Proc. Natl. Acad. Sci. USA
Vol. 88, pp. 3088–3091, April 1991
Medical Sciences

Vaccination against autoimmune mouse diabetes with a T-cell epitope of the human 65-kDa heat shock protein

(peptide vaccination/T-cell vaccination)

DANA ELIAS*, TAMARA RESHEF*, OHAD S. BIRK*, RUURD VAN DER ZEE†, MICHAEL D. WALKER‡, AND IRUN R. COHEN*§

Departments of *Cell Biology and ‡Biochemistry, Weizmann Institute of Science, Rehovot 76100, Israel; and †Laboratory of Bacteriology, National Institute of Public Health and Environmental Protection, Bilthoven, The Netherlands

Communicated by Michael Sela, December 27, 1990 (received for review December 3, 1990)

ABSTRACT **Insulin-dependent diabetes mellitus is caused by autoimmune destruction of the insulin-producing beta cells resident in the pancreatic islets. We recently discovered that the pathogenesis of diabetes in NOD strain mice was associated with T-cell reactivity to an antigen cross-reactive with a mycobacterial 65-kDa heat shock protein. To identify peptide epitopes critical to the insulin-dependent diabetes mellitus of NOD mice, we studied the specificities of helper T-cell clones capable of causing hyperglycemia and diabetes. We now report the identification of a functionally important peptide within the sequence of the human variant of the 65-kDa heat shock protein molecule. T-cell clones recognizing this peptide mediate insulitis and hyperglycemia. Alternatively, the T cells can be attenuated and used as therapeutic T-cell vaccines to abort the diabetogenic process. Moreover, administration of the peptide itself to NOD mice can also down-regulate immunity to the 65-kDa heat shock protein and prevent the development of diabetes. Thus, T-cell vaccination and specific peptide therapy are feasible in spontaneous autoimmune diabetes.**

Insulin-dependent diabetes mellitus (IDDM) of both humans and NOD strain mice becomes clinically overt after most of the beta cells in the islets have been destroyed by an autoimmune process (1). The destruction of the beta cells seems to be caused by autoimmune T cells (2–4) that may recognize a processed peptide antigen presented by a major histocompatibility complex (MHC) class II molecule (5).

To identify a peptide epitope important in the IDDM of NOD mice, we investigated the antigen specificity recognized by diabetogenic T-cell clones responding to the 65-kDa heat shock protein (hsp65) of *Mycobacterium tuberculosis* (MT-hsp65). We earlier reported that diabetogenic T cells recognized an epitope on this molecule (6). We now report that the target epitope is present in the sequence of the human hsp65 (H-hsp65) molecule, that the T cells responding to this epitope can therapeutically vaccinate mice against IDDM, and that the peptide epitope itself can be used to treat the disease.

MATERIALS AND METHODS

Mice. E. Leiter (The Jackson Laboratory) kindly supplied breeding nuclei of the spontaneously diabetic NOD/Lt (NOD) strain and of the nondiabetic NON.H-2^{NOD} strain. The NON.H-2^{NOD} mice were in their 11th backcross generation and were congenic at the H-2 complex with the NOD mice.

Antigens. Recombinant H-hsp65, recombinant MT-hsp65, recombinant mycobacterial 70-kDa heat shock protein (hsp70), and control *Escherichia coli* antigen were prepared as described (6–8). Control *E. coli* were transfected with the pEX2 plasmid that did not contain the hsp65 genes. The H-hsp65 gene was the gracious gift of Richard A. Young (Massachusetts Institute of Technology, Cambridge). Peptides p277 and p278 were synthesized by Ora Goldberg (Biological Services Laboratory of the Weizmann Institute of Science) with an automated synthesizer and were purified on a Biogel p-4 column (50 × 1.5 cm; Bio-Rad). The sequence of p277 residues (437–460 in the H-hsp65 sequence) is VLGGGCALLRCIPALDSLTPANED (9). The sequence of p278 residues (458–474 in the H-hsp65 sequence) is NEDQKIGIEIIKRTLKI. Bovine serum albumin (BSA) was purchased from Sigma.

T Cells and T-Cell Proliferation. T-cell clones were prepared as described (6). T-cell lines were prepared from the spleen cells of 3-month-old prediabetic female NOD mice that were unprimed or were primed by i.p. immunization with H-hsp65, hsp70, or BSA and 50 μg of antigen in incomplete Freund's adjuvant (oil). The spleen cells were raised as lines by repeated selection (three times) with specific antigen as described (10). Proliferative responses done in triplicate are shown in the tables and figure as the stimulation index (SI): ratio of cpm of [^{3}H]thymidine incorporated in spleen cells incubated with test antigen to cpm of [^{3}H]thymidine incorporated in control cultures without added antigen (6). The cpm of control cultures was 5000–7000 and the SD from the mean cpm was always <10% of the mean.

Transfer of IDDM. The ability of T cells to transfer diabetes was tested by activating them by culture with concanavalin A for 48 hr (11). Groups of prediabetic, 1-month-old female recipient mice were inoculated i.p. with 5 × 10^6 cells. The recipient mice were scored for the development of acute diabetes 7 days later manifested by glycosuria and hyperglycemia (blood glucose, >200 mg/dl; range, 220–320 mg/dl) and histologic evidence of insulitis as described (6).

T-Cell Vaccination. T-cell vaccines were constructed from the T-cell lines and clones. Before vaccination, the T cells were activated by incubation for 72 hr *in vitro* with the respective antigen (5 μg/ml) as described (6). At the end of 72 hr the T-cell blasts were isolated by centrifugation on a Ficoll/Hypaque layer. The T cells were then attenuated with γ irradiation (3000 R; 1 R = 0.258 mC/kg) as described (10). Groups of 15–25 5-week-old prediabetic NOD female mice were then left unvaccinated or were vaccinated by i.p. inoculation with 10^7 T cells. At the age of 3.5 months, 5 mice from each group were studied for the proliferative responses of their splenic T cells to H-hsp65, shown as the SI. The control cpm without added antigen was 2465 ± 235 and 2246

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: IDDM, insulin-dependent diabetes mellitus; MHC, major histocompatibility complex; BSA, bovine serum albumin; SI, stimulation index.

§To whom reprint requests should be addressed.

± 185 for unvaccinated and vaccinated mice, respectively (see Table 2). The remaining mice were bled for determination of antibodies to H-hsp65 in a solid-phase radioimmunoassay as described (6). The antibody titer signifies the cpm of binding to H-hsp65 of serum diluted 1:100. The mean cpm of anti-H-hsp65 serum antibody obtained from nondiabetic 1-month-old NOD mice was 1450 ± 194 cpm. Sera of the mice were assayed for the presence of hsp65 cross-reactive antigen by a solid-phase radioimmunoassay as described (6). Briefly, microtiter wells were coated with 50 μl of serum diluted 1:5 and then overlaid with rabbit anti-hsp65 immunoglobulin diluted 1:100. This anti-hsp65 immunoglobulin recognizes both MT-hsp65 and H-hsp65 in Western blots and binds to islets of healthy rats in immunofluorescence assays (unpublished data). Binding was measured with ^{125}I-labeled goat anti-rabbit immunoglobulin. Diabetes at 7 months was indicated by blood glucose levels of >200 mg/dl (range, 350–600 mg/dl).

Peptide Vaccination. Four-week-old female NOD mice received a single injection i.p. of 50 μg of peptide 277 or 278 emulsified in incomplete Freund's adjuvant (Difco). Some groups of mice were tested for spontaneous diabetes (insulitis and blood glucose, >200 mg/dl; range, 450–600 mg/dl) at the age of 8 months. Other groups of mice were challenged to induce diabetes 2 weeks later either by inoculation with 50 μg of MT-hsp65 emulsified in incomplete Freund's adjuvant, or with 5×10^6 activated clone C9 T cells. Induced diabetes was detected by hyperglycemia and insulitis as described (6). Control mice received an emulsion of phosphate-buffered saline in incomplete Freund's adjuvant. At the age of 3 months, two mice of each group were studied for proliferative responses of splenic T cells to H-hsp65, and all mice were bled to determine antibodies to hsp65 and the presence of hsp65 cross-reactive antigen in the blood.

RESULTS

Diabetogenic T Cells Recognize Peptide of H-hsp65. Table 1 shows that four of five clones originally selected for their responsiveness to MT-hsp65 (6) actually respond more strongly to H-hsp65 than they do to the MT-hsp65 antigen. There was no response to a control preparation of *E. coli*.

Clones C9, C7, and 27, which are relatively vigorous in their response to H-hsp65, produced hyperglycemia and insulitis within 1 week after their transfer into 4-week-old, prediabetic NOD mice. Clones 21 and 4, weak responders to H-hsp65, did not produce diabetes in recipient mice. Note that clone 4 was a strong responder to MT-hsp65.

Lines of NOD T cells responsive to other antigens such as BSA or recombinant hsp70 failed to produce diabetes (data not shown). Thus, the ability of NOD T cells to cause diabetes in young, prediabetic NOD recipients was associated with the responsiveness of the T cells to H-hsp65.

To identify the portion of the H-hsp65 molecule critical to IDDM, we cloned the hsp65 molecule from a mouse beta cell tumor and found that the T cells of NOD mice responded only to fusion fragments of H-hsp65 distal to position 437 in the sequence (O.S.B., D.E., M.D.W., and I.R., unpublished data). We therefore chose to synthesize peptide segments from position 437 to the carboxyl end of the H-hsp65 molecule. Fig. 1 shows the T-cell responses of spleen cells obtained from 3-month-old prediabetic female NOD mice to two of these peptides: p277 and p278. The amino acids at positions 437–460 in the sequence of H-hsp65 (9) constituted p277. Peptide p278 was composed of amino acids 458–474. The response to MT-hsp65 is also included in Fig. 1. The NOD splenic T cells responded strongly to p277, weakly to MT-hsp65, and not at all to p278.

Table 1 includes the responses of T-cell clones to the peptides. Diabetogenic clones 27, C7, and C9 responded strongly to p277, but not at all to p278. Clones 21 and 4 did not respond to p277, although clone 4 was a good responder to MT-hsp65. Thus the p277 peptide contains an epitope recognized by T cells capable of producing diabetes.

The key epitope in the p277 peptide appears to be contained within the 12 amino acids at positions 449–460 (PALDSLTPANED). Splenocytes of 3-month-old female NOD mice and clone C9 were assayed for proliferation. The spleen cells responded to the 12-amino acid peptide with $51 \pm 4 \times 10^3$ cpm, to the whole p277 peptide with $48 \pm 5 \times 10^3$ cpm, and to the p278 control peptide with $5 \pm 0.4 \times 10^3$ cpm; clone C9 responded to the 12-amino acid peptide with $25 \pm 2 \times 10^3$ cpm and to the p278 control peptide with $2 \pm 0.1 \times 10^3$ cpm.

Diabetes in Nondiabetic Mice. It was conceivable that NOD mice are uniquely susceptible to anti-H-hsp65 T cells because such mice are genetically programmed to develop IDDM. To test this possibility, we administered clone C9 T cells to mice of the nonobese normal NON.H-2^{NOD} strain. These mice do not develop IDDM spontaneously despite the fact that the H-2^{NOD} alleles of NOD mice have been bred into them. The NON.H-2^{NOD} mice therefore serve as MHC-compatible recipients of NOD T cells. Intraperitoneal injections of activated C9 cells (5×10^6 cells) produced hyperglycemia (the blood glucose ranged from 253 to 355 mg/dl) and insulitis within 1 week in six of eight NON.H-2^{NOD} mice. Thus, anti-H-hsp65 T

Table 1. T-cell clones recognizing H-hsp65 and its p277 peptide cause diabetes

T-cell clone	Proliferative response, SI: To antigen H-hsp65	To antigen MT-hsp65	To control *E. coli*	To peptide p277	To peptide p278	Transferred diabetes, incidence
27	16.9	7.2	0.9	11.3	1.0	9/11
C7	23.8	6.7	1.1	8.5	1.5	10/12
C9	38.5	5.8	1.2	20.0	1.4	10/15
21	5.3	2.8	1.0	1.2	1.1	0/13
4	3.1	7.5	1.1	1.5	1.3	0/15

T-cell clones were tested for their proliferative responses. The responses of clones 27, C1, C9, and 21 to H-hsp65 were significantly greater ($P < 0.001$) than their responses to MT-hsp65 or to the *E. coli* control by Student's *t* test. The diabetogenic clones 27, C1, and C9 responded significantly more to peptide p277 than they did to peptide p278 ($P < 0.001$).

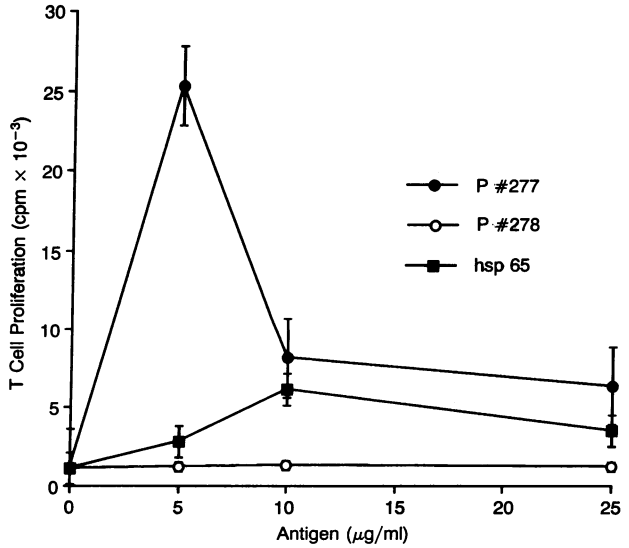

FIG. 1. T-cell responses to synthetic peptides. MT-hsp65 and the peptides p277 and p278 were tested in a T-cell proliferation assay with spleen cells of 3-month-old female NOD mice.

Table 2. T-cell vaccination against anti-H-hsp65 immunity and inhibition of diabetes

T-cell vaccine		Anti-H-hsp65 immunity at 3.5 months			Incidence of diabetes at 7 months
Attenuated T cells	Specificity	T cell, SI	Antibody titer, cpm ± SD × 10^{-3}	Blood hsp65, cpm ± SD × 10^{-3}	
None	—	11.3	4.9 ± 1.5	3.8 ± 0.7	16/20
Spleen	hsp65	2.5*	1.5 ± 0.3*	0.9 ± 0.2*	1/15*
Spleen	hsp70	10.5	5.2 ± 1.4	4.2 ± 1.1	15/20
Spleen	BSA	9.3	4.7 ± 1.5	4.5 ± 0.9	7/10
Clone C9	hsp65	1.7*	1.1 ± 0.4*	0.7 ± 0.3*	0/10*
Clone 21	hsp65	8.7	3.9 ± 0.8	3.7 ± 0.8	8/10

T-cell vaccines were prepared from T-cell clones or from splenic T-cell lines and 1-month-old female NOD mice were vaccinated i.p. with 1×10^7 irradiated T cells. T-cell and antibody immunity to H-hsp65 and blood hsp65 were measured at 3.5 months, and diabetes was scored at 7 months.
*Differed significantly by Student's t test from the other groups ($P < 0.01$).

cells can produce diabetes even in mice that do not develop IDDM spontaneously. Moreover, active immunization of NON.H-2^{NOD} with H-hsp65 (50 μg in oil given once i.p.) induced hyperglycemia (blood glucose, 250–380 mg/dl) and insulitis, detected 3 weeks later in five of five NON.H-2^{NOD} mice. Hence, H-hsp65 is diabetogenic even in mice that are not programmed to develop IDDM spontaneously.

T-Cell Vaccination. We previously reported that autoimmune helper T cells, attenuated to blunt their virulence, may be used as T-cell vaccines to prevent or treat experimental autoimmune encephalomyelitis (12), experimental autoimmune thyroiditis (13), or adjuvant arthritis (14). Therefore, we irradiated (3000 R) activated clones C9 or 21, or activated cells from lines responsive to the antigens H-hsp65, hsp70, or BSA, and administered the cells to prediabetic NOD mice. Table 2 shows that attenuated anti-H-hsp65 cells or clone C9 T cells successfully vaccinated the NOD mice against spontaneous diabetes measured at the age of 7 months. Inhibition of diabetes was accompanied by inhibition at 3.5 months of age of the spontaneous immune responses to hsp65 characteristic of NOD mice developing IDDM (6). The amounts of anti-H-hsp65 T cells and antibodies were significantly reduced. In addition, there was a decrease in hsp65 antigen appearing spontaneously in the blood of the vaccinated mice. It was shown previously that the destruction of beta cells is associated with the appearance in blood of endogenous hsp65 (6). Therefore, the decrease in endogenous hsp65 probably resulted from a decrease in destruction of beta cells.

Various manipulations of the immune system unrelated to specific antigens have been shown to interfere with the development of IDDM in NOD mice (reviewed in ref. 1): transplantation of bone marrow, neonatal thymectomy, administration of interleukin 2, or treatment with antibodies to T cells, with antibodies to MHC antigens, or with drugs such as cyclosporine or nicotinamide. In contrast to these manipulations, T-cell vaccination appears to be immunologically specific. Only T cells strongly reactive to hsp65 successfully vaccinated the mice against IDDM. Ineffective were clones 21 or 4, weakly reactive to H-hsp65 (see Table 1), or T-cell lines strongly responsive to BSA or to hsp70.

Peptide Vaccination. If the immune response to the p277 epitope is critical to the autoimmune process of NOD mice, it might be possible to either cause or prevent diabetes by administering the p277 peptide itself. Accordingly, we treated groups of seven 1-month-old prediabetic NOD mice with 50 μg of p277 or p278 in incomplete Freund's adjuvant (oil) and measured the blood glucose 3 weeks later. None of the mice manifested a glucose concentration of >130 mg/dl. In other words, the peptides themselves were not diabetogenic.

However, administration of peptide p277 protected the NOD mice against each of three types of diabetes; that induced by active immunization to MT-hsp65, that produced by adoptive transfer of virulent T-cell clones, and that appearing spontaneously by 8 months of age (Table 3). Peptide p278 had no effect. Similar to the results with T-cell vaccination, inhibition of spontaneous IDDM by administration of p277 was associated with a decrease in spontaneous T-cell and antibody reactivity to H-hsp65 and with a disappearance of blood hsp65. Thus, peptide p277 could vaccinate NOD mice against diabetes.

DISCUSSION

The earlier observation of the involvement of MT-hsp65 in IDDM (6) can now be explained by cross-reactivity of MT-hsp65 with the H-hsp65 molecule. The mouse hsp65 molecule we have cloned has ≈97% homology at the amino acid level (90% at the DNA level) with the H-hsp65 molecule and only one amino acid difference in the p277 sequence (K for T at position 455). Hence, the immune response to H-hsp65 probably reflects the autoimmune response of the mouse to its own hsp65 (unpublished data).

Targeting of anti-H-hsp65 T cells to the islets could be explained by local expression of hsp65, either constitutive or associated with beta cell stress genetically programmed in NOD mice. However, the fact that clone C9 caused diabetes in NON.H-2^{NOD} mice, which do not develop IDDM spontaneously, suggests that beta cell stress may not be required for anti-hsp65 T cells to find and attack the islets. This implies that healthy islets may constitutively express the necessary

Table 3. Vaccination with peptide p277 induces resistance to IDDM

Peptide vaccination	Incidence of diabetes			Anti-hsp65 immunity at 3 months		
	Induced by MT-hsp65	Transferred by clone C9	Spontaneous at 8 months	T cell, SI	Antibody titer, cpm ± SD × 10^{-3}	Blood hsp65, cpm ± SD × 10^{-3}
None	8/10	10/15	8/10	10.6	5.1 ± 1.2	4.2 ± 1.2
p278	6/7	8/10	8/10	9.2	4.8 ± 0.9	3.9 ± 1
p277	0/7*	0/10*	4/13*	2.1*	2.1 ± 0.2*	1.1 ± 0.2*

One-month-old female NOD mice were inoculated i.p. with p278 or p277 (50 μg) in incomplete Freund's adjuvant. Two weeks later, the mice were challenged with MT-hsp65 in oil to induce active diabetes (6) or were recipients of activated C9 T-cell clone cells (5×10^6 cells i.p.) to induce transferred diabetes. Anti-hsp65 immunity and blood hsp65 were measured at 3 months. Spontaneous diabetes was scored at 8 months.
*Differed significantly by Student's t test from the other groups ($P < 0.01$).

amount of hsp65. Alternatively, it is conceivable that during evolution the segment of the hsp65 gene encoding the p277 epitope has been duplicated and used as part of a non-hsp molecule with a specific function in beta cells. An example of the appropriation of hsp genes for non-hsp functions is the use of an hsp gene to construct the lens crystallin of the eye (15). How the p277 epitope is processed and presented by the MHC class II molecules in the islets is unknown.

Serologic studies have demonstrated an association between IDDM and antibodies to the enzyme glutamic acid decarboxylase of molecular mass 64 kDa present in neurons and in beta cells (16, 17). In addition, a molecule of 38 kDa identified in secretory granules of beta cells was found to be recognized by T cells of IDDM patients (18). Neither the 64- nor the 38-kDa molecule has been tested functionally. We do not know whether they contain an epitope cross-reactive with the p277 epitope.

Immunity to hsp65 has previously been demonstrated in arthritis: anti-MT-hsp65 T cells are associated with adjuvant arthritis in rats (8) and with rheumatoid arthritis in humans (19). Paradoxically then, two autoimmune diseases, IDDM and arthritis, may be related to hsp65 molecules, at least in mice. However, it seems that the critical epitopes are different for the two diseases. A T-cell epitope in adjuvant arthritis has been identified as the 180–188 sequence of MT-hsp65 (8), which is a variable part of the molecule; H-hsp65 lacks this peptide (see ref. 9). Consequently, rat arthritogenic clone A2b, unlike the mouse diabetogenic clones, does not respond to H-hsp65 or to p277 (unpublished data).

Irrespective of its role in the pathogenesis of diabetes, we have shown here that immunity to the p277 peptide is essential for development of NOD diabetes and that the p277 peptide can be exploited to regulate the spontaneous autoimmune process of beta cell destruction. Similarly, vaccination with a synthetic polypeptide called COP1 has been used to treat multiple sclerosis (20).

It is conceivable that vaccination with peptide p277 or with anti-p277 T cells induces antiidiotypic (21, 22) and antiergotypic T cells (23) to quell the autoimmunity responsible for IDDM. Indeed, we find that vaccination of NOD mice with attenuated clone C9 or with peptide p277 induces antiidiotypic T cells specifically responsive to clone C9 (unpublished data).

We thank Mrs. Doris Ohayon for preparing the manuscript. Parts of this study were supported by grants from the Juvenile Diabetes Foundation International, by the Minerva Foundation, and by a gift from Mr. Rowland Schaefer. Recombinant hsp65 and hsp70 were made available with support from the UNDP/World Bank/World Health Organization Special Program for Research and Training in Tropical Diseases. I.R.C. is the incumbent of the Mauerberger Chair of Immunology.

1. Castano, L. & Eisenbarth, G. S. (1990) *Annu. Rev. Immunol.* **8,** 647–679.
2. Wicker, L. S., Miller, B. J. & Mullen, Y. (1986) *Diabetes* **35,** 855–860.
3. Bendelac, A., Carnaud, C., Boitard, C. & Bach, J. F. (1987) *J. Exp. Med.* **166,** 823–832.
4. Haskins, K., Porats, M., Bergman, B., Lafferty, K. & Bradley, B. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 8000–8004.
5. Todd, J. A., Bell, J. I. & McDevitt, H. O. (1987) *Nature (London)* **329,** 599–604.
6. Elias, D., Markovits, D., Reshef, T., van der Zee, R. & Cohen, I. R. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 1576–1580.
7. Thole, J. E. R., Keulen, W. J., Kolk, A. H. J., Groothius, D. G., Berwald, L. G., Tiesjema, R. H. & van Embden, J. D. A. (1987) *Infect. Immun.* **55,** 1466–1475.
8. van Eden, W., Thole, J. E. R., van der Zee, R., Noordzij, A., van Embden, J. D. A., Hensen, E. J. & Cohen, I. R. (1988) *Nature (London)* **331,** 171–173.
9. Jindal, S., Dudani, A. K., Harley, C. B., Singh, B. & Gupta, R. S. (1989) *Mol. Cell. Biol.* **9,** 2279–2283.
10. Ben-Nun, A. & Cohen, I. R. (1982) *J. Immunol.* **129,** 303–308.
11. Naparstek, Y., Ben-Nun, A., Holoshitz, J., Reshef, T., Frenkel, A., Rosenberg, M. & Cohen, I. R. (1983) *Eur. J. Immunol.* **13,** 418–423.
12. Ben-Nun, A., Wekerle, H. & Cohen, I. R. (1981) *Nature (London)* **292,** 60–61.
13. Maron, R., Zerubavel, R., Friedman, A. & Cohen, I. R. (1983) *J. Immunol.* **131,** 2316–2322.
14. Lider, O., Karin, N., Shinitzky, M. & Cohen, I. R. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 4577–4580.
15. de Jong, W. W., Hendriks, W., Mulders, J. W. M. & Bloemendal, H. (1989) *Trends Biochem. Sci.* **14,** 365–368.
16. Baekkeskov, S., Nielson, J. H., Masner, B., Bilde, T., Ludvigsson, J. & Lernmark, A. (1982) *Nature (London)* **298,** 167–169.
17. Baekkeskov, S., Aanstoot, H.-J., Christgau, S., Reetz, A., Solimena, M., Cascalho, M., Folli, F., Richter-Olesen, H. & Camilli, P. D. (1990) *Nature (London)* **347,** 151–156.
18. Roep, B. O., Arden, S. A., de Vries, R. R. P. & Hutton, J. C. (1990) *Nature (London)* **345,** 632–634.
19. Res, P. C. M., Schaar, C. G., Breedveld, F. C., van Eden, W., van Embden, J. D. A., Cohen, I. R. & de Vries, R. R. P. (1988) *Lancet* **ii,** 478–480.
20. Bornstein, M. B., Miller, A., Slagle, S., Weitzman, M., Crystal, H., Drexler, E., Keilson, M., Merriam, A., Wassertheil-Smoller, S., Spada, V., Weiss, W., Arnon, R., Jacobsohn, I., Teitelbaum, D. & Sela, M. (1987) *N. Engl. J. Med.* **317,** 408–414.
21. Lider, O., Reshef, T., Beraud, E., Ben-Nun, A. & Cohen, I. R. (1988) *Science* **239,** 181–183.
22. Sun, D., Quin, Y., Chluba, J., Epplen, J. T. & Wekerle, H. (1988) *Nature (London)* **332,** 843–845.
23. Lohse, A. W., Mor, F., Karin, N. & Cohen, I. R. (1989) *Science* **244,** 820–822.