

Peptide p277 of HSP60 signals T cells: inhibition of inflammatory chemotaxis

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Keywords: chemotaxis, delayed-type hypersensitivity, toll-like receptors, type 1 diabetes mellitus

Abstract

Peptide p277 is a 24-amino acid fragment of the heat shock protein 60 molecule, first discovered to be an antigen for diabetogenic T-cell clones in non-obese diabetic (NOD) mice. Therapeutic vaccination with p277 can arrest the spontaneous diabetogenic process both in NOD mice and in humans associated with a T_{H1} to T_{H2} cytokine shift specific for the autoimmune T cells. We now report that p277 can directly signal human T cells via innate toll-like receptor (TLR)-2, leading to up-regulation of integrin-mediated adhesion to fibronectin, and inhibition of chemotaxis to the chemokine SDF-1 α *in vitro*. Resting CD45RA⁺ T cells responded to lower concentrations of p277 than resting CD45RO⁺ T cells, but activation of CD45RO⁺ T cells greatly increased their sensitivity to p277. Mouse T cells, but not macrophages, were also sensitive to the innate effects of peptide p277, and adoptive transfer of diabetes by splenic T cells from NOD mice could be inhibited by p277 treatment before transfer. Thus, T cells do respond innately to p277, and signaling by soluble p277 through TLR2 could contribute to the treatment of type 1 diabetes; p277 may stop the destruction of β cells by signaling in concert both innate and adaptive receptors on T cells.

Introduction

Peptide p277 is composed of 24 amino acids from the sequence (437–460) of the human 60-kDa heat shock protein (HSP60), with the two cysteine residues of the native sequence replaced by valines (1). Peptide p277 was detected as a target of the diabetogenic C9 clone of T cells derived from non-obese diabetic (NOD) mice spontaneously developing type 1 diabetes (2). Nevertheless, a single injection of p277 in incomplete Freund's adjuvant was found to arrest the progression of diabetes in NOD mice, associated with a shift from a T_{H1} to T_{H2} cytokine profile of specific autoimmune T cells (3). Indeed, p277 was effective even when the treatment was initiated in mice that were already clinically diabetic; p277 could be administered as a therapeutic vaccine (1).

It appeared that p277 might be relevant to human diabetes: the HLA susceptibility locus DQ8, like the NOD mouse Ia^{g7}, features a peptide-binding motif that accommodates p277 (4), and humans with type 1 diabetes manifest T-cell reactivity to HSP60 and p277 (5). Peptide p277 was administered in vegetable oil four times over a year in a randomized, placebo-controlled phase 2 study in adults newly diagnosed

with type 1 diabetes. This treatment led to preservation of residual C peptide, and was also associated with a selective T_{H1} to T_{H2} shift in the autoimmune T-cell cytokine response (6).

The functional outcome of T-cell activation by antigens is greatly influenced by activation of innate immune receptors including toll-like receptors (TLRs); ligands for innate immune receptors can orchestrate the biological consequences that follow antigen recognition (7). The present studies were done to learn whether peptide p277 might be able to activate innate receptors, and not only serve as an antigen.

The parent molecule of p277—HSP60—has been reported to function as a ligand for innate receptors, as well as an antigen for T cells or antibodies (8, 9). HSP60 has been reported to activate macrophages via TLR4 to produce IL-12, tumor necrosis factor α and other pro-inflammatory mediators (9, 10). These effects of HSP60 on macrophages have been attributed by some investigators to LPS associated with the HSP60 (11). However, we found that HSP60, apparently free of LPS, was able to directly activate T cells via TLR2 to down-regulate homing into inflammatory sites (12, 13).

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Transmitting editor: L. Steinman

Received 4 December 2005, accepted 7 July 2006

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Here we tested whether soluble peptide p277 might affect the ability of T cells to adhere to fibronectin (FN) and migrate to an inflammatory site. We also studied the effects of soluble p277 on the ability of T cells to adoptively transfer diabetes.

Methods

Reagents

The following reagents and chemicals were purchased from the sources indicated: RPMI-1640 and DMEM (Gibco BRL, Paisley, UK), FCS, HEPES buffer, antibiotics, sodium pyruvate (Kibbutz Beit-Haemek, Israel); FN (Chemicon, Temecula, CA, USA); recombinant human IL-2 (Chiron, Amsterdam, The Netherlands) and SDF-1 α (R&D Systems, Minneapolis, MN, USA). mAbs to human β 1 integrins (CD29, clone 3SS), and to their α -chain subunits α_3 , α_4 , α_5 and α_6 (clones AK7, 17C6, HP2/1 and JBSS, respectively), were obtained from Serotec (Oxford, UK) and mAbs to TLR2 or TLR4 were obtained from eBioscience (San Diego, CA, USA). Human HSP60 and HSP70 were cloned into the mammalian expression vector pCDNA3 as described (14) and cell-free expression was induced using the rabbit reticulocyte lysate expression system according to the manufacturer's instructions (Promega, WI, USA). LPS was obtained from Sigma and phosphorothioated CpG oligonucleotide was synthesized at the Weizmann Institute. Peptides were synthesized using standard Fmoc chemistry as previously described (5). The following peptides were used: p277, VLGGGVALLRVIPALDSLKPANED; the *Mycobacterium tuberculosis* homologue of p277, MT277, VAGGGVTLQAAPTLDLKLKLEGDE and p3 of HSP60, KFGA-DARALMLQGVDLLADA.

Human T cells

T cells were purified from healthy human donor peripheral blood; several donors (3–7) were compared for each experiment to control for donor variability. The cells were isolated on Ficol-Histopaque pre-packed columns and washed. B cells and monocytes were depleted as previously described (12). Next, CD3⁺ T cells were isolated by negative selection using a mouse anti-human antibody cocktail (Pan T-cell kit, Miltenyi Biotec, Germany) containing mAb to CD11b, CD16, CD19, CD36 and CD56. The antibody-labeled cells were then passed through separation columns (MidiMACS columns, Miltenyi Biotec). In a second round of purification, CD3⁺ T cells (>95% CD3⁺ T cells) were marked for negative selection with magnetically coupled mAb to CD45RA⁺ and CD45RO⁺ (Miltenyi Biotec). The purified cells (usually >97% CD45RO⁺ or CD45RA⁺ T cells) were cultured in RPMI containing antibiotics and 10% heat-inactivated FCS.

Mice

Female and male NOD/Ltj (NOD) and female C3H/HeJ (bearing non-functional, mutated TLR4) and C3HEB/FeJ (bearing functional, wild-type TLR4) mice were obtained from Jackson Laboratory (Bar Harbor, ME, USA). The mice were maintained under specific pathogen-free conditions and experiments were approved by the Institutional Animal Care and Use Committee.

Mouse spleen cells, splenic T cells and macrophages

Single-cell suspensions were prepared from the spleens of recently diabetic NOD mice. Splenic T cells were isolated by negative selection with magnetically labeled anti-mouse antibodies (mouse Pan T-cell kit; Miltenyi Biotec) and purity was verified by FACS (>96%). Peritoneal macrophages were prepared by peritoneal lavage on day 4 after administration of 2.5 cc 4% thioglycollate (Aldrich, USA) to the peritoneum. The murine macrophage cell line RAW 264.7 was obtained from the European Collection of Cell Cultures (Wiltshire, UK).

Macrophage activation

Freshly isolated peritoneal cells or RAW 264.7 cells were washed and plated at 2×10^5 per well in 96-well plates in DMEM 10% FCS overnight. The following day, the cells were washed and stimulants were added in triplicate in serum-free medium. The culture supernatants were sampled at 24 h based on previously established time-response curves. Supernatants were tested for the presence of IL-12 by sandwich ELISA (Pharmingen, San Diego, CA, USA) and compared with recombinant murine IL-12 (Pharmingen).

T-cell adhesion and migration assays

Analysis of T-cell adhesion to extracellular matrix (ECM) components was determined as previously described (12). Briefly, flat-bottom microtiter well plates were pre-coated with FN, laminin or collagen type I ($10 \mu\text{g ml}^{-1}$) and the remaining binding sites were blocked with 1% BSA. Next, ^{51}Cr -labeled T cells were re-suspended in RPMI medium supplemented with 1% HEPES buffer and 0.1% BSA (adhesion medium), pre-incubated (30 min, 37°C, 7% CO₂ in a humidified atmosphere) with peptides, and the cells were then added to the wells. The plates containing the test materials were further incubated (30 min, 37°C in a 7% CO₂ humidified atmosphere) and then gently washed. The adherent cells were lysed (1 M NaOH, 0.1% Triton X-100 in H₂O), removed and counted using a γ -counter (Packard, Downers Grove, IL, USA). The results are expressed as the mean [\pm standard deviation (SD)] percentage of bound T cells from quadruplicate wells.

Chemotaxis experiments using human CD45RA⁺ or CD45RO⁺ T cells or mouse splenic T cells were conducted using costar transwells (Cambridge, MA, USA; 6.5-mm diameter, 5- μm pore), as previously described (13). Briefly, 100 μl of chemotaxis buffer (RPMI-1640, 1% FCS) containing 2×10^5 ^{51}Cr -labeled T cells were added to the upper chamber, and 0.6 ml of chemotaxis buffer with or without SDF-1 α (125 ng ml^{-1}) were added to the bottom chamber. After 3 h, migrating (bottom chamber) and non-migrating (upper chamber) cells were collected, lysed (1 M NaOH, 0.1% Triton X-100 in H₂O) and counted using a γ -counter.

Adoptive transfer of diabetes

Pooled splenocytes or purified splenic T cells were prepared from recently diabetic female NOD mice. The cells were plated at $2 \times 10^6 \text{ ml}^{-1}$ in RPMI 10% FCS with or without the addition of $10 \mu\text{g ml}^{-1}$ of peptides p277 or MT277 and incubated overnight at 37°C 7.5% CO₂. The cells were collected and washed with PBS twice prior to intravenous injection. Male 7-week old NOD recipients were irradiated (700 rad) 24 h

before cell transfer, and divided into experimental groups. In the case of splenocyte transfer, the mice received 3×10^8 cells per mouse by intravenous injection. In the case of purified T-cell transfer, recipient mice were injected intravenously with 3×10^6 cells per mouse. Following transfer, blood glucose was measured weekly using a Beckman Glucose Analyzer II (Beckman Instruments, Brea, CA, USA) as previously described (15). A mouse was considered diabetic when it manifested a blood glucose level $>300 \text{ mg dl}^{-1}$ on two consecutive weekly readings. Survival was recorded twice weekly.

Statistical analyses

Data are expressed as the mean \pm range or SD, or standard error. Statistical comparisons of means were performed using a two-tailed unpaired Student's *t*-test. Time to diabetes and survival in the adoptive transfer groups were compared using log rank analysis carried out with the Prism 4 program (GraphPad Software, San Diego, CA, USA).

Results

Peptide p277 does not activate mouse macrophages

It has been reported that HSP60 can activate macrophages (9, 10), so we tested whether peptide p277 might also activate macrophages. Figure 1(A) shows that recombinant human HSP60 or HSP70 ($1 \mu\text{g ml}^{-1}$) did activate a mouse macrophage cell line to secrete IL-12, but the macrophage line did not respond to peptide p277 ($1 \mu\text{g ml}^{-1}$). We synthesized HSP60 using a cell-free mammalian reticulocyte lysate to avoid potential contamination with LPS or other bacterial products. Additional experiments demonstrated that whole HSP60, but not peptide p277, could activate macrophages to secrete IL-6 (data not shown). Similarly, we have not been able to detect induction by p277 of the anti-inflammatory cytokines IL-10 and IL-4 (data not shown), and free p277 in molar excess was not able to block activation of IL-12 by HSP60 (data not shown). Peritoneal macrophages too did not respond to peptide p277 (Fig. 1B), although as expected, macrophages from C3H/HeB mice bearing wild-type TLR4 responded to whole HSP60; macrophages from C3H/HeJ mice bearing mutated TLR4 did not respond. We therefore have no evidence that p277 interacts with innate receptors on macrophages. Indeed, the failure of our preparation of synthetic p277 to activate macrophages indicates that the peptide was not contaminated with LPS or with other stimulatory bacterial molecules; macrophages can serve as an exquisitely sensitive bioassay for LPS-like materials.

Peptide p277 activates adhesion of human T cells to FN

To detect innate effects of p277 on T cells, we purified T cells from the peripheral blood of healthy human donors, fractionated the T cells into CD45RA⁺ and CD45RO⁺ sub-populations (12) and incubated the T cells with various concentrations of p277. We first studied the effect of p277 on T-cell adhesion, an early event in T-cell activation that is critical to T-cell migration during inflammation. Figure 2(A) shows the effects of p277 on T-cell adhesion to FN, measured as the percentage of adherent T cells (background adhesion: $7 \pm 3\%$). It can be seen that the

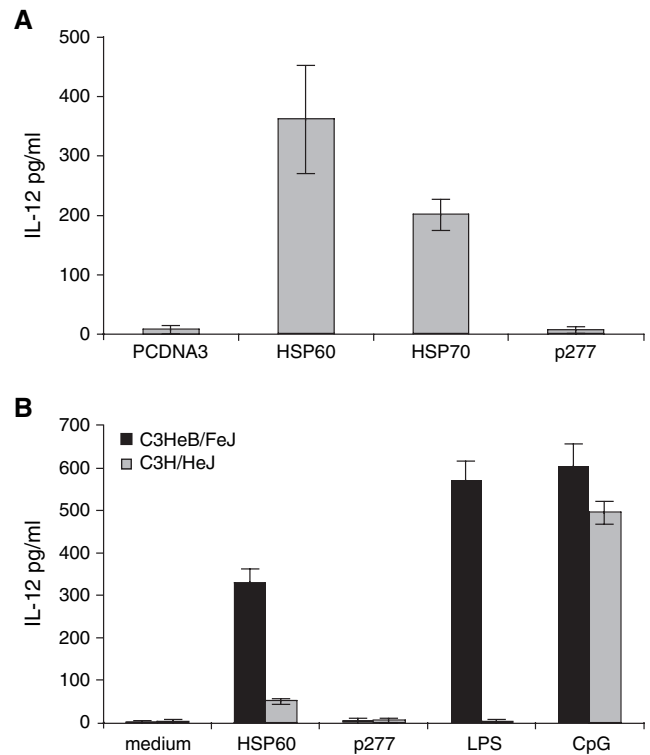


Fig. 1. p277 does not stimulate murine macrophages. (A) RAW 264.7 cells were stimulated with $1 \mu\text{g ml}^{-1}$ of synthetic peptide p277 versus HSP60 and HSP70 synthesized *in vitro* using erythrocyte lysates. The PCDNA3 vector used to express these proteins does not show an inflammatory effect. (B) Thioglycollate elicited peritoneal macrophages from C3H/HeB (bearing wild-type TLR4) and C3H/HeJ (bearing mutated TLR4) mice were stimulated for 24 h and supernatants were collected for IL-12 measurement.

CD45RA⁺ population responded to relatively low concentrations of p277 ($0.1\text{--}1.0 \text{ ng ml}^{-1}$). Adhesion of CD45RA⁺ T cells decreased at concentrations of $10\text{--}100 \text{ ng ml}^{-1}$, and then increased again at concentrations of $1000\text{--}5000 \text{ ng ml}^{-1}$ —a bell-shaped dose–response relationship similar to that observed in response to whole HSP60 (12). The CD45RO⁺ population responded mostly to the higher concentrations ($>100 \text{ ng ml}^{-1}$) of p277. However, the CD45RO⁺ population gained sensitivity to p277 following activation of the T cells by overnight incubation with IL-2; resting CD45RO⁺ T cells responded optimally to 1000 ng ml^{-1} of p277, but activating the T cells with IL-2 amplified the induction of optimal adhesion by four orders of magnitude; 0.1 ng ml^{-1} of p277 were now effective (Fig. 2B). Thus, the state of T-cell activation can determine the sensitivity of CD45RO⁺ T cells.

Human T-cell adhesion induced by p277 depends on $\beta 1$ integrins and TLR2

T-cell interactions with ECM components are mediated by $\beta 1$ integrins on the T-cell surface (16). Figure 3(A) shows that adhesion induced by p277 was significantly inhibited by mAbs to VLA4 and VLA5—the specific $\alpha 4$ and $\alpha 5$ chains of the $\beta 1$ integrins needed for adherence to FN (16). In contrast, control mAbs to VLA3 and VLA6 did not affect adhesion induced by p277. Thus, p277, like whole HSP60 (12), activates T-cell

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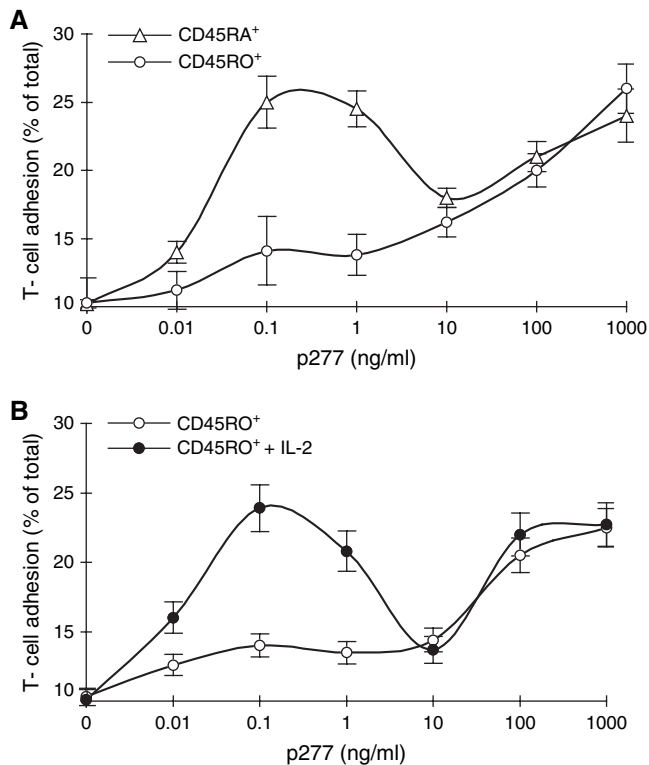


Fig. 2. p277 induces adhesion to fibronectin (FN) of CD45RA⁺ and CD45RO⁺ human T cells. (A) Purified human CD45RA⁺ and CD45RO⁺ T cells were incubated (24 h; tissue culture conditions), washed and labeled with ⁵¹[Cr]. Cells were then pre-treated (30 min) with different concentrations of p277 and seeded onto FN-coated microtiter wells for an additional 30 min. The nonadherent cells were washed away, and the remaining FN-bound cells were lysed. Radioactivity of lysates, representing the amount of FN-adherent cells, was determined. The mean \pm standard deviation (SD) of six independent experiments, using different donor bloods, is shown. (B) Purified human CD45RO⁺ T cells were incubated with or without IL-2 (10 ng ml⁻¹) overnight and then exposed to different concentrations of p277 for 30 min. Adhesion was determined as in (A). The mean \pm SD of five independent experiments is shown.

adhesion to FN by activating β 1 integrins. Control peptide p3 of HSP60 (17) was unable to enhance T-cell adherence.

Recent findings have indicated that the effects of HSP60 on T-cell functions depend on TLR2 (12). To test whether TLR2 or TLR4 might be functionally involved in the activation of T-cell adhesion by p277, we assayed the effect of pre-incubating CD45RA⁺ T cells with blocking antibodies to these TLR molecules. Figure 3(B) shows that T-cell adhesion to FN induced by p277 was inhibited by the antibody to TLR2, but not by the antibody to TLR4.

Peptide p277 inhibits human T-cell chemotaxis to SDF-1 α

The ability of T cells to navigate through the ECM into inflammatory sites depends on combined signals mediated by pro-adhesive mediators such as cytokines and chemoattractants that are associated with ECM glycoproteins and proteoglycans (18). Blocking antibodies to SDF-1 α reduce diabetes incidence in female NOD mice (19), and SDF-1 α gene variants may be associated with age of onset of type 1

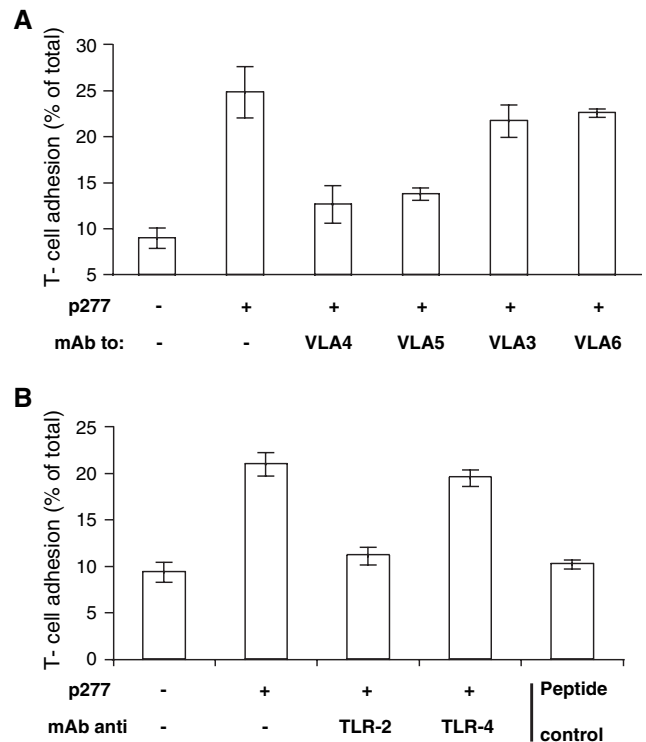


Fig. 3. p277-induced T-cell adhesion depends on β 1 integrins and TLR2, but not TLR4. (A) CD45RA⁺ T cells were pre-treated with the indicated mAb (10–15 μ g ml⁻¹) and then with p277 (0.1 ng ml⁻¹). The mean \pm standard deviation (SD) of three independent experiments performed with different donors is shown. (B). CD45RA⁺ T cells were pre-treated with anti-TLR2 or anti-TLR4 (20 μ g ml⁻¹). The cells were then treated with p277 (0.1 ng ml⁻¹), and percent adhesion to fibronectin was determined. The mean \pm SD of three independent experiments performed with different donors is shown. CD45RA⁺ T cells were treated with control peptide p3 (see text) using the same protocol as treatment with p277.

diabetes (20). These findings suggest that the chemokine SDF-1 α may play a significant role in diabetes. We previously found that T-cell migration to SDF-1 α could be inhibited by pre-incubation with HSP60 (12). Here we incubated CD45RO⁺ and CD45RA⁺ T cells with p277 for 1 h and measured chemotaxis induced by SDF-1 α across a FN-coated membrane. Figure 4(A) shows that p277 inhibited chemotaxis of CD45RA⁺ T cells with a bell-shaped dose–response curve; CD45RO⁺ T cells were less sensitive, but activation of CD45RO⁺ T cells by IL-2 increased their sensitivity to p277 (data not shown). Thus, concentrations of p277 that induced T-cell adhesion (see Fig. 2) also inhibited T-cell migration towards SDF-1 α .

Peptide p277 inhibits mouse T-cell chemotaxis in vitro

Insulinitis and the destruction of β cells involve migration to the islets of T cells that produce IFN γ and other inflammatory cytokines (21). We studied the effects of p277 on the chemotaxis of mouse T cells to SDF-1 α in the transwell assay system. Figure 4(B) shows that both p277 and its parent molecule HSP60 inhibit the migration of purified T cells from diabetic NOD female mice ($P < 0.006$ and $P < 0.02$, respectively). The *M. tuberculosis* peptide MT277, partially homologous to p277, did not affect migration.

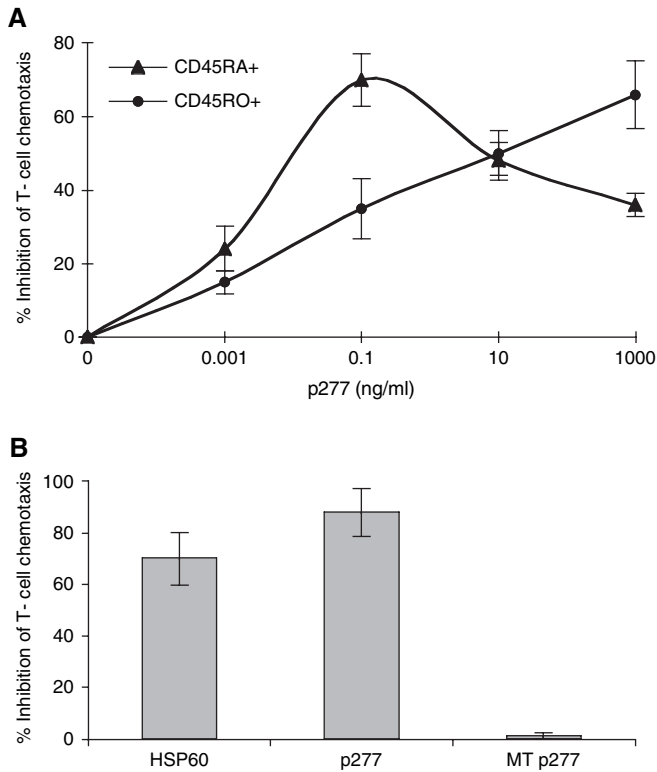


Fig. 4. p277 inhibits human and mouse T-cell migration induced by SDF-1 α . Purified human CD45RA⁺ and CD45RO⁺ T cells were treated with indicated concentrations of p277 (A), or purified splenic T cells from newly diabetic female NOD mice were treated with 0.1 ng ml⁻¹ of HSP60, p277 or MT p277 (B), washed and labeled with ⁵¹[Cr]. Cells were added to the upper chamber of transwell plates consisting of two chambers separated by a porous membrane coated with fibronectin. The lower chambers contained medium with SDF-1 α (250 ng ml⁻¹). After 3 h, the cells that had transmigrated into the lower wells were collected, centrifuged and lysed, and the radioactivity in the resulting supernatants was determined. Percent inhibition of T-cell chemotaxis was calculated as described in Methods.

Peptide p277 inhibits adoptive transfer of NOD diabetes

T cells from the spleens of diabetic NOD female mice can migrate to the islets and adoptively transfer diabetes to irradiated male NOD mice (22). Purified diabetogenic T cells (>97% purity) were incubated overnight with peptides p277 or MT277 (10 μ g ml⁻¹) before adoptive transfer to irradiated NOD males. Peptide p277 effected a significant reduction in diabetes mortality: ~40% of the mice receiving the MT277-treated T cells died of diabetes within 100 days of cell transfer; at that time, there were no deaths from diabetes in the recipients of the T cells that had been treated with peptide p277 (Fig. 5). Relative protection from diabetic mortality by p277 was preserved through day 103 ($P < 0.006$). Thus, incubation with soluble p277 *in vitro* impaired transiently the ability of purified NOD T cells to adoptively transfer diabetes *in vivo*. We also found that p277 significantly inhibited ($P < 0.0025$) the ability of the un-separated spleen cells to mediate lethal diabetes in the recipient mice (data not shown).

Discussion

Peptide p277 was first identified as a target antigen for a diabetogenic clone of T cells isolated from NOD mice (2).

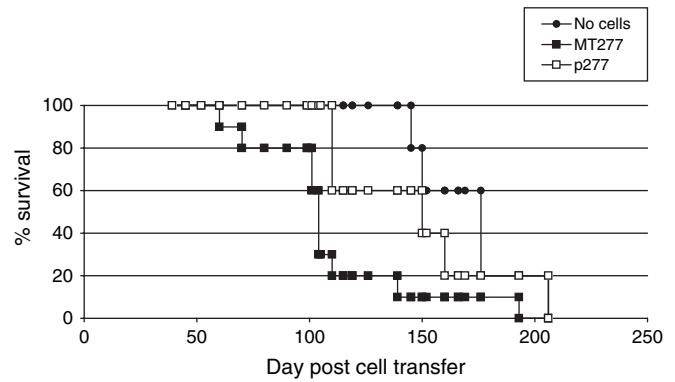


Fig. 5. *In vitro* p277 treatment inhibits adoptive transfer diabetes. Purified splenic T cells from newly diabetic NOD females were treated with p277 versus MT277 (18 h) and then washed extensively prior to transfer to irradiated male NOD mice. Mortality was significantly reduced by p277 treatment compared with MT277 ($P = 0.006$). Mortality in the p277-treated group did not differ from background diabetes mortality in control irradiated mice treated with PBS ($P = 0.49$).

Peptide p277 as an antigen is recognized by the responding TCR when presented on MHC II molecules by antigen-presenting cells (APC). Despite being a target antigen, a single administration of p277 in adjuvant aborted the diabetogenic process in NOD mice (1). A shift of the autoimmune process from a damaging T_H1 type to a suppressive T_H2 type of cytokine profile seemed to account for the therapeutic effect of p277 in NOD mice (3). A phase 2 clinical trial in new onset human disease also showed preservation of β -cell function associated with a specific T_H2 shift (6). Both in mice and in humans, the effects of p277 on immune function were immunologically specific; there was no modification of T_H1 immunity to foreign antigens (6, 23). Specific immunological memory was also evident in the persistence of the T_H2 shift after administration of the short-lived p277 peptide.

The results reported here show that p277, like its parent HSP60 molecule, can also affect T cells by serving as a ligand that activates innate TLR2 signaling (12, 13). In contrast to presentation of p277 by APC to the TCR, the effects of p277 on innate receptors of T cells do not appear to require APC and MHC presentation; soluble peptide alone affected the purified T cells. The effect of this innate signaling reported here is to activate integrin-mediated adhesion to FN and to down-regulate T-cell chemotaxis to the inflammatory chemokine SDF-1 α . The recruitment of T lymphocytes from the blood into a target organ requires both adhesion and chemotaxis; adhesion without chemotaxis will not suffice. So the ability of soluble p277 to inhibit chemotaxis is a reasonable explanation for the ability of soluble p277 to inhibit adoptive transfer of diabetes by diabetogenic T cells from NOD mice. T-cell adhesion and chemotaxis are critical for the development of autoimmune diseases in animals (24, 25) and in humans, recently shown using an anti-integrin antibody in the treatment of multiple sclerosis (26).

It remains to be seen how p277 activates the TLR2 pathway in T cells. TLR molecules were first thought to function exclusively as receptors for pathogen molecules and not for self-molecules (27). It has been reported, however, that self-HSP60 can trigger macrophages by way of both TLR4 and

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TLR2 (9, 28, 29). However, the exquisite sensitivity of macrophages to LPS makes it difficult to rule out LPS contamination, and the activation of macrophages attributed to HSP60 might actually involve microbial molecules such as LPS (30). Nevertheless, other endogenous TLR-signaling molecules have been identified (31–33). Indeed, the innate effects of HSP60 on T cells appear to be independent of LPS (12, 13). Moreover, the p277 used in the present report was produced as a purified synthetic peptide, and so is essentially free of LPS and other bacterial products. Indeed, unlike macrophages, T cells are much more sensitive to HSP60 (10 nM) or p277 (50–100 nM; ng ml⁻¹ concentrations) than they are to LPS (roughly 100 mM; µg ml⁻¹ concentrations). Purified human T cells did not respond to bacterial variants of HSP60 (12, 13), although they did respond to very small amounts of human HSP60 (12, 13) or peptide p277, as shown here. Similarly, macrophages did not respond to peptide p277 (Fig. 1), further suggesting that p277 was free of functionally significant amounts of LPS. Thus, it is not reasonable to attribute the results reported here on T cells to LPS or other bacterial molecules.

We recently reported that denatured HSP60 was ineffective on purified human T cells (12, 13). However, the present findings indicate that HSP60-derived peptide, p277, induces T-cell adhesion and inhibits T-cell chemotaxis, as well as the whole molecule (Figs 2 and 4). A possible explanation for this phenomenon is that even after denaturation of HSP60 by boiling, the p277 epitope is not revealed, or there is some steric interference to an optimal response of T cells to denatured HSP60.

TLR molecules are not the primary receptors for binding of either HSP60 (34) or bacterial products such as LPS (35). Instead, TLR molecules are essential components of signaling complexes activated by HSP60 or LPS interacting with other, primary receptors. In the case of LPS, at least three additional components, LPS-binding protein, CD14 and MD-2, have been identified. It is not unlikely that p277 too interacts with a primary receptor, yet unknown, before activating TLR2 and additional molecules to trigger the T-cell response.

Although NOD T-cell reactivity originally led us to synthesize peptide p277 artificially (1), it would seem that a p277-like epitope must be available to the immune system *in vivo*. We have found that NOD mice spontaneously make antibodies that bind to p277 when their development of diabetes has been aborted by treatment with CpG oligonucleotide (15). Moreover, humans with new-onset type 1 diabetes also make antibodies to p277 spontaneously (Bart Roep, personal communication). Thus, p277, or something cross-reactive immunologically, must be cleaved from HSP60 or otherwise present in the body to activate B cells to make specific antibodies. Hypothetically, an internal p277 ligand could function to regulate inflammatory T-cell migration physiologically, a possibility that is under study.

Does innate activation contribute to the arrest of β-cell destruction by p277? The effect of p277—administered infrequently and subcutaneously as we have done in mice and humans—is both long-term and immunologically specific (1, 6); despite enhancement of T_H2 reactivity to diabetes-associated antigens, immunity to bacterial antigens remains in the T_H1 mode following p277 treatment (6). Innate signal-

ing, in contrast to the observed effects of p277 vaccination on the disease process, has no immunological specificity and no long-term memory.

Nevertheless, it is conceivable that the innate effects of even very small amounts of p277 might be targeted synergistically to clones of T cells active in β-cell destruction. CD45RO⁺ cells are thought to include memory T cells, and specific clones of such T cells must get activated in the course of autoimmune destruction of β cells. Certainly, vaccination with p277 would activate T cells with antigen receptors for p277. Figure 2(B) shows that CD45RO⁺ T cells markedly increased their sensitivity to p277 innate signaling when they were activated by IL-2. Thus, anti-p277 clones of T cells involved in the autoimmune process of type 1 diabetes are likely to be more sensitive to innate effects of free p277 than would be memory T cells specific for other antigens. Recently, TLR2 was reported to function as a co-stimulatory receptor for T-cell activation and cytokine secretion by pathogen-derived TLR2 ligands (36). In addition, we recently reported that HSP60 via TLR2-dependent signaling shifts the cytokine secretion profile towards T_H2 in human T cells (37). It remains to be determined whether p277 has an innate effect through TLR2 on the T_H2 cytokine switch.

Acknowledgements

I.R.C. is the incumbent of the Mauerberger Chair of Immunology and the Director of the Center for the Study of Emerging Diseases, Jerusalem. G.N. was supported by a Juvenile Diabetes Research Foundation International post-doctoral fellowship.

Abbreviations

APC	antigen-presenting cells
ECM	extracellular matrix
FN	fibronectin
HSP	heat shock protein
NOD	non-obese diabetic

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