-Aβ^{g7} peptides (50 μg/ml) corresponding to regions 1–14, 54–76, 84–91 and the control OVA (323–339) peptides were added to the MLR cultures containing NOD T cells (responders) and BALB/c spleen cells (stimulators). After 72 hr, cultures were harvested and ^{3}H-thymidine uptake was measured as described in Materials and Methods. Results represent triplicate culture ± SD.

observed that TGF-β1 significantly inhibited the response of NOD T cells to BALB/c spleen cells. This inhibition was similar to that observed in the presence of I-Aβ^{g7} (54–76) peptide-primed cell culture supernatants (Fig. 6). These results show that TGF-β is responsible for the inhibition of the T cell response. However, the concentration of TGF-β1 required to achieve inhibition similar to the supernatants was much higher than the concentration of TGF-β present in the supernatants. This suggests that a combination of cytokines present in the supernatants may contribute to the inhibition.

Generation of an I-Aβ^{g7} (54–76) peptide-specific T cell line

We have shown previously that I-Aβ^{g7} (54–76) peptide is naturally processed and presented by APCs in NOD mice. Tolerance to the I-Aβ^{g7} (54–76) peptide is broken with age, suggesting a role for peptide-specific T cells in the pathogenesis of disease in NOD mice. To further characterize the cells generated in response to I-Aβ^{g7} (54–76) peptide, T cell lines were established from young NOD mice immunized with I-Aβ^{g7} (54–76) peptide and unprimed diabetic NOD mice as described in Materials and Methods. To check the specificity of the I-Aβ^{g7} (54–76) peptide-specific T cell lines, cells were cultured in the presence or absence of I-Aβ^{g7} (54–76) peptide and proliferation was assayed as described in Materials and Methods. The T cell lines generated from both young NOD mice (I-A.Y) and diabetic mice (I-A.D) proliferated strongly in response to I-Aβ^{g7} (54–76) peptide suggesting that the cells are specific for the peptide (Fig. 7). Some response was also observed when T cells were cultured with irradiated spleen cells in the absence of peptide and this response was higher in I-A.D T cells. This response could be attributed to the endogenous presentation of the peptide by the APCs.

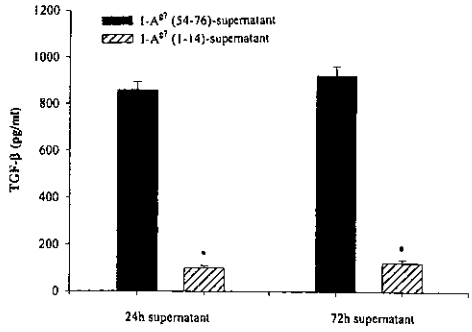

Fig. 5 I-Aβ^{g7} (54–76) peptide-primed cells secrete large amounts of TGF-β *in vitro*. Female NOD mice (4-6 wk old) were immunized with I-Aβ^{g7} (54–76) peptide or I-Aβ^{g7} (1–14) peptide (50 μg/footpad) emulsified in IFA. After 10 days, popliteal lymph nodes were harvested and cells (2 x 10^6) were cultured in the presence of either I-Aβ^{g7} (54–76) peptide or I-Aβ^{g7} (1–14) peptide (50 μg/ml). Culture supernatants were collected after 24 hr and 72 hr and secretion of TGF-β was measured as described in Materials and Methods. Statistical analysis was performed using Student's *t* test, in which $p<0.001$ for supernatant from I-Aβ^{g7} (54–76) peptide-primed cells compared to supernatant from I-Aβ^{g7} (1–14) peptide-primed cells for both time points.

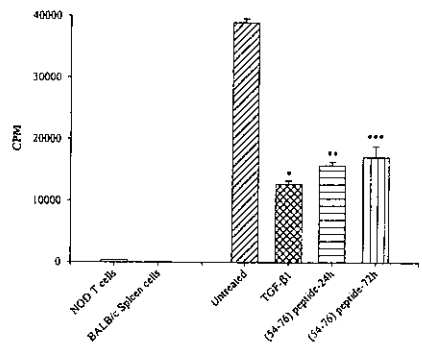

Fig. 6 TGF-β inhibits MLR of NOD responders to BALB/c stimulators. Recombinant TGF-β1 (final concentration of 10 ng/well) was added to NOD T cells as responders and irradiated BALB/c spleen cells as stimulators in a ratio of 1:2. Supernatants from I-Aβ^{g7} (54–76) peptide-primed cells collected after 24 and 72 hr were also added to the MLR cultures. After 72 hr, cultures were harvested and ^{3}H-thymidine uptake was measured as described in Materials and Methods. Statistical analysis was performed using Student's *t* test, in which *$p<0.0005$ for TGF-β1, **$p<0.0007$ for I-Aβ^{g7} (54–76) peptide-24 supernatant, ***$p<0.0008$ for I-Aβ^{g7} (54–76) peptide-72 hr supernatant compared with untreated responders and stimulators (1:2).

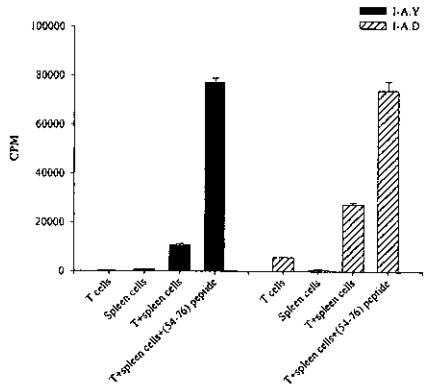

Fig. 7 Generation of I-Aβ^{g7} (54–76) peptide specific T cell lines I-A.Y and I-A.D from NOD mice. T cell lines specific for I-Aβ^{g7} (54–76) peptide were established from young NOD mice immunized with I-Aβ^{g7} (54–76) peptide (I-A.Y) and unprimed diabetic NOD (I-A.D) mice as described in Materials and Methods. To check the specificity of the I-Aβ^{g7} (54–76) peptide-specific T cell lines, cells were cultured in the presence or absence of I-Aβ^{g7} (54–76) peptide and proliferation was assayed. After 72 hr, cultures were harvested and ^{3}H-thymidine uptake was measured as described in Materials and Methods. Results represent triplicate culture ± SD.

Cytokine response of I-Aβ^{g7} (54–76) peptide-specific T cell lines

To further characterize the I-Aβ^{g7} (54–76) peptide-specific T cell lines, I-A.Y and I-A.D, cells were cultured in the presence of I-Aβ^{g7} (54–76) peptide and the culture supernatants were tested for the presence of IL-4, IFN-γ, TGF-β and IL-10. The data presented in Fig. 8 show that T cell lines generated from young mice (I-A.Y) secreted high levels of IL-4 and TGF-β, but no IFN-γ. On the other hand, T cell lines generated from diabetic (I-A.D) NOD mice secreted significant amounts of IFN-γ, but no IL-4 or TGF-β. Both cell lines secreted large amounts of IL-10. This suggests that immunization with I-Aβ^{g7} (54–76) peptide at a young age induces a Th3/Tr1 type response in contrast to a Th1/Tr1 type response generated as a result of spontaneous breakdown of tolerance with age and disease status.

Adoptive transfer of I-A.Y and I-A.D T cells into NOD/SCID mice

Further experiments were performed using an adoptive transfer protocol to find out if the T cell lines generated from young (I-A.Y) and diabetic (I-A.D) NOD mice specific for I-Aβ^{g7} (54–76) peptide can induce diabetes in NOD/SCID mice. NOD/SCID mice were injected with either I-A.Y or I-A.D T cell lines or spleen cells from diabetic NOD mice as described in Materials and Methods. NOD/SCID recipients were followed for development of diabetes for the next 8 wk. It was observed that the mice injected with only diabetogenic spleen cells started developing diabetes two weeks after the cell transfer and all of the mice gradually became diabetic by 5 wk (Fig. 9). NOD/SCID mice injected with the I-A.D T cell line also started developing diabetes two weeks after the cell transfer and all the mice became diabetic similar to the

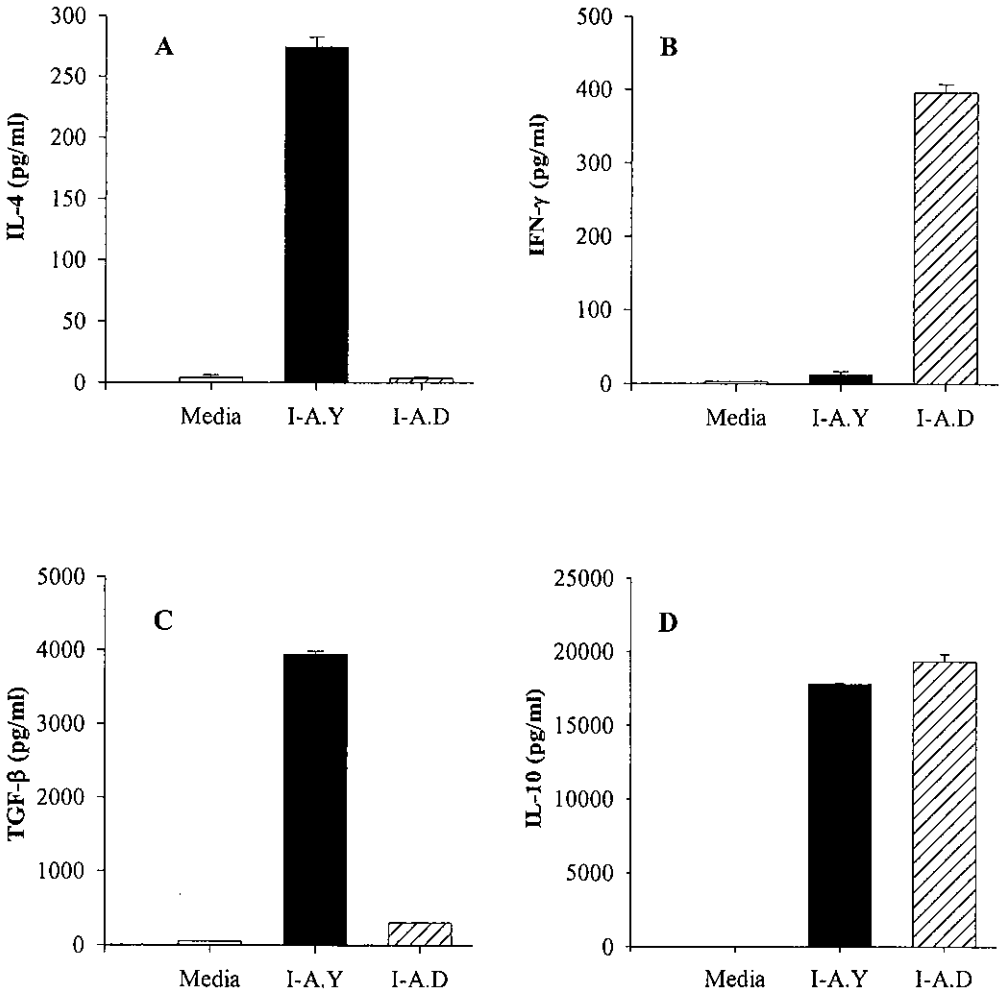

Fig. 8 Cytokine profile of I-Aβ^{g7} (54–76) peptide specific T cell lines. I-Aβ^{g7} (54–76) peptide-specific T cell lines, I-A.Y and I-A.D, were cultured in the presence of I-Aβ^{g7} (54-76) peptide and the culture supernatants collected after 72 hr were tested for the presence of IL-4 (**A**), IFN-γ (**B**), TGF-β (**C**) and IL-10 (**D**) as described in Materials and Methods.

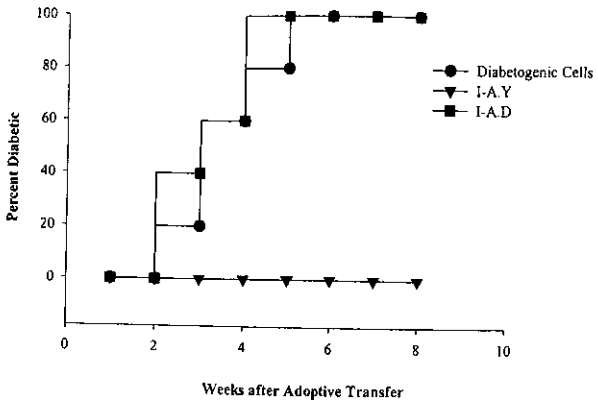

Fig. 9 I-A.D T cell line is diabetogenic while I-A.Y T cell line is non-diabetogenic. NOD/SCID mice were injected intravenously with either I-Aβ^{g7} (54–76) peptide-specific T cell line I-A.Y or I-A.D or spleen cells from diabetic NOD mice (10 x 10^6/ mouse). NOD/SCID recipients were followed for development of diabetes for the next 8 wk. Recipient (n= 10/group) mice were then monitored twice per week for glycosuria and regarded as overtly diabetic based on two consecutive positive (>11.5 mmol) glycosuria tests.

mice given diabetogenic cells only. On the other hand, NOD/SCID mice injected with the I-A.Y T cell line did not develop diabetes. These results confirmed that cells generated spontaneously in response to I-Aβ^{g7} (54–76) peptide after the breakdown of tolerance are involved in diabetes pathogenesis. However, cells generated after immunization of young NOD mice (I-A.Y) with I-Aβ^{g7} (54–76) peptide are not pathogenic and do not cause disease.

DISCUSSION

Thymic or central deletion of autoreactive T cell clones is the major mechanism for maintaining tolerance to self. T cell selection involves the interaction of the TCR with a MHC-peptide complex. T cells with high affinity for self-MHC-peptide complexes are deleted and cells with low affinity are positively selected and transported to the periphery. Defective negative selection in the thymus is one of the possible mechanisms leading to autoimmune diseases. T1D is one of the autoimmune diseases mediated by T cells (4,14). Susceptibility to T1D is strongly associated with the expression of a unique MHC class II (I-A^{g7}) in NOD mice and HLA-DQ0302 in humans (25,48,49,57). Both class II molecules I-A^{g7} and DQ0302 cannot form stable dimmers in SDS-PAGE gels and they both preferably bind peptides with acidic residues near the C-terminus (10,15,28,53). These properties of DQ0302 and I-A^{g7} may also lead to a less diverse peptide repertoire being presented to developing T cells compared to other MHC class II molecules. It has been shown that DQ0302 can accommodate peptides with no negative charge at position 9 but the interactions are of low affinity (29). These low affinity interactions may lead to selection of self-reactive T cells that are transported to the periphery. These cells may become activated in the periphery by recognizing a cross-reactive antigen (molecular mimicry) leading to the breakdown of tolerance to autoantigens (2).

We have shown earlier that there is a spontaneous breakdown of tolerance to self-I-Aβ^{g7} (54–76) peptide in NOD mice with age suggesting a role for this peptide in the pathogenesis of T1D. Tolerance to this peptide can also be broken at an earlier age by immunizing mice with the peptide. Immunization of young NOD mice with the peptide induced a Th2 response and prevented the development of diabetes in spontaneous, accelerated (cyclophosphamide) and adoptive transfer models (11). The present study was undertaken to further characterize the cells generated in response to self-I-Aβ^{g7} (54–76) peptide and understand the mechanism involved in the prevention of diabetes.

Experiments were done to find out if the breakdown of tolerance to this self-I-Aβ^{g7} (54–76) peptide observed in NOD mice occurs in non-autoimmune BALB/c mice as well. It was observed that T cells from young NOD mice did not respond to self-I-Aβ^{g7} (54–76) peptide but T cells from BALB/c mice proliferated in response to I-Aβ^{d} (62–78) peptide. On the other hand, T cells from unprimed old BALB/c and diabetic NOD mice responded to I-Aβ^{d} (62–78) or I-Aβ^{g7} (54–76) peptide respectively although the response was stronger in NOD mice. The response to self-MHC peptide in BALB/c mice can be explained by the possibility that during selection in the thymus T cells may not have seen the I-Aβ^{d} (62–78) peptide. Therefore, when this peptide is presented in the periphery, T cells mount an immune response. Further, BALB/c mice were immunized with I-Aβ^{d} (62–78) peptide to induce a breakdown of tolerance. We found that the response of T cells primed with I-Aβ^{d} (62–78) peptide was similar to that observed with unprimed T cells, whereas T cells isolated from NOD mice primed with I-Aβ^{g7} (54–76) peptide showed strong proliferative response. These results suggest that I-Aβ^{d} (62–78) peptide is not very immunogenic in BALB/c mice as opposed to I-Aβ^{g7} (54–76) peptide in autoimmune NOD mice. Also, the increase in response to this peptide with age in both NOD and BALB/c mice indicates that there is a breakdown of tolerance to this immunodominant epitope with age. However, in BALB/c mice this breakdown does not lead to autoimmunity but in NOD mice it contributes to the development of diabetes. The possible reason for this is, these cells are kept under constant control by regulatory cells in BALB/c mice. The development of diabetes in both humans and NOD mice is associated with deficiency in the CD4$^+$CD25$^+$ regulatory T cell compartment. Another regulatory T cell subset, natural killer T cells (NKT), is also impaired in both NOD mice and human

T1D patients (39,62). Due to these defects in the regulatory cell populations the autoreactive response to self-MHC peptide can lead to autoimmunity in NOD mice.

Previously, we have reported that T cells isolated from young NOD mice immunized with self-I-Aβ^{g7} (54–76) peptide prevent transfer of disease by diabetogenic cells. Also, peptide-specific antibodies inhibit antigen-specific T cell responses (11). Further experiments were done to understand the mechanism of disease prevention by peptide-specific cells. First, we wanted to see if the cytokines secreted by peptide-specific cells have any effect on NOD T cell response in an allogeneic-MLR using BALB/c spleen cells as stimulators. Surprisingly, the supernatant from I-Aβ^{g7} (54–76) peptide-primed cells inhibited the T cell response significantly when added to the MLR while the supernatant from control peptide treated cells did not have any significant effect. To rule out the possibility that I-Aβ^{g7} (54–76) peptide present in culture supernatants may be responsible for the observed suppression, we added various I-Aβ^{g7} peptides to the MLR cultures. None of the peptides have any effect on the T cell response to allogeneic-MHC suggesting that the inhibition is mediated by a soluble factor secreted by I-Aβ^{g7} (54–76) peptide-primed cells.

Kitani *et al.* (27) have described human self-MHC-reactive T cell clones with suppressor effect. They showed that suppression of non-T cells was mediated by TGF-β alone but the suppression of primary T cells and antigen-specific T cells was mediated by both IL-10 and TGF-β (27). Cytokine analysis of the supernatant from I-Aβ^{g7} (54–76) peptide-primed cells showed the presence of IL-4 (11) and in this study we found secretion of large amounts of TGF-β and small amounts of IL-10 (data not shown). TGF-β1 is a pluripotent cytokine that controls peripheral T cell tolerance. It has been shown earlier that TGF-β can inhibit the allogeneic-MLR and prevent graft *versus* host disease (7,36,64). To confirm that TGF-β is responsible for the inhibition of MLR, we added TGF-β1 to the MLR cultures. We found that TGF-β1 inhibited T cell response in MLR similar to culture supernatant from peptide-primed cells. However, the concentration of TGF-β1 required to get similar suppression was higher than the TGF-β concentration in supernatants (1:2 dilution). It is possible that a combination of cytokines present in supernatants (IL-10 and IL-4) is more effective than TGF-β alone in suppressing the allogeneic-MLR. It has been shown earlier that self-MHC class II reactive suppressor T cells inhibit the proliferation of effector T cells from NOD mice. These cells also prevented spontaneous diabetes and recurrent diabetes in islet transplanted NOD mice. The suppressive activities of self-MHC class II reactive suppressor T cells were mediated by secretion of TGF-β (22). Another possibility is the involvement of some other cytokine, possibly macrophage-colony stimulating factor, that has been shown to inhibit allogeneic-MLR responses (44).

To further characterize the T cells generated in response to self-I-Aβ^{g7} (54–76) peptide, T cell lines specific for the I-Aβ^{g7} (54–76) peptide were established from young immunized NOD mice and unprimed diabetic NOD mice. These T cell lines proliferated in response to I-Aβ^{g7} (54–76) peptide showing the specificity of the response. These cells also proliferated when cultured with syngeneic APCs in the absence of peptide and this response was stronger in I-A.D T cells. This response could be due to two possibilities, first, the I-Aβ^{g7} (54–76) peptide is naturally processed and presented by APCs and second, these T cells recognize the 54–76 region on intact MHC molecules. Indeed, this region has been implicated in contacting the TCR during the interaction with MHC-peptide complexes (30). Further, the peptide-specific T cell line, I-A.Y, secreted large amounts of IL-4, TGF-β and IL-10 suggesting the generation of Th3/Tr1 cells. The I-A.D T cell line, on the other hand, secreted large amounts of IL-10 and IFN-γ suggesting generation of Th1/Tr1 cells. The I-A.D T cell line induced diabetes when transferred into NOD/SCID mice but the I-A.Y T cell line was found to be non-pathogenic and did not induce disease. TGF-β can regulate autoreactive T cell responses by preventing the maturation of APCs and differentiation of naive $CD4^+$ T cells (18,19,24). The presence of IL-10 can prevent the migration of dendritic cells and the production of pro-inflammatory chemokines (13,34). The combination of TGF-β and IL-10 production by I-A.Y T cells may be responsible for their inability to induce diabetes in NOD/SCID mice. It has been shown that IL-10 can inhibit the activation of autoreactive T cells by down-regulating IL-12 , TNF-α and MHC class II (34). It has also been shown earlier that Th2 cells can induce acute pancreatitis and autoimmune diabetes in NOD/SCID recipients via production of IL-10 (38). IL-10 can also induce recruitment, proliferation and cytotoxicity of $CD8^+$ T cells and can up-regulate the cytotoxicity of NKT cells when combined with IL-18 (9,34). Possibly, in the absence of the regulatory effects of TGF-β in combination with IFN-γ, IL-10 secretion by I-A.D T cells leads to the destruction of β cells resulting in the development of diabetes in NOD/SCID mice. Further experiments are being done to examine the effect of supernatants from I-A.Y and I-A.D T cell lines on antigen-specific and non-antigen-specific T cell responses. Also, to see if the I-A.Y cell line can block disease transfer by diabetogenic cells, adoptive transfer experiments will be done after mixing the I-A.Y T cell line with diabetogenic cells.

Positive selection of T cells in the thymus is facilitated by self-MHC-peptide complexes, these complexes may also be present in the periphery. It has been shown that mature T cells express 5- to 10-fold more TCR on their surface (42). As a result, the interactions of TCR and self-

MHC-peptide complexes may become stronger than that in the thymus leading to the activation of autoreactive T cells. Normally, these responses are inhibited by a population of $CD4^+CD25^+$ regulatory T cells that inhibit the response of other T cells (43,50). Another possibility is that the activation threshold of mature T cells is dynamically controlled by self-MHC-peptide complexes in the periphery so that the interaction of TCR with self-MHC-peptide complexes is not sufficient to activate autoreactive T cells (12 ,20,21,37,47,51,63). Any defect in the regulatory compartment leads to the development of autoimmunity. We have recently shown that the dendritic cells in NOD mice have elevated co-stimulatory and Th1 inducing ability (32). This may prevent activation of regulatory T cells such as $CD4^+CD25^+$ cells. Moreover, TGF-β has a direct role in blocking the maturation of APCs and Th1 cells (18). Correcting the defects in immunoregulatory mechanisms using self-MHC peptides may be one of the approaches to prevent autoimmune diseases like T1D.

Acknowledgments – The authors thank Edwin Lee-Chan (Department of Microbilogy and Immunology, University of Western Ontario, London, Canada) for synthesis and purification of the peptides used in this study. We thank Tracey Stephens (Department of Microbilogy and Immunology, University of Western Ontario, London, Canada) for helpful comments and critical reading of the manuscript. This work was supported by grants from the Canadian Institutes of Health Research (CIHR), Juvenile Diabetes Research Foundation (JDRF) and the Canadian Diabetes Association (CDA).

REFERENCES

1. Agrawal, B., Manickasundari, M., Fraga, E. and Singh, B., T cells that recognize peptide sequences of self MHC class II molecules exist in syngeneic mice. *J. Immunol.* 1991, **147**: 383-390.
2. Albert, L.J. and Inman, R.D., Molecular mimicry and autoimmunity. *N. Engl. J. Med.* 1999, **341**: 2068-2074.
3. Atlan-Gepner, C., Naspetti, M., Valero, R., Barad, M., Lepault, F., Vialettes, B. and Naquet, P., Disorganization of thymic medulla precedes evolution towards diabetes in female NOD mice. *Autoimmunity* 1999, **31**: 249-260.
4. Bach, J.F., Insulin-dependent diabetes mellitus as an autoimmune disease. *Endocr. Rev.* 1994, **15**: 516-542.
5. Bach, J.F., Insulin-dependent diabetes mellitus as a beta-cell targeted disease of immunoregulation. *J. Autoimmun.* 1995, **8**: 439-463.
6. Benichou, G., Takizawa, P.A., Ho, P.T., Killion, C.C., Olson, C.A., McMillan, M. and Sercarz, E.E., Immunogenicity and tolerogenicity of self-major histocompatibility complex peptides. *J. Exp. Med.* 1990, **172**: 1341-1346.
7. Boussiotis, V.A., Chen, Z.M., Zeller, J.C., Murphy, W.J., Berezovskaya, A., Narula, S., Roncarolo, M.G. and Blazar, B.R., Altered T cell receptor + CD28-mediated signaling and blocked cell cycle progression in interleukin 10 and transforming growth factor-beta-treated alloreactive T cells that do not induce graft-*versus*-host disease. *Blood* 2001, **97**: 565-571.
8. Brown, J.H., Jardetzky, T., Saper, M.A., Samraoui, B., Bjorkman, P.J. and Wiley, D.C., A hypothetical model of the foreign antigen binding site of class II histocompatibility molecules. *Nature* 1988, **332**: 845-850.
9. Cai, G., Kastelein, R.A. and Hunter, C.A., IL-10 enhances NK cell proliferation, cytotoxicity and production of IFN-gamma when combined with IL-18. *Eur. J. Immunol.* 1999, **29**: 2658-2665.
10. Carrasco-Marin, E., Shimizu, J., Kanagawa, O. and Unanue, E.R., The class II MHC I-Ag7 molecules from non-obese diabetic mice are poor peptide binders. *J. Immunol.* 1996, **156**: 450-458.
11. Chaturvedi, P., Agrawal, B., Zechel, M., Lee-Chan, E. and Singh, B., A self MHC class II beta-chain peptide prevents diabetes in non-obese diabetic mice. *J. Immunol.* 2000, **164**: 6610-6620.
12. Cyster, J.G. and Goodnow, C.C., Tuning antigen receptor signaling by CD22: integrating cues from antigens and the microenvironment. *Immunity* 1997, **6**: 509-517.
13. D'Amico, G., Frascaroli, G., Bianchi, G., Transidico, P., Doni, A., Vecchi, A., Sozzani, S., Allavena, P. and Mantovani, A., Uncoupling of inflammatory chemokine receptors by IL-10: generation of functional decoys. *Nat. Immunol.* 2000, **1**: 387-391.
14. Delovitch, T.L. and Singh, B., The nonobese diabetic mouse as a model of autoimmune diabetes: immune dysregulation gets the NOD. *Immunity* 1997, **7**: 727-738.
15. Ettinger, R.A., Liu, A.W., Nepom, G.T. and Kwok, W.W., Beta 57-Asp plays an essential role in the unique SDS stability of HLA-DQA1*0102/DQB1*0602 alpha beta protein dimer, the class II MHC allele associated with protection from insulin-dependent diabetes mellitus. *J. Immunol.* 2000, **165**: 3232-3238.
16. Feili-Hariri, M., Kao, H., Mietzner, T.A. and Morel, P.A., Functional consequences of the binding of MHC class II-derived peptides to MHC class II. *Int. Immunol.* 1996, **8**: 1857-1865.
17. Gombert, J.M., Herbelin, A., Tancrede-Bohin, E., Dy, M., Carnaud, C. and Bach, J.F., Early quantitative and functional deficiency of $NK1^+$-like thymocytes in the NOD mouse. *Eur. J. Immunol.* 1996, **26**: 2989-2998.
18. Gorelik, L., Constant, S. and Flavell, R.A., Mechanism of transforming growth factor beta-induced inhibition of T helper type 1 differentiation. *J. Exp. Med.* 2002, **195**: 1499-1505.
19. Gorelik, L. and Flavell, R.A., Transforming growth factor-beta in T cell biology. *Nat. Rev. Immunol.* 2002, **2**: 46-53.
20. Grossman, Z. and Paul, W.E., Adaptive cellular interactions in the immune system: the tunable activation threshold and the significance of subthreshold responses. *Proc. Natl. Acad. Sci. USA* 1992, **89**: 10365-10369.
21. Grossman, Z. and Singer, A., Tuning of activation thresholds explains flexibility in the selection and development of T cells in the thymus. *Proc. Natl. Acad. Sci. USA* 1996, **93**: 14747-14752.
22. Han, H.S., Jun, H.S., Utsugi, T. and Yoon, J.W., A new type of $CD4^+$ suppressor T cell completely prevents spontaneous autoimmune diabetes and recurrent diabetes in syngeneic islet-transplanted NOD mice. *J. Autoimmun.* 1996, **9**: 331-339.
23. Hattori, M., Buse, J.B., Jackson, R.A., Glimcher, L., Dorf, M.E., Minami, M., Makino, S., Moriwaki, K., Kuzuya, H., Imura, H., Strauss, W.M., Seidman, J.G. and Eisenbarth, G.S., The NOD mouse: recessive diabetogenic gene in the major histocompatibility complex. *Science* 1986, **231**: 733-735.
24. Heath, V.L., Murphy, E.E., Crain, C., Tomlinson, M.G. and O'Garra, A., TGF-beta1 down-regulates Th2 development and results in decreased IL-4-induced STAT6 activation and GATA-3 expression. *Eur. J. Immunol.* 2000, **30**: 2639-2649.
25. Horn, G.T., Bugawan, T.L., Long, C.M. and Erlich, H.A., Allelic sequence variation of the HLA-DQ loci: relationship to serology and to insulin-dependent diabetes susceptibility. *Proc. Natl. Acad. Sci. USA* 1988, **85**: 6012-6016.
26. Kishimoto, H. and Sprent, J., A defect in central tolerance in NOD mice. *Nat. Immunol.* 2001, **2**: 1025-1031.
27. Kitani, A., Chua, K., Nakamura, K. and Strober, W., Activated self-MHC-reactive T cells have the cytokine phenotype of Th3/T

regulatory cell 1 T cells. *J. Immunol.* 2000, **165**: 691-702.
28. Kwok, W.W., Domeier, M.L., Raymond, F.C., Byers, P. and Nepom, G.T., Allele-specific motifs characterize HLA-DQ interactions with a diabetes-associated peptide derived from glutamic acid decarboxylase. *J. Immunol.* 1996, **156**: 2171-2177.
29. Kwok, W.W., Reijonen, H., Falk, B.A., Koelle, D. M. and Nepom, G.T., Peptide binding affinity and pH variation establish functional thresholds for activation of HLA-DQ-restricted T cell recognition. *Hum. Immunol.* 1999, **60**: 619-626.
30. Lee, J.M., McKean, D.J. and Watts, T.H., Functional mapping of MHC class II polymorphic residues. The alpha-chain controls the specificity for binding an Ad-*versus* an A k-restricted peptide and the beta-chain region 65-67 controls T cell recognition but not peptide binding. *J. Immunol.* 1991, **146**: 2952-2959.
31. Maloy, K.J. and Powrie, F., Regulatory T cells in the control of immune pathology. *Nat. Immunol.* 2001, **2**: 816-822.
32. Marleau, A.M. and Singh, B., Myeloid dendritic cells in non-obese diabetic mice have elevated costimulatory and T helper-1-inducing abilities. *J. Autoimmun.* 2002, **19**: 23-35.
33. Miller, J.F. and Basten, A., Mechanisms of tolerance to self. *Curr. Opin. Immunol.* 1996, **8**: 815-821.
34. Moore, K.W., de Waal, M.R., Coffman, R.L. and O'Garra, A., Interleukin-10 and the interleukin-10 receptor. *Annu. Rev. Immunol.* 2001, **19**: 683-765.
35. Naquet, P., Naspetti, M. and Boyd, R., Development, organization and function of the thymic medulla in normal, immunodeficient or autoimmune mice. *Semin. Immunol.* 1999, **11**: 47-55.
36. Narumoto, K., Saibara, T., Maeda, T., Onishi, S., Hayashi, Y., Miyazaki, E., Hiroi, M., Enzan, H. and Kobayashi, N., Transforming growth factor-beta 1 derived from biliary epithelial cells may attenuate alloantigen-specific immune responses. *Transpl. Int.* 2000, **13**: 21-27.
37. Nicholson, L.B., Anderson, A.C. and Kuchroo, V.K., Tuning T cell activation threshold and effector function with cross-reactive peptide ligands. *Int. Immunol.* 2000, **12**: 205-213.
38. Pakala, S.V., Kurrer, M.O. and Katz, J.D., T helper 2 (Th2) T cells induce acute pancreatitis and diabetes in immune-compromised nonobese diabetic (NOD) mice. *J. Exp. Med.* 1997, **186**: 299-306.
39. Poulton, L.D., Smyth, M.J., Hawke, C.G., Silveira, P., Shepherd, D., Naidenko, O.V., Godfrey, D.I. and Baxter, A.G., Cytometric and functional analyses of NK and NKT cell deficiencies in NOD mice. *Int. Immunol.* 2001, **13**: 887-896.
40. Ridgway, W.M., Fasso, M. and Fathman, C.G., A new look at MHC and autoimmune disease. *Science* 1999, **284**: 749-751.
41. Ridgway, W.M. and Fathman, C.G., The association of MHC with autoimmune diseases: understanding the pathogenesis of autoimmune diabetes. *Clin. Immunol. Immunopathol.* 1998, **86**: 3-10.
42. Robey, E. and Fowlkes, B.J., Selective events in T cell development. *Annu. Rev. Immunol.* 1994, **12**: 675-705.
43. Sakaguchi, S., Regulatory T cells: key controllers of immunologic self-tolerance. *Cell* 2000, **101**: 455-458.
44. Sakurai, T., Yamada, M., Simamura, S. and Motoyoshi, K., Recombinant human macrophage-colony stimulating factor suppresses the mouse mixed lymphocyte reaction. *Cell Immunol.* 1996, **171**: 87-94.
45. Savino, W., Boitard, C., Bach, J.F. and Dardenne, M., Studies on the thymus in nonobese diabetic mouse. I. Changes in the microenvironmental compartments. *Lab. Invest.* 1991, **64**: 405-417.
46. Savino, W., Carnaud, C., Luan, J.J., Bach, J.F. and Dardenne, M., Characterization of the extracellular matrix-containing giant perivascular spaces in the NOD mouse thymus. *Diabetes* 1993, **42**: 134-140.
47. Sebzda, E., Kundig, T.M., Thomson, C.T., Aoki, K., Mak, S.Y., Mayer, J.P., Zamborelli, T., Nathenson, S.G. and Ohashi, P.S., Mature T cell reactivity altered by peptide agonist that induces positive selection. *J. Exp. Med.* 1996, **183**: 1093-1104.
48. Segall, M., HLA and genetics of IDDM. Holism *vs* reductionism? *Diabetes* 1988, **37**: 1005-1008.
49. Sheehy, M.J., Scharf, S.J., Rowe, J.R., Neme de Gimenez, M.H., Meske, L.M., Erlich, H.A. and Nepom, B.S., A diabetes-susceptible HLA haplotype is best defined by a combination of HLA-DR and -DQ alleles. *J. Clin. Invest* 1989, **83**: 830-835.
50. Shevach, E.M., Certified professionals: $CD4^{(+)}CD25^{(+)}$ suppressor T cells. *J. Exp. Med.* 2001, **193**: F41-F46.
51. Smith, K., Seddon, B., Purbhoo, M.A., Zamoyska, R., Fisher, A.G. and Merkenschlager, M., Sensory adaptation in naive peripheral CD4 T cells. *J. Exp. Med.* 2001, **194**: 1253-1261.
52. Suarez-Pinzon, W.L. and Rabinovitch, A., Approaches to type 1 diabetes prevention by intervention in cytokine immunoregulatory circuits. *Int. J. Exp. Diabetes Res.* 2001, **2**: 3-17.
53. Suri, A., Vidavsky, I., van der Drift, K., Kanagawa, O., Gross, M.L. and Unanue, E.R., In APCs, the autologous peptides selected by the diabetogenic I-Ag7 molecule are unique and determined by the amino acid changes in the P9 pocket. *J. Immunol.* 2002, **168**: 1235-1243.
54. Thomas-Vaslin, V., Damotte, D., Coltey, M., Le Douarin, N.M., Coutinho, A. and Salaun, J., Abnormal T cell selection on nod thymic epithelium is sufficient to induce autoimmune manifestations in C57BL/6 athymic nude mice. *Proc. Natl. Acad. Sci. USA* 1997, **94**: 4598-4603.
55. Thorsby, E. and Ronningen, K.S., Particular HLA-DQ molecules play a dominant role in determining susceptibility or resistance to type 1 (insulin-dependent) diabetes mellitus. *Diabetologia* 1993, **36**: 371-377.
56. Tisch, R. and McDevitt, H., Insulin-dependent diabetes mellitus. *Cell* 1996, **85**: 291-297.
57. Todd, J.A. and Bain, S.C., A practical approach to identification of susceptibility genes for IDDM. *Diabetes* 1992, **41**: 1029-1034.
58. Todd, J.A., Bell, J.I. and McDevitt, H.O., HLA-DQ beta gene contributes to susceptibility and resistance to insulin-dependent diabetes mellitus. *Nature* 1987, **329**: 599-604.
59. Undlien, D.E. and Thorsby, E., HLA associations in type 1 diabetes: merging genetics and immunology. *Trends Immunol.* 2001, **22**: 467-469.
60. Verge, C.F. and Eisenbarth, G.S., Strategies for preventing type I diabetes mellitus. *West J. Med.* 1996, **164**: 249-255.
61. Wicker, L.S., Todd, J.A. and Peterson, L.B., Genetic control of autoimmune diabetes in the NOD mouse. *Annu. Rev. Immunol.* 1995, **13**: 179-200.
62. Wilson, S.B., Kent, S.C., Patton, K.T., Orban, T., Jackson, R.A., Exley, M., Porcelli, S., Schatz, D.A., Atkinson, M.A., Balk, S.P., Strominger, J.L. and Hafler, D.A., Extreme Th1 bias of invariant Valpha24JalphaQ T cells in type 1 diabetes. *Nature* 1998, **391**: 177-181.
63. Wong, P., Barton, G.M., Forbush, K.A. and Rudensky, A.Y., Dynamic tuning of T cell reactivity by self-peptide-major histocompatibility complex ligands. *J. Exp. Med.* 2001, **193**: 1179-1187.
64. Zeller, J.C., Panoskaltsis-Mortari, A., Murphy, W.J., Ruscetti, F.W., Narula, S., Roncarolo, M.G. and Blazar, B.R., Induction of $CD4^+$ T cell alloantigen-specific hyporesponsiveness by IL-10 and TGF-beta. *J. Immunol.* 1999, **163**: 3684-3691.
65. Zuklys, S., Balciunaite, G., Agarwal, A., Fasler-Kan, E., Palmer, E. and Hollander, G.A., Normal thymic architecture and negative selection are associated with Aire expression, the gene defective in the autoimmune-polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED). *J. Immunol.* 2000, **165**: 1976-1983.