Proc. Natl. Acad. Sci. USA
Vol. 87, pp. 1576–1580, February 1990
Medical Sciences

Induction and therapy of autoimmune diabetes in the non-obese diabetic (NOD/Lt) mouse by a 65-kDa heat shock protein

(T cells/anti-idiotypic antibodies/autoantibodies/tolerance)

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Communicated by Michael Sela, December 1, 1989 (received for review September 14, 1989)

ABSTRACT **Insulin-dependent diabetes mellitus is caused by autoimmune destruction of the insulin-producing beta cells of the pancreas. The results described here indicate that a beta-cell target antigen in non-obese diabetic (NOD/Lt) mice is a molecule cross-reactive with the 65-kDa heat shock protein (hsp65) of *Mycobacterium tuberculosis*. The onset of beta-cell destruction is associated with the spontaneous development of anti-hsp65 T lymphocytes. Subsequently hsp65 cross-reactive antigen becomes detectable in the sera of the prediabetic mice and some weeks later anti-hsp65 antibodies, anti-insulin antibodies, and anti-idiotypic antibodies to insulin antibodies become detectable. The hsp65-cross-reactive antigen, the autoantibodies, and the T-cell reactivity then decline with the development of overt insulin-dependent diabetes. The importance of hsp65 in the pathogenesis of insulin-dependent diabetes was confirmed by the ability of clones of anti-hsp65 T cells to cause insulitis and hyperglycemia in young NOD/Lt mice. Moreover, hsp65 antigen could be used either to induce diabetes or to vaccinate against diabetes, depending on the form of its administration to prediabetic NOD/Lt mice. Other antigens such as the 70-kDa heat shock protein (hsp70) had no effect on the development of diabetes.**

Type 1 or insulin-dependent diabetes mellitus (IDDM) is caused in most cases by autoimmune destruction of the insulin-producing beta cells resident in the islets of the pancreas (1). It is thought that once the autoimmune process takes root, it progresses relentlessly without causing symptoms until the number of beta cells irreversibly destroyed is so large, perhaps 90% of the beta-cell mass, that the individual suffers a derangement in glucose homeostasis and requires an exogenous supply of insulin to sustain life.

The non-obese diabetic (NOD/Lt) mouse is a useful experimental model of IDDM (1). NOD/Lt mice spontaneously develop inflammation of the islets, insulitis, beginning at 4–6 weeks of age which progresses to overt IDDM at 4–5 months of age. Autoimmune T lymphocytes would seem to be the cause of beta-cell destruction because IDDM can be adoptively transferred to very young prediabetic NOD/Lt mice with T lymphocytes from older mice (2).

Identification of target antigens recognized in the pathogenesis of IDDM is important for at least two reasons: specific antigens would facilitate the early diagnosis of preclinical IDDM and they might be used to abort the destructive autoimmune process through modification of the autoimmune response. For example, copolymer 1 (COP 1), a synthetic peptide immunologically cross-reactive with myelin basic protein has been used to alter the course of multiple sclerosis (3).

We now show that a beta-cell antigen cross-reactive with a 65-kDa heat shock protein (hsp65) of *Mycobacterium tuberculosis*, termed hsp65 cross-reactive (hsp65-CR) antigen, is involved in the pathogenesis of NOD/Lt mouse IDDM.

MATERIALS AND METHODS

Mice. The breeding nucleus of NOD/Lt mice was a gift of E. Leiter (Jackson Laboratories). The incidence of IDDM developing by 7 months of age in the colony at the Weizmann Institute is 80% for females and 40% for males.

Antigens. Cloned recombinant hsp65 antigen and control antigen from *Escherichia coli* transfected without the hsp65 gene (4) and cloned recombinant 70-kDa heat shock protein (hsp70) antigen (5) were produced at the National Institute of Public Health and Environmental Protection (Bilthoven, the Netherlands) as described. Beef insulin and bovine serum albumin were purchased from Sigma.

Immunizations. Rabbit anti-hsp65 immunoglobulin was raised by immunizing two outbred New Zealand White rabbits s.c. with 500 μg of hsp65 emulsified in an incomplete Fruend's adjuvant (IFA; Difco). The rabbits were given booster injections twice at monthly intervals with 500 μg of hsp65 in IFA. One month later the rabbits were bled and the immunoglobulin fraction of the serum was partially purified by standard precipitation in ammonium sulfate (45% of saturation at 4°C). Control rabbit immunoglobulins were prepared from sera obtained before immunization to hsp65.

Mice were immunized by a single i.p. inoculation of hsp65, hsp70, or bovine serum albumin (50 μg) in either isotonic phosphate-buffered saline (PBS) or in IFA. The development of IDDM was documented by blood glucose concentration and histological evidence of insulitis (hematoxylin/eosin and light green staining done at the Histology Laboratory of The Weizmann Institute). Grading of insulitis was done by an individual blinded to the identity of the test slides as follows: ±, an occasional perivascular mononuclear cell infiltrate in the vicinity of islets; +, most islets bordered by mononuclear cell infiltrates, but no penetration of the islet substance; ++, mononuclear cell penetration and replacement of normal islet tissue.

Blood Glucose. Blood was removed from the tail vein of individual mice at about 0900 and the concentration of glucose was measured using a Diascan glucose meter and test strips (Behringswerke). A mouse was considered to be diabetic if the blood glucose was >3 standard deviations above the mean of that measured in nondiabetic or prediabetic control mice without insulitis, ≈200 mg/dl or greater.

T-Cell Proliferation and T-Cell Clones. Spleen-cell suspensions obtained from female NOD/Lt mice were assayed for

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Abbreviations: hsp65, 65-kDa heat shock protein of *Mycobacterium tuberculosis*; hsp70, 70-kDa heat shock protein; hsp65-CR, molecule cross-reactive with hsp65; IDDM, insulin-dependent diabetes mellitus; IFA, incomplete Freund's adjuvant.

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T-cell proliferation, essentially as described for T-cell responses to thyroglobulin (6). Briefly, the cells at 1×10^6 cells per ml were incubated in triplicate or quadruplicate for 72 hr in 0.2 ml of culture medium in microtiter wells in the presence or absence of various antigens at 5 μg/ml: hsp65, hsp70, or control *E. coli* lysate. Proliferation was measured by the incorporation of [^{3}H]thymidine into DNA during the final 12 hr of incubation. The results were computed as the stimulation index: the ratio of the mean test cpm obtained in the presence of antigen to the mean background cpm obtained in the absence of antigen. Standard deviations were always <10% of the mean cpm. The backgrounds varied between 5000 and 7000 cpm.

T-cell lines were obtained by repeatedly stimulating the spleen cells with hsp65 (5 μg/ml) as described (6). Clones were isolated by limiting dilution of the line cells (7). All the clones obtained were positive for the CD4 marker of helper T cells and negative for the CD8 marker of cytotoxic/suppressor T cells, assayed as described (6, 7). The ability of the T clones to induce diabetes was tested by inoculating 1-month-old female NOD/Lt mice i.p. with 5×10^6 cells after activation for 72 hr *in vitro* with hsp65 (5 μg/ml) as described (6). The recipient mice were bled to determine their blood glucose concentrations and sacrificed to assay them for insulitis 2 weeks later.

Radioimmunoassay for Antibodies and hsp65-CR Antigen in Serum. A standard solid-phase RIA test was used (8). To detect serum antibodies, the microtiter plates were coated by incubation (50 μg/ml) with hsp65 (for anti-hsp65), insulin (for anti-insulin), or guinea pig anti-insulin positive for the DM idiotype (for anti-idiotypic antibody; refs. 8 and 9). The presence of antibodies to these antigens was detected by incubating the coated wells with test mouse sera (diluted 1:50) and developing the test with ^{125}I-labeled goat anti-mouse immunoglobulin (Amersham; 10^5 cpm per well). To detect hsp65-CR antigen, the wells were incubated with 1.5 μl of test serum diluted 1:5 and then overlayed with rabbit anti-hsp65 immunoglobulin (diluted 1:100). Binding was measured using ^{125}I-labeled goat anti-rabbit immunoglobulin (Amersham).

RESULTS

Serum hsp65-CR and Autoantibodies Precede IDDM. Because heat shock or stress proteins have been associated with autoimmune diseases (4), we looked for hsp65-CR antigen in the blood of NOD/Lt mice. A group of 18 NOD/Lt mice were bled serially for ≈200 days, beginning at 20 days of age. Fourteen of the mice eventually developed IDDM and 4 escaped disease. The sera of the individual mice were tested for hsp65-CR antigen and anti-hsp65 antibodies. In addition we tested the sera for insulin antibodies and for anti-idiotypic antibodies to anti-insulin antibodies of the DM idiotype, since these autoantibodies seem to be markers of developing

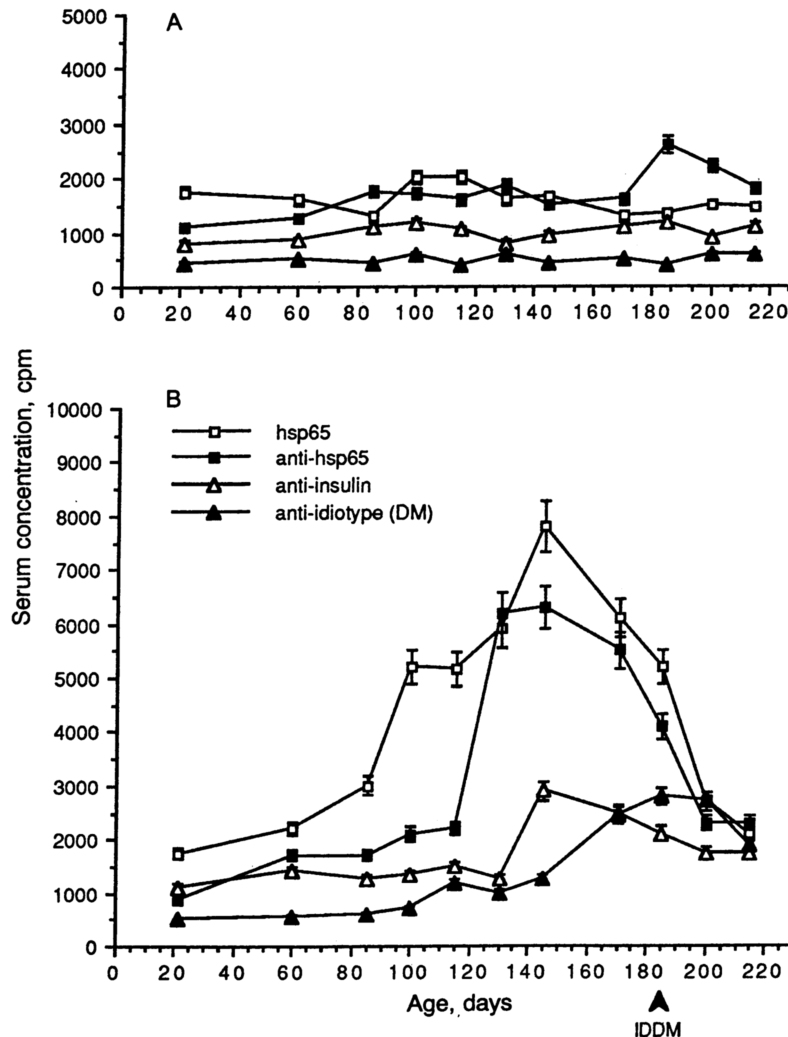

FIG. 1. Serum hsp65-CR antigen and antibodies to hsp65, to insulin, and to anti-insulin antibodies (DM) precede the development of IDDM. (*A*) NOD/Lt mouse that did not develop IDDM. (*B*) Mouse that developed clinical IDDM on day 185 of life (arrow). Data are mean ± SE.

IDDM (9). Fig. 1*B* shows the results from a mouse that developed overt IDDM at 185 days of age. Similar to the other diabetic mice this mouse showed an increasing amount of hsp65-CR antigen in the serum several months before overt IDDM. Antibodies to hsp65, antibodies to insulin, and DM anti-idiotypic antibodies appeared later. The amounts of hsp65-CR antigen and the antibodies decreased markedly as IDDM appeared. The well mouse (Fig. 1*A*) did not show these positive serum reactivities.

The mean age of onset of IDDM for all 14 diabetic NOD/Lt mice was 150.5 ± 8.3 days; hsp65-CR antigen appeared in the sera 72.5 ± 6.5 days before IDDM; anti-hsp65 preceded IDDM by 44 ± 4.3 days; anti-insulin appeared 29 ± 5.5 days before IDDM; and anti-idiotypic antibodies preceded IDDM by 19 ± 2.7 days.

Anti-hsp65 T Cells Precede IDDM. T lymphocytes cause IDDM in NOD/Lt mice (2); do NOD/Lt T lymphocytes recognize hsp65? Table 1 shows that T lymphocytes of NOD/Lt mice were negative at 1 month of age but become spontaneously reactive to hsp65 antigen at 2 months of age, the time of early insulitis and just before the appearance of hsp65-CR antigen in the serum. T-lymphocyte reactivity increased at 2.5 months, became strong at 3 months, about 2 months before the outbreak of overt IDDM, and declined just before the appearance of disease (4.5 months). The reactivity was specific for hsp65; there was no response to hsp70 or control *E. coli* antigen. Thus, insulitis and the appearance of hsp65-CR antigen in the blood is preceded by T-lymphocyte reactivity specific for hsp65.

Anti-hsp65 T Cells Cause Diabetes. To investigate whether T-lymphocyte reactivity to hsp65 could produce diabetes, we isolated clones of helper T lymphocytes from 3-month-old NOD/Lt mice specifically reactive to hsp65 by limiting dilution of an anti-hsp65 T lymphocyte line. Prediabetic NOD/Lt mice, 1.5 months old, were injected i.v. with activated clone cells and the mice were examined for diabetes. Table 2 shows that clones 21 and 26, but not clone 1, caused insulitis and a significant increase in blood glucose within 2 weeks after inoculation. In other experiments, anti-hsp65 T-cell clones were found to cause hyperglycemia in as short a time as 2–4 days after i.p. inoculation (data not shown). Thus some anti-hsp65 T lymphocytes appear to be capable of causing early diabetes in prediabetic NOD/Lt mice.

Administration of hsp65 Can Induce or Prevent Diabetes. If the hsp65-CR antigen is an important target of autoimmune attack in diabetes, then administration of hsp65 in immunogenic or nonimmunogenic forms might be expected to influence the development of IDDM. Protein antigens administered in adjuvants tend to be immunogenic, while the same antigens in soluble form are usually not immunogenic and often are tolerogenic (10). Accordingly we treated 30-day-old NOD/Lt mice with hsp65 in PBS (nonimmunogenic) or in IFA (immunogenic) and observed the mice for T-cell responses to hsp65 and for induced IDDM appearing as hyperglycemia and insulitis at age 60 days (30 days later) and for

Table 1. T-lymphocyte proliferation to hsp65 antigen precedes IDDM

	Proliferative response (stimulation index)				
Antigen	1	2	2.5	3	4.5
hsp65	1.0	2.3	3.5	7.0	2.4
hsp70	ND	ND	1.2	1.2	ND
E. coli	1.2	1.0	1.1	1.1	1.0

T-lymphocyte proliferative responses to hsp65, hsp70, and *E. coli* lysate were measured in NOD/Lt female mice at various ages (as indicated in months) before the onset of IDDM (around 5 months). The mean stimulation indices of three to five mice at each age are shown. ND, not determined.

Table 2. Clones of anti-hsp65 T lymphocytes cause diabetes

Anti-hsp65 clone	Blood glucose, mg/dl	Insulitis
None	110 ± 2	±
1	196 ± 4*	++
26	210 ± 3*	++
21	114 ± 3	±

Groups of five to seven 1-month-old female NOD/Lt mice were inoculated i.p. with 5×10^6 activated anti-hsp65 T-clone cells. Blood glucose concentration and histologic examination for insulitis were assayed 2 weeks later. ±, An occasional perivascular mononuclear cell infiltrate in the vicinity of islets; ++, mononuclear cell penetration and replacement of normal islet tissue.
*$P < 0.001$ compared to clone 21 or "none."

spontaneous IDDM appearing at ages 180 and 250 days. Control mice treated once with PBS or with the control antigen bovine serum albumin in IFA showed at 60 days of age the expected low degree of T-lymphocyte reactivity to hsp65 and no early diabetes. These mice developed the expected incidence of late spontaneous IDDM (Table 3, groups A and B). Administration of nonimmunogenic hsp65 in PBS (Table 3, group C) did not induce an increased T-lymphocyte response or diabetes at day 60. However, this treatment reduced significantly the severity of spontaneous diabetes developing later ($P < 0.001$); the diabetic mice treated with hsp65 in PBS developed blood glucose concentrations of 212–215 mg/dl compared to 439–545 mg/dl in the control mice.

Adminstration of immunogenic hsp65 in IFA (Table 3, group D) induced an augmented anti-hsp65 T-lymphocyte response and diabetes detected 30 days later by hyperglycemia (mean blood glucose = 279 mg/dl) and insulitis. However, the diabetes induced by hsp65 in IFA was transient; the mice recovered and actually resisted the development of spontaneous IDDM appearing at age 180 or 250 days. Both incidence and severity of IDDM were significantly reduced ($P < 0.01$).

Group E in Table 3 shows the effect of treating NOD/Lt mice with hsp65 in PBS followed 14 days later by hsp65 in IFA. The hsp65 antigen in PBS partially prevented the augmented T-lymphocyte response to hsp65 and protected the mice against early diabetes otherwise inducible by hsp65 in IFA; only 1 of 18 mice became diabetic compared to 12 of 15 mice given hsp65 in IFA alone ($P < 0.01$). Moreover, the combination of hsp65 in PBS followed by hsp65 in IFA vaccinated the NOD/Lt mice against the spontaneous development of IDDM at 180 and 250 days of age.

Table 4 shows that the ability of hsp65/IFA to induce acute diabetes and prevent spontaneous IDDM was specific; administration of hsp70/IFA had neither effect.

DISCUSSION

The above results indicate that T-lymphocyte immunity to a beta-cell self-antigen cross-reactive with mycobacterial hsp65 has an important function in diabetogenesis in NOD/Lt mice. T lymphocytes responding to the hsp65 antigen are detectable at the onset of insulitis and it is likely that these T lymphocytes can also recognize the beta-cell hsp65-CR antigen. Indeed, some clones of anti-hsp65 T lymphocytes can produce insulitis and hyperglycemia in very young NOD/Lt mice within several days after intravenous inoculation.

Although their functions are not understood, heat shock proteins are among the most conserved of all gene products; the human homologue of hsp65 has ≈50% homology with the mycobacterial molecule (11). The hsp65-CR antigen critical in IDDM might be a member of the heat shock protein family with tissue-specific expression in islets, or it might be an

Table 3. Effect of administering hsp65 on development of diabetes

	Treatment		Induced diabetes (day 60)			Spontaneous IDDM			
						Day 180		Day 250	
Group	First	Second	Incidence	Blood glucose, mg/dl	T-cell response to hsp65 (SI)	Incidence	Blood glucose, mg/dl	Incidence	Blood glucose, mg/dl
A	PBS	None	0/10	120 ± 20	2.2	8/10	439 ± 106	9/10	505 ± 58
B	BSA/IFA	None	0/16	118 ± 15	2.4	13/16	502 ± 77	14/16	545 ± 45
C	hsp65/PBS	None	0/14	116 ± 19	2.1	6/14	212 ± 9	7/14	215 ± 8
D	hsp65/IFA	None	12/15	279 ± 33	6.3	2/15	214 ± 8	4/15	218 ± 12
E	hsp65/PBS	hsp65/IFA	1/18	213	3.3	2/18	216 ± 12	5/18	220 ± 9

Four- to 5-week-old NOD/Lt female mice were treated by i.p. inoculation with PBS or with 50 μg of the indicated antigens in PBS or IFA. After 21 days, the mice in group E were inoculated with hsp65 in IFA. On day 60 of life, 30 days after the first treatment, the mice were studied for induced diabetes (blood glucose > 200 mg/dl). The blood glucose concentrations of groups D and E are shown for the diabetic mice only. Additional groups of four to six mice were sacrificed and their T-lymphocyte responses to hsp65 were measured as the mean stimulation index (SI). The SI of the individual mice in each group differed from the mean by no more than 0.2. Spontaneous IDDM was assayed at ages 180 and 250 days as hyperglycemia. The mean blood glucose concentrations for spontaneous IDDM were computed excluding the nondiabetic mice (blood glucose < 200 mg/dl). BSA, bovine serum albumin.

otherwise unrelated beta-cell molecule that happens to share the key epitope with hsp65. In either case, the immunologic similarity of hsp65 to the beta-cell molecule could account for the development of autoimmunity in individuals with immune response genes (12) abetting T-lymphocyte immunity to self-mimicking epitopes on cross-reactive molecules present in the environment. Homologues of hsp65 are ubiquitous among prokaryotes and eukaryotes and diabetogenic immunization need not depend on mycobacterial infection. Once activated, the specific T lymphocytes could attack and damage beta cells leading to leakage of beta-cell hsp65-CR antigen into the blood and induction of anti-hsp65 antibodies.

A 64-kDa beta-cell antigen has been detected by antibodies obtained form IDDM patients (13). However, the beta-cell molecule detected by anti-hsp65 antibodies appears to be slightly smaller (62 kDa; unpublished data) and its nature will become clearer upon determination of the full amino acid sequence.

Reactivity to the hsp65 antigen has been associated with adjuvant arthritis in rats (4) and with rheumatoid arthritis in humans (14). The hsp65 molecule features multiple T-lymphocyte epitopes (15) and it is conceivable that among them is one cross-reactive with a beta-cell antigen and another cross-reactive with an antigen in the joints. Indeed, the hsp65 epitope critical for adjuvant arthritis (amino acids 180–188) appears more homologous to a sequence in the link protein of the cartilage proteoglycan (4, 16) than it is to a sequence in the mammalian heat shock protein molecule (see ref. 11). Hence, responses to different hsp65 epitopes might lead either to arthritis or to diabetes. Further characterization of the key epitopes will help resolve the paradox of different autoimmune diseases seemingly associated with a single hsp65 antigen.

Preliminary studies indicate that spontaneously diabetic BB rats and newly diagnosed human IDDM patients also feature hsp65-CR antigen and hsp65 antibodies in the blood (unpublished data). Thus, it is conceivable that hsp65-CR antigen and/or hsp65 antibody might be used to detect persons with preclinical autoimmune beta-cell destruction.

Table 4. Effects of administrating hsp65 or hsp70 on diabetes

	Induced diabetes (day 56)		Spontaneous IDDM (day 150)	
Treatment	Incidence	Blood glucose, mg/dl	Incidence	Blood glucose, mg/dl
None	0/10	142 ± 31	5/10	411 ± 48
hsp70/IFA	0/12	135 ± 24	6/12	320 ± 31
hsp65/IFA	8/10	289 ± 20*	0/10	165 ± 15*

Groups of 5-week-old NOD/Lt female mice were or were not treated with 50 μg of hsp65 or hsp70 in IFA i.p. Twenty-one days later (day 56 of life), the mice were examined for induced diabetes (blood glucose > 200 mg/dl). Spontaneous IDDM was assayed at age 150 days.

*$P < 0.01$ compared to untreated mice.

Other antibodies whose presence we show here to be associated with developing IDDM are antibodies to insulin and anti-idiotypic antibodies to the DM idiotype. Induction of autoantibodies to insulin might be triggered by immunogenic insulin crystals leaking from damaged beta cells. Insulin autoantibodies have been detected previously in humans developing IDDM (17). Anti-idiotypic antibodies to insulin antibodies of the DM idiotype were observed to arise spontaneously in mice immunized to insulin (8). These anti-idiotypes mimic the receptor binding epitope of the insulin molecule and behave as anti-insulin receptor antibodies (18). Their presence in mice was associated with peripheral insulin resistance due to down regulation and desensitization of the insulin receptor (19). Hence these anti-idiotypic antibodies could contribute to the diabetic process.

Of particular interest was the observation that immunogenic hsp65 administered in IFA-induced anti-hsp65 T lymphocytes and a self-limited form of IDDM. Indeed, the diabetes produced by T-lymphocyte clones also may be transient (unpublished data). Thus, autoimmune T lymphocytes might effect reversible beta-cell dysfunction and not only cause beta-cell death. Moreover, it seems that an acute diabetogenic immunization may itself mobilize regulatory mechanisms capable of inhibiting spontaneous chronic autoimmune insulitis.

The effects of immunogenic hsp65 were specific; hsp70 administered in oil neither induced acute transient diabetes or prevented spontaneous IDDM. Thus, the effects on diabetes of hsp65 cannot be explained as a mere result of immunization to any bacterial hsp.

Fortunately, vaccination against chronic spontaneous IDDM was also obtainable without the penalty of acute induced IDDM; hsp65 in PBS protected the mice against the anti-hsp65 T-lymphocyte response to hsp65 in IFA, and the combination effectively aborted the chronic spontaneous disease. The nature of the immunological regulation of anti-hsp65 T lymphocytes induced by administration of hsp65 in its various forms needs extensive study. Nevertheless, the feasibility of early diagnosis and therapy of preclinical IDDM in NOD/Lt mice suggests that human IDDM too may be susceptible to early diagnosis and cure.

We thank Dr. Ayala Frenkel for raising the anti-hsp65 antibody. Mrs. Batya Fromm and Mrs. Doris Ohayon prepared the manuscript. Part of this work was supported by a grant from the Juvenile Diabetes Foundation International. Recombinant hsp65 and hsp70 were made available with support from the UNDP/World Bank/WHO Special

Programme for Research and Training in Tropical Diseases. I.R.C. is the incumbent of the Mauerberger Chair of Immunology.

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