

T-Cell Epitope Analysis on the Autoantigen Phogrin (IA-2 β) in the Nonobese Diabetic Mouse

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The protein tyrosine phosphatases (PTPs) IA-2 and phogrin (IA-2 β) are major autoantigens in type 1 diabetes that possess common serological epitopes in their COOH termini. The epitopes recognized by the T-cells that cause the disease, however, remain to be defined. Eight phogrin-specific T-cell clones were generated from NOD mice, and their epitopes were mapped. The mapping was performed initially with recombinant glutathione S-transferase-phogrin COOH deletion constructs and ultimately with overlapping synthetic peptides. Two dominant epitopes were identified: one (aa 629–649) immediately adjacent to the transmembrane domain (aa 604–628) and the second (aa 755–777) lying in the NH₂-terminal region of the conserved PTP domain. T-cells that are specific to either of these peptides and that could destroy islet tissue in vivo through spontaneous T-cell proliferative responses were observed in prediabetic female NOD splenocytes only to the aa 755–777 epitope. In NOD female mice immunized with the epitope peptide, intramolecular determinant spreading occurred from the aa 629–649 epitope to the aa 755–777 epitope but not in the opposite direction. We concluded that the initial T-cell response to phogrin is restricted to a small number of dominant peptides and that it subsequently spreads to other regions of the molecule, including those containing the major humoral epitopes that are highly conserved between IA-2 and phogrin. *Diabetes* 50:1729–1734, 2001

IA-2 and phogrin (IA-2 β), two structurally related members of the protein tyrosine phosphatase (PTP) family, are expressed in neuroendocrine tissues, including the pancreatic islets (1,2). A role as major autoantigens in type 1 diabetes is suggested by the presence of autoantibodies to these proteins in ~70% of new-onset type 1 diabetic patients and their first-degree relatives (3). T-cell proliferative responses to IA-2 from peripheral blood mononuclear cells of diabetic patients have also been reported (4). Both proteins are comprised of a signal peptide, a 20-kDa luminal region, a transmem-

brane domain, and a 42-kDa cytosolic region, the latter incorporating a single 18-kDa PTP domain at the COOH terminus. The epitopes recognized by autoantibodies have been mapped to the cytosolic region on both antigens (5), where they are distributed throughout the conserved (79% aa identity) PTP domain and the preceding juxtamembrane segment (60% aa identity). Autoantibodies to phogrin appear to be a subset of IA-2 autoantibodies, and individuals with IA-2- or phogrin-specific autoantibodies alone are relatively rare (10 and 1% of antibody-positive sera, respectively). Studies performed with deletion constructs and IA-2/phogrin chimeras indicate the existence of determinant spreading and changes in the epitope specificity of antibodies throughout the progression of preclinical and postclinical diabetes in humans (5–7).

We generated a series of phogrin-specific T-cell clones from young NOD female mice by immunization with recombinant phogrin cytosolic region in complete Freund's adjuvant (CFA). All T-cell clones were of the CD4+/T-helper 1 phenotype, with diverse patterns of V β chain usage in their T-cell receptors (TCRs). Three of these T-cell clones, when tested in vivo, destroyed islet tissue from islet transplant recipients (8). The present study aimed to evaluate the diversity of T-cell epitopes recognized by these clones, to map the dominant determinants with synthetic peptides, and to evaluate the hierarchy of epitope responses by challenging prediabetic animals with the respective peptides. Remarkably, only two specificities were evident in the whole 42-kDa region, one of which appeared dominant, as evidenced in prediabetic NOD mice by the presence of spontaneous T-cell proliferative responses to this epitope.

RESEARCH DESIGN AND METHODS

Animals. NOD/Bdc mice and BALB/c mice were bred in the Barbara Davis Center Animal Colony, maintained under specific pathogen-free conditions, and manipulated in accordance with University of Colorado Health Sciences Center institutional animal care and use protocols.

Antigens

Recombinant phogrin. The cytosolic region of the rat phogrin molecule (aa 629–1,003) was subcloned into pGEX-3X to produce a soluble *Escherichia coli* glutathione S-transferase (GST) fusion protein (9). Fusion protein was purified with glutathione agarose affinity resin (Sigma, St. Louis, MO) and digested with factor Xa (Boehringer Mannheim, Indianapolis, IN), and the antigen was isolated by a further round of affinity chromatography (8).

Deletion constructs. A series of phogrin COOH-terminal deletion constructs were generated from the rat phogrin sequence by polymerase chain reaction using oligonucleotides that introduced both a *Bam*HI restriction site into the truncated 3' end and an in-frame *Eco*RI restriction site at the 5' end. Gel-purified *Bam*HI/*Eco*RI fragments were cloned into pGEX-3X and expressed as GST fusion proteins in *E. coli*. Soluble recombinant proteins were purified with glutathione agarose affinity resin; insoluble inclusion bodies were purified with a detergent-based purification method (10). The proteins were introduced into T-cell proliferation assays at a concentration of 100

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Received for publication 27 November 2000 and accepted in revised form 23 April 2001.

CCM, complete Click's medium; CFA, complete Freund's adjuvant; DMEM, Dulbecco's modified Eagle's medium; EAE, experimental autoimmune encephalitis; FBS, fetal bovine serum; GST, glutathione S-transferase; IL-2, interleukin-2; MHC, major histocompatibility complex; PTP, protein tyrosine phosphatase; SI, stimulation index; TCR, T-cell receptor.

TABLE 1

Proliferative responses of phogrin-specific clones to the deletion proteins A through I and to phogrin cytosolic domain (FL)

Clone	I	H	G	F	E	D	C	B	A	FL
4	1.3	1.1	1.1	6.1	6.2	6.0	6.4	4.6	6.8	4.2
6	11.0	6.4	6.7	5.7	4.5	6.0	5.3	5.8	6.3	4.9
12	1.1	1.7	1.4	8.1	6.7	8.3	7.2	8.3	5.1	8.2
13	15.0	10.0	17.3	18.0	13.0	21.0	14.0	14.5	19.0	11.6
14	1.7	1.1	1.0	17.5	20.0	21.0	27.0	20.0	11.0	21.0
15	60.0	53.0	52.0	61.0	44.0	58.0	63.0	60.0	45.0	47.0
18	3.3	1.0	3.0	67.0	51.0	84.0	58.0	58.0	60.0	53.0
19	2.2	1.5	2.7	18.0	98.0	95.0	103.0	23.0	30.0	27.0

Responses are given in SI relative to incubation without antigen.

$\mu\text{g/ml}$. Control experiments showed no nonspecific responses to either the contaminating bacterial proteins or the GST moiety.

Synthetic peptides. A series of synthetic 20mer peptides, each overlapping by 10 amino acids and spanning two distinct epitope regions on the phogrin C terminus, were obtained from BioWorld (Dublin, OH) (Table 1). They were introduced into T-cell proliferation assays as aqueous solutions at 30–100 $\mu\text{g/ml}$.

Phogrin-specific T-cell clones and lines. Phogrin-specific T-cell clones were previously obtained by limiting dilution cloning of T-cell lines established from lymph node cells of young NOD female mice primed with recombinant rat phogrin in CFA (8). Lines and clones were propagated by serial stimulation in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) (Hyclone Laboratories, Logan, UT) and 50 U/ml recombinant mouse interleukin-2 (IL-2) (DMEM/FBS/IL-2) in the presence of 2.5×10^7 irradiated NOD spleen cells (3,500 Rad) and 5 $\mu\text{g/ml}$ recombinant phogrin.

Immunization of mice with phogrin peptides. Mice were immunized with 50 μg of either peptide 2 or peptide 7 emulsified in CFA (Sigma, St. Louis, MO) (50 μl total volume) at the base of the tail. Draining lymph nodes (inguinal and periaortic) were removed 7 days postimmunization, and the dissociated cells were washed and cultured in duplicate in 96-well microculture plates (1.0×10^6 cells per well). The cells were cultured in Click's medium (Irvine Scientific, Santa Anna, CA) supplemented with 0.5% NOD mouse serum, 0.86% NaHCO_3 , 50 $\mu\text{mol/l}$ 2-mercaptoethanol, 10 mmol/l HEPES, and 50 $\mu\text{g/ml}$ gentamicin and glutamine (complete Click's medium [CCM]) as well as 10–100 $\mu\text{g/ml}$ antigen with a pulse 6 h before harvest of 0.5 μCi [^3H]thymidine. Peptide-responsive lines were maintained by in vitro stimulation of 5×10^5 T-cells with 2×10^7 irradiated NOD spleen cells in 20 ml DMEM/FBS/IL-2 on a 14-day cycle.

T-cell line and clone proliferation assays. T-cell lines and clones were assayed by coculture of 2.5×10^4 T-cells with 1.0×10^6 irradiated NOD spleen cells and 10–100 $\mu\text{g/ml}$ antigen in DMEM/FBS for 72 h with the addition of 0.5 μCi [^3H]thymidine 6 h before harvest.

Detection of spontaneous T-cell proliferative responses to phogrin peptides in NOD and BALB/c mice. A total of 1.0×10^6 spleen cells of individual female NOD and BALB/c mice (8–12 weeks of age) were cultured in duplicate for 6 days in CCM with 10–100 $\mu\text{g/ml}$ peptide. Cultures were pulsed with 0.5 μCi [^3H]thymidine 24 h before harvest. Results were evaluated in the context of individual mice. An average of 40 individual mice of both strains were screened with this assay.

TABLE 2

Synthetic 20mer peptides and the responding phogrin-specific clones

Peptides	Sequences	Clones/lines positive
Epitope region aa 629–664		
Phogrin 1 (629–649)	RHNSHYLKEKLSGLGADPS	—
Phogrin 2 (640–659)	KLSGLGADPSADATEAYQEL	6, 13, 15, 18, and 19
Phogrin 3 (650–669)	ADATEAYQELCRQRMVVRPQ	—
Epitope region aa 725–776		
Phogrin 4 (725–745)	EDHLKKNRLEKEWEALCAY	—
Phogrin 5 (735–755)	EKEWEALCAYQAEPDSSLVA	—
Phogrin 6 (745–765)	QAEPDSSLVAQREENAPKRN	—
Phogrin 7 (755–777)	QREENAPKNRSLAVLTYDHASRI	4, 12, 18, and 19

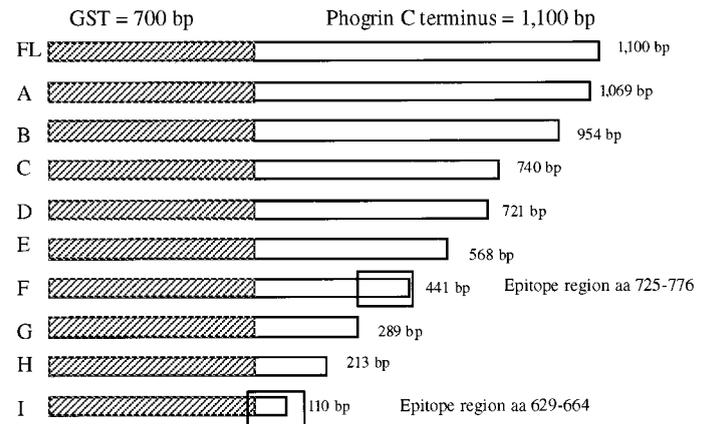


FIG. 1. Epitope regions on the phogrin COOH terminus. DNA deletion constructs A through I were derived from phogrin aa 604–1,004 using a common 5' primer and specific 3' primers inserted into *Bam*HI/*Eco*RI-cut pGEX3X with 3'-terminal stop codon. Purified recombinant GST-fusion proteins were used as antigens in T-cell proliferation assays (see Table 1) and revealed two distinct epitope regions (aa 629–664 and aa 725–776).

RESULTS

Identification of peptide epitopes of the phogrin-specific T-cell clones. COOH-terminally truncated forms of the rat phogrin cytosolic domain were produced in the form of recombinant GST fusion proteins in *E. coli* (Fig. 1). Of the nine proteins generated, two were soluble, and seven others formed insoluble inclusion bodies that could be purified by a combination of detergent extraction and differential centrifugation. T-cell clone proliferation assays using 100 $\mu\text{g/ml}$ of each construct (Table 1) revealed the presence of two distinct epitope regions, corresponding to the shortest construct (aa 629–664, deletion protein I) (Fig. 1) and the nonoverlapping region between proteins G and F (aa 725–776) (Fig. 1). All of the clones recognized the recombinant phogrin antigen, and none of them proliferated to GST alone. Insulin-specific NOD CD4+ T-cells did not respond to these proteins, indicating that the response was antigen-specific (not shown).

Synthetic 20mer peptides, each overlapping by 10 amino acids, were generated to the two epitope regions (aa 629–664 and aa 725–776) (Table 2), and proliferative responses were tested with the phogrin-specific T-cell clones and lines (Fig. 2). Three clones (6, 13, and 15) gave maximal proliferative responses to peptide 2, three other

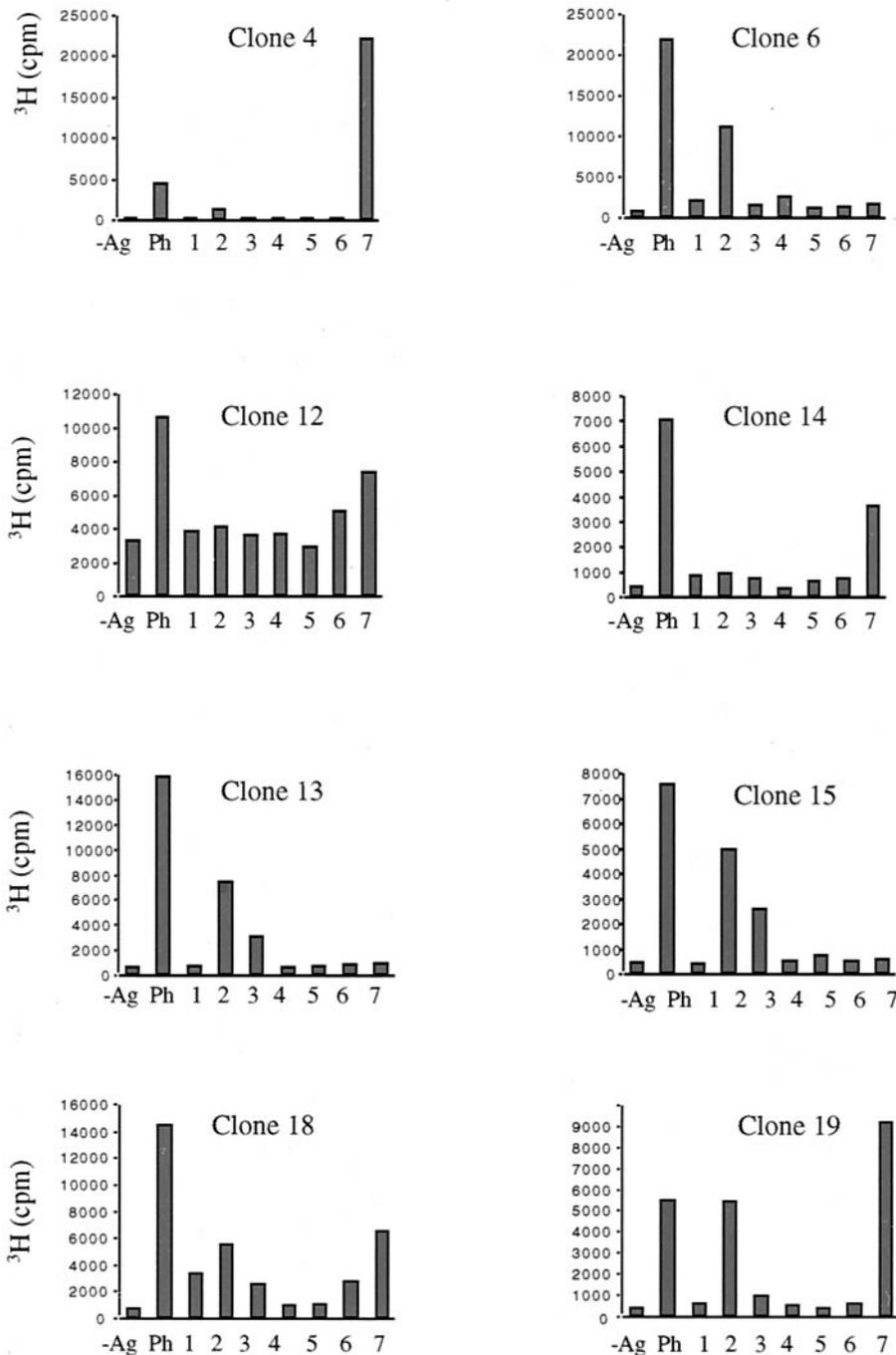


FIG. 2. Proliferative responses of phogrin-specific T-cell clones and lines to phogrin (Ph), phogrin peptides 1-7, and the absence of antigen (-Ag). Proliferation assays were performed as described in RESEARCH DESIGN AND METHODS.

clones (4, 12, and 14) gave maximal responses to peptide 7, and two clones (18 and 19) gave responses to both peptide 2 and 7. We noted that when the latter were tested with the recombinant proteins (Table 1), they retained marginal reactivity toward protein I, suggesting that they were possibly oligoclonal. As a consequence, they were not studied further. The data with both the recombinant proteins and the synthetic peptides suggest that the induced immune response to phogrin is restricted to two small regions represented by peptides 2 and 7. Peptide 2 reactivity was usually accompanied by a lesser reactivity to peptide 3, indicating that major determinants lay within the 10-amino acid overlap of the peptides (ADATEAYQEL).

Sequences of the two phogrin epitopes are conserved between rat and mouse. The phogrin-specific T-cell clones used in these studies were generated from NOD mice immunized with rat phogrin. Although rat and mouse phogrin are 95% identical in terms of amino acid sequence, there is a concern that the induced T-cell responses might focus on the residues, which differ between the two species. Both of the peptide epitopes are identical between mouse and rat. Compared with human phogrin, peptide 2 has three amino acid differences, and peptide 7 has two. IA2, which is 75% identical to phogrin over the entire COOH terminus, was more divergent in the region of peptides 2 and 7. Mouse IA-2, which shares only 8 of the 20 positions (in bold type), has an additional histidine residue

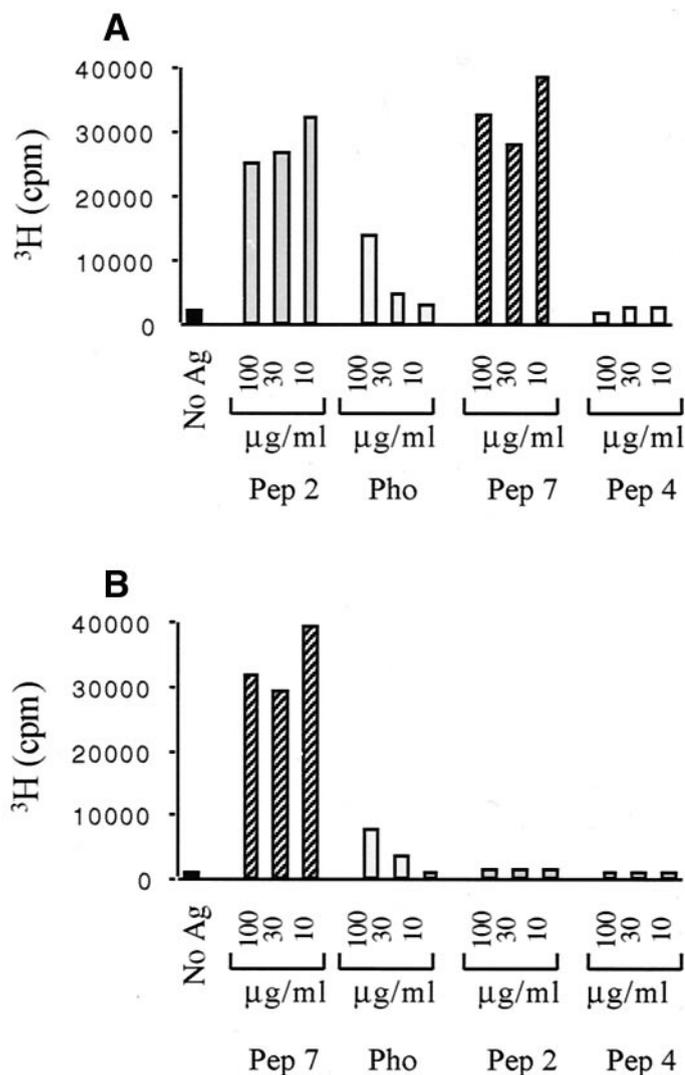


FIG. 3. Proliferative recall responses of regional lymph node cells of 12-week-old NOD mice 7 days postimmunization with peptide 2 (A) or peptide 7 (B). Proliferation assays were performed as described in RESEARCH DESIGN AND METHODS. Ag, antigen; Pep, peptide; Pho, phogrin.

in peptide 2 (RLAALGPEGAHGDTTFEYQDL); 12 of the 20 residues of mouse IA2 are shared in peptide 7 (QDESNIKKNRHPFLPYDHARI). Mouse IA-2 did not stimulate any of the above phogrin T-cell clones presented in the form of soluble recombinant COOH fragments.

Phogrin peptides 2 and 7 elicit primed T-cell proliferative responses in NOD mice. Draining lymph nodes from NOD mice immunized with peptide 2 showed vigorous T-cell proliferative responses when challenged with peptide 2, and they showed a lesser response to the phogrin COOH terminus at the same protein concentration (Fig. 3). No response to a control peptide (peptide 4) was seen, although remarkably, a vigorous response to peptide 7 was observed. When peptide 7 was used for immunization, a vigorous proliferative response to peptide 7 occurred along with a lesser response to phogrin. In this case, however, no response was seen to either peptide 2 or 4.

T-cell lines were established from the lymph nodes of these immunized mice by a round of *in vitro* restimulation and expansion. At this stage, they responded only to the respective immunogen (peptide 2 or 7), thus indicating

that the immunization with peptide 2 elicits two independent T-cell populations but that the peptides are not cross-reactive (Fig. 4).

Spontaneous T-cell proliferative responses to phogrin epitope peptides. Splenocytes from 8- to 12-week-old female NOD mice exhibited a significant spontaneous T-cell proliferative response to peptide 7 (stimulation index [SI] = 4) but not to peptide 2 or control peptide 4 (Fig. 5). BALB/c females of the same age showed no responses to any of the peptides. Results were evaluated in the context of individual mice. An average of 40 mice were screened in each strains, with ~90% of NOD and 0% of BALB/c mice showing significantly increased T-cell responses to peptide 7.

DISCUSSION

The natural history of type 1 diabetes in humans is characterized by initial serological reactivity to a limited number of antigens and epitopes, a situation that may persist without clinical sequelae for many years (11). This is followed by the emergence of autoantibodies directed at multiple autoantigens and the rapid progression to clinical

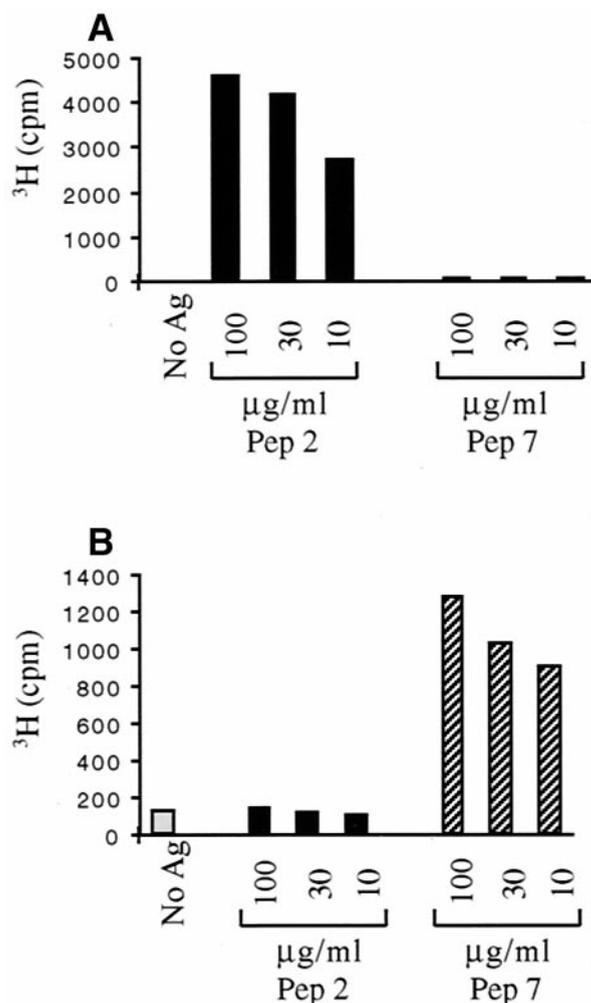


FIG. 4. Proliferative recall responses of T-cell lines generated from lymph nodes of NOD mice primed with either peptide 2 (A) or 7 (B) in CFA and maintained *in vitro* with the corresponding peptide (25 µg/ml) in DMEM/FBS/IL-2 together with irradiated NOD antigen-presenting cells. Proliferation assays were performed as described in RESEARCH DESIGN AND METHODS. Ag, antigen; Pep, peptide.

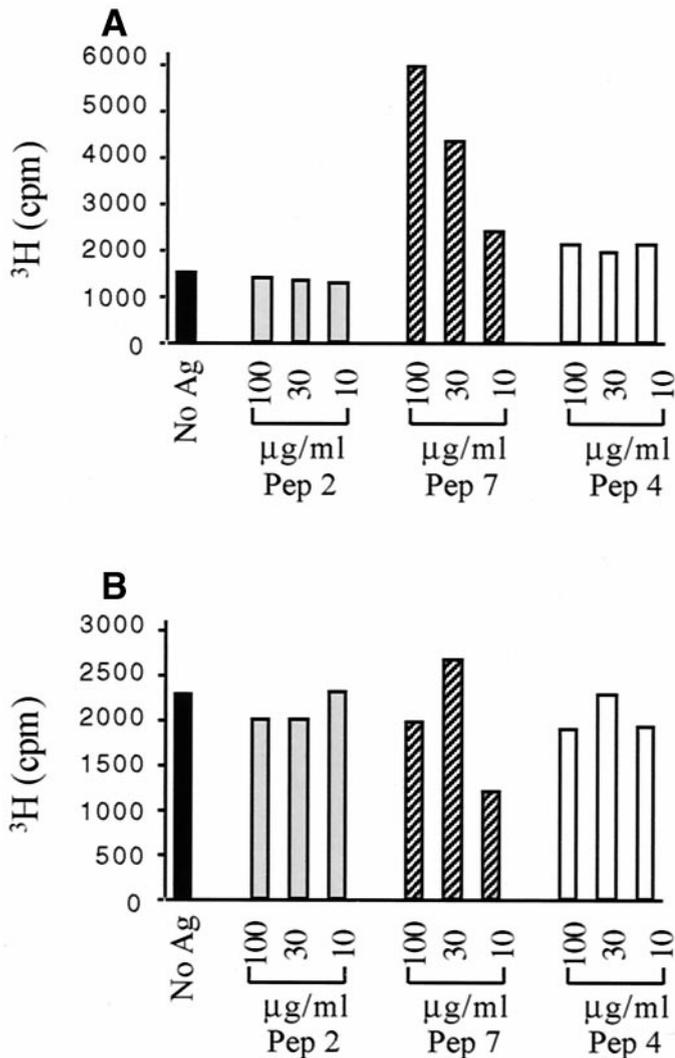


FIG. 5. Spontaneous T-cell proliferative responses of spleen cells of female NOD (A) and BALB/c mice (B) to phogrin peptides. A total of 1.0×10^6 spleen cells of individual mice were cultured in duplicate in the presence of serial dilutions of phogrin peptides or without antigen for 5 days, with a pulse of $1 \mu\text{Ci } ^3\text{H}$ thymidine per well before harvest. Ag, antigen; Pep, peptide.

disease. What happens within the effector and regulatory T-cell population is essentially unknown, especially at the level of islet infiltrates. Based on data from the NOD mouse, one surmises that disease progresses from a benign peri-islet mononuclear infiltration to an invasive destructive lesion over the course of several months or years (12,13). The major targets of humoral autoimmunity in human type 1 diabetes (i.e., insulin, GAD65, and IA-2/phogrin) also appear to be targets of cellular autoimmunity both in humans and in the NOD mouse. With regard to either insulin or GAD65, whether the same peptide epitopes are involved in human and mouse diabetes is unclear (14–18). An immunodominant epitope sequence on the IA-2 C terminus (aa 841–850) that was recognized by T-cell lines derived from peripheral blood of type 1 diabetic patients has been reported; however, a group of nondiabetic siblings responded in a similar manner (19), suggesting that it was not disease-related. Peptide sequences on IA-2 that were preferentially processed and presented in the context of the human HLA DR-4 molecule have also

been characterized (20). Neither study, however, provides any information on either the destructive potential to islet tissue carried by T-cells specific to these epitopes or their participation in spontaneous disease pathogenesis.

Our previous studies demonstrated that phogrin-specific T-cell clones derived from immunized NOD mice are capable of causing diabetes in an islet transplant model and that spontaneous T-cell reactivity to the phogrin cytosolic domain is detectable in lymph nodes draining the prediabetic NOD pancreas (8). Analysis of these T-cell clones using expressed phogrin deletion constructs proved a sensitive means of mapping the boundaries of epitopes and provided evidence for the existence of only two major epitopes. Synthetic peptides narrowed the regions to two distinct T-cell epitopes represented by peptide 2 (aa 629–649) in the first epitope region and peptide 7 (aa 755–777) in the second. The phogrin-specific T-cell clones were generated from NOD mice immunized with phogrin COOH terminus from the rat; nevertheless, the T-cell epitope sequences appeared to be 100% conserved between rat and mouse, indicating that they are potentially related to autoimmunity.

Immunization of NOD mice with either of the two epitope peptides resulted in vigorous T-cell proliferation to the epitope peptide itself and also to phogrin. Remarkably, peptide 2 immunization resulted in a response to peptide 7, but the converse was not true. Spleen cells of prediabetic NOD female mice showed spontaneous T-cell proliferative responses to peptide 7 but not to peptide 2, a control peptide (peptide 4), or phogrin itself (data not shown). The data suggest that NOD animals are not tolerant to phogrin and that more specifically, they are not tolerant to either peptide 2 or 7. It appears that an expanded population of T-cells reactive to phogrin peptide 7 exist in the prediabetic NOD mouse and are probably present at a higher precursor frequency than cells reactive to peptide 2 or any other phogrin epitope. We speculate that after immunization with peptide 2, endogenous phogrin is processed and presented, resulting in the further expansion of other autoreactive clones, notably peptide 7-reactive T-cells. Both peptides 2 and 7 bear consensus sequences for I-A^{g7} major histocompatibility complex (MHC) binding (21), and peptide 7 overlaps with a region of IA-2 that can be presented by human DR4 MHC (20). Peptide 7 is located in a region that is highly conserved between phogrin and IA-2, and the epitope itself exhibits 60% identity. IA-2-specific T-cell hybridomas generated from NOD mice after immunization with their IA-2 COOH domain also respond to this general region, although the exact epitope remains to be determined (K.K., D.R.W., J.C.H., unpublished results). We currently have no examples of crossreactive T-cell clones that respond to both phogrin and IA-2 epitopes, but we presume that these may emerge during the course of disease and, furthermore, that along with changes in the affinity of cognate TCRs (22), they contribute to progression to clinical diabetes.

Intramolecular epitope spreading has been demonstrated as an important component of pathogenicity in several models of autoimmune disease, including lupus (23), diabetes (24), and experimental autoimmune encephalitis (EAE) (25). As shown in EAE, an effect on the progress of the disease can be achieved if tolerance is induced to the determinant that appears as the last specificity at the end

of the chain of determinant spreading (26). The identification of the T-cell epitopes on the phogrin C terminus may thus facilitate the design of diabetes intervention strategies based on native epitopes or altered peptide ligands.

ACKNOWLEDGMENTS

This work was supported by grants from the Juvenile Diabetes Foundation (1-1998-222) and the Barbara Davis Center Diabetes and Endocrinology Research Center (P30 DK57516). K.K. is the recipient of an American Diabetes Association mentor-based postdoctoral fellowship.

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