Cellular Immune Response to Phogrin in the NOD Mouse

Cloned T-Cells Cause Destruction of Islet Transplants

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The ability of nonobese diabetic (NOD) mice to mount a cellular immune response to the secretory granule protein tyrosine phosphatase (PTP), phogrin was evaluated by immunization of 8- to 12-week-old animals with recombinant phogrin in complete Freund's adjuvant. Draining lymph nodes displayed a robust proliferative response to the protein, as did derived T-cell lines and clones. Ten clones obtained by limiting dilution were all CD4$^+$ and of a T-helper-1–like phenotype, but showed variation in their Vβ usage. Of the 10 clones, 3 responded to endogenous antigens in rat islets. Two of these caused the destruction of rat islets that had been transplanted under the kidney capsule of streptozotocin-treated NOD *scid* mice without affecting adjacent thyroid implants. The results demonstrate the feasibility of generating antigen-specific diabetes-inducing CD4$^+$ cells by direct immunization of NOD mice and their potential use for further studies of the antigenic epitopes in the PTP family members. The conclusion, based on serological studies, that PTP members do not play a role in the pathogenesis of type 1 diabetes in rodent models needs reevaluation in light of these findings. *Diabetes* 48:1529–1534, 1999

Insulin-dependent diabetes (type 1 diabetes) in humans and experimental models, such as the NOD mouse and the BB rat, arises from cell-mediated autoimmune destruction of the islet β-cell. Experiments in NOD and NOD *scid* mice indicate that individual CD4$^+$ T-cell clones derived from spleen or inflamed islets of diabetic animals can be sufficient to cause disease in adoptive transfer models (1–3). The emergence of spontaneous disease nevertheless depends on an interaction of CD8 and CD4 cells of both regulatory and destructive phenotypes (4–6). Few of the molecular targets of autoreactive T-cells have been identified in humans or experimental animals (7,8). It is unclear whether the antigenic targets differ between species and to what extent there is overlap with the major autoantigens identified by serological analyses, namely insulin (9), GAD65 (10), and the protein tyrosine phosphatase (PTP) family members islet cell antibody 512 (IA-2) (11–13) and phogrin (IA-2β) (14–16).

The role for the PTP members as T-cell autoantigens, in particular, has not been extensively explored, yet these molecules are expressed at much higher levels than GAD65 in islets and are, like so many of the candidate autoantigens, found exclusively in dense core secretory granules (13,17). IA-2 and phogrin were initially described as autoantibody-reactive 40- and 37-kDa tryptic fragments derived from a non-GAD 64-kDa precursor immunoprecipitated from radiolabeled β-cells (14,18). They were subsequently cloned from various tissue sources by subtractive and expression screening strategies (11,12,17). Both proteins are type 1 transmembrane receptor-like glycoproteins of similar size, domain structure, and topology and show 80 and 26% identity, respectively, in their cytosolic and lumenal domains. The major serological determinants in human type 1 diabetes are confined to the cytosolic domain and show considerable overlap (19–21). Approximately 60–70% of sera of new-onset type 1 diabetic patients (16,18,22) react to both molecules, as do 40–50% of sera from first-degree relatives starting from an early stage in the disease process. Increased proliferative T-cell responses to IA-2 have also been reported in peripheral blood of recent-onset type 1 diabetic patients and first-degree relatives (23,24).

The role of IA-2 and phogrin as autoantigenic targets in the NOD mouse is more controversial. Initial serological studies (25) discounted their importance; more recently it has been shown that antibodies directed at the cytosolic domain of IA-2 appear transiently immediately before the onset of hyperglycemia (26). Although T-cell responses to both GAD (27,28) and insulin (3) have been documented in NOD mice, similar data for IA-2 or phogrin are lacking. In the present study, we have characterized the T-cell response to phogrin in the NOD mouse in response to immunization with the COOH-terminal fragment of the molecule that bears the major antigenic epitopes identified in serological studies in humans. Phogrin-specific CD4$^+$ T-cell clones of a T-helper-1 (TH-1) phenotype were readily obtained from these animals, and were shown to recognize the endogenous antigens in islets and to mediate islet-specific destruction in vivo using transplanted NOD *scid* recipients.

RESEARCH DESIGN AND METHODS

DA rats, NOD/Bdc mice, and NOD/*scid* mice were bred in the Barbara Davis Center animal colony, maintained under specific pathogen–free or sterile conditions

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APC, antigen-presenting cell; Bt, biotin-conjugated; CFA, complete Freund's adjuvant; CMC, complete Click's medium; DMEM, Dulbecco's modified Eagle's medium; ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; GST, glutathione S-transferase; IFN-γ, γ-interferon; IL, interleukin; PTP, protein tyrosine phosphatase; SI, stimulation index; TCR, T-cell receptor; TH, T-helper.

(*scid* animals), and manipulated in accordance with University of Colorado Health Sciences Center Institutional Animal Care and Use Committee protocols. Pancreatic islets were isolated from adult DA rats by collagenase digestion (29) and Ficoll gradient purification (30) and were used directly for transplantation or dissociated into a single cell preparation with trypsin/EDTA for in vitro studies.

The cytosolic region of the rat phogrin molecule (amino acids 629–1003) was amplified by polymerase chain reaction, and the sequence was verified and subcloned into the Bam HI/Eco RI site in the expression vector pGEX-3X to produce a soluble *Escherichia coli* recombinant glutathione S-transferase (GST) fusion protein (31). Fusion protein from 500 ml bacterial culture was bound to 1 ml (bed volume) glutathione agarose affinity resin (Sigma, St. Louis, MO), washed with 10 vol of phosphate-buffered saline, and then cleaved in situ for 18 h at 4°C with biotinylated Factor Xa (Boehringer Mannheim, Indianapolis, IN) (25 µg/ml in 1 ml 50 mmol/l Tris-HCl, 0.1 mol/l NaCl, 1 mmol/l $CaCl_2$, pH 8.0). The column eluate (4 bed vol of the same buffer) was passed through glutathione agarose and Streptavidin agarose (Boehringer Mannheim) columns to remove residual GST complexes and Factor Xa, respectively. Approximately 2.5 mg of purified cleaved recombinant protein was obtained per liter of bacterial culture

Phogrin-reactive T-cells were generated from 8- to 12-week-old NOD females injected at the base of the tail with 12.5 µg of purified recombinant phogrin in complete Freund's adjuvant (CFA) (F5881; Sigma) in a total volume of 50 µl. Inguinal and periaortic lymph nodes were harvested after 1–2 weeks, and 1×10^7 lymph node cells were cultured for 6 days with 10 µg/ml phogrin in 24-well plates in Click's medium (Irvine Scientific, Santa Anna, CA) supplemented with 0.5% NOD mouse serum, 0.86% $NaHCO_3$, 50 µmol/l 2-mercaptoethanol, 10 mmol/l HEPES, 50 µg/ml gentamicin, and glutamine (complete Click's medium [CMC]), followed by a 9-day culture 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 interleukin (IL)-2 (Pharmingen, San Diego, CA). Subsequent maintenance of the phogrin-specific lines was achieved by in vitro stimulation of 5×10^5 T-cells with 2×10^7 irradiated (3,500 rad) NOD spleen cells in 20 ml DMEM/FBS/IL-2 on a 14-day cycle. Phogrin-specific T-cell clones were generated by limiting dilution in 96-well plates using average cell/well densities of 0.3, 1.0, 3.0, and 10. Wells showing T-cell growth by 14 days were expanded for subsequent analysis.

Lymph node proliferation assays were performed in duplicate with 1×10^6 freshly harvested cells cultured in 0.2 ml CMC for 6 days with threefold serial dilutions of phogrin or GST. Cultures were pulsed with 0.5 µCi [^{3}H]thymidine 24 h before harvest. T-cell lines and clones were assayed by co-culture of 2.5×10^4 T-cells with 1×10^6 irradiated (3,500 rad) NOD spleen cells and antigen in DMEM/FBS for 72 h with the addition of 0.5 µCi [^{3}H] thymidine 6 h before harvest. NOD and DA rat islet antigens were assayed using 5,000 and 50,000 islet cells/well, respectively.

Flow cytometry was performed on T-cell clones (5×10^5 cells) using fluorescein isothiocyanate (FITC)-conjugated rat anti-mouse $CD4^+$ (Pharmingen) and Cy-2–conjugated rat anti-mouse CD8 (Pharmingen) monoclonal antibodies. Selected clones were typed for T-cell receptor (TCR) Vβ chains using primary monoclonal antibodies to Vβ 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 13, 14, and 17 and detection with a secondary antibody cocktail of FITC-anti-rat Ig (Rockland, Gilbertville, PA) and FITC-anti-mouse Ig (Boehringer-Mannheim). Analyses were performed on a Coulter (Fullerton, CA) Epics Elite instrument.

Cytokine production was determined by culturing 5×10^5 T-cells with 1×10^7 irradiated (3,500 rad) NOD splenocytes and 3 µg/ml phogrin in 2 ml DMEM/FBS for 48 h. Supernatants were assayed in duplicate for γ-interferon (IFN-γ), IL-4, and IL-10 by two-site enzyme-linked immunosorbent assay (ELISA) using three different sample dilutions. Plates were coated with rat anti-mouse IFN-γ (2 µg/ml, 18181D; Pharmingen), rat anti-mouse IL-4 (0.5 µg/ml, 18191D; Pharmingen), or rat anti-mouse IL-10 (2 µg/ml, 18141D; Pharmingen) and incubated for 2 h at room temperature with sample followed by biotin-conjugated (Bt) rat anti-mouse IFN-γ (0.5 µg/ml, 18112D; Pharmingen), Bt rat anti-mouse IL-4 (0.5 µg/ml, 18042D; Pharmingen), or Bt rat anti-mouse IL-10 antibody (0.25 µg/ml, 18152D; Pharmingen), respectively. Second antibody binding was determined spectrophotometrically using streptavidin-alkaline phosphatase conjugate (Pharmingen) by monitoring the hydrolysis of p-nitrophenol phosphate (Sigma) at 405 nm. In vivo pathogenicity of the T-cell clones was examined in adoptive transfer experiments using a previously described (32) diabetes-recurrence model. NOD/*scid* males of 10–14 weeks of age were rendered diabetic with a single intravenous injection of streptozotocin (150 mg/ml) and subsequently transplanted with 450 DA rat islets under the kidney capsule to restore normoglycemia. Phogrin-specific T-cell clones were prepared for injection by culture with antigen and irradiated splenocytes for 4 days (see above), followed by expansion for 10 days in 150 ml of DMEM/FBS/IL-2. The T-cells (1×10^7) were injected intravenously in Hank's buffered saline solution, and the animals' blood glucose was monitored every 2 days (Precision Q.I.T. Sensor; Abbot Laboratories, Abbott Park, IL). Animals were killed either 3 days after diabetes onset (defined by three consecutive readings >15 mmol/l) or after 6 weeks, at termination of the experiment. Control animals received streptozotocin treatment and an islet transplant, but no T-cells. As a tissue specificity control, a number of treated and control animals also received a single DA rat thyroid lobe under the same kidney capsule as the islet. Tissue from the site of engraftment in each case was fixed in formalin, paraffin-embedded, and stained with hematoxylin-eosin, or processed for immunocytochemistry using guinea pig anti-insulin (Dako, Carpinteria, CA), rabbit anti-glucagon (Dako), or a rabbit anti-phogrin (lumenal domain) antibody produced in our laboratory. The multispecies streptavidin/peroxidase kit (Signet Laboratories, Dedham, MA) was used to detect antibody binding.

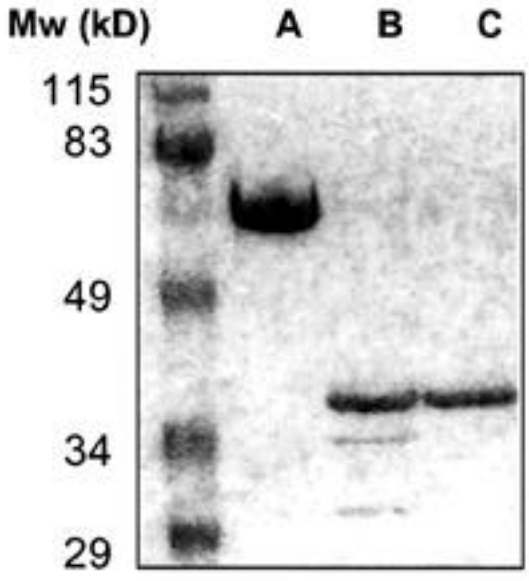

FIG. 1. Preparation of recombinant phogrin COOH-terminal. Recombinant phogrin-GST fusion protein was affinity purified on glutathione agarose (*A*), digested with Factor Xa–digested phogrin (*B*), and then subjected to an additional glutathione agarose affinity step and streptavidin agarose to remove contaminants (*C*).

RESULTS

Production of purified phogrin for use in T-cell assays. The use of recombinant fusion proteins for immunological assays can present problems relating to reactivity to the fusion partner or contaminant bacterial proteins and from the presence of mitogens. Preparations of the phogrin COOH-terminal domain using glutathione-agarose affinity chromatography and on-column cleavage with Factor Xa (Fig. 1) required additional passage through glutathione-agarose column and streptavidin chromatography to remove GST and the protease. The 42-kDa product was homogeneous on SDS-PAGE and neither elicited a proliferative response from an irrelevant insulin-responsive T-cell clone nor inhibited the clone's response to its cognate antigen. It was thus deemed to be free from mitogens or inhibitors and suitable for T-cell studies.

Spontaneous T-cell proliferative response to phogrin in NOD mice. Mesenteric lymph node cells of young NOD females (12–13 weeks of age) exhibited a pronounced T-cell proliferative response to both recombinant mouse and rat phogrin with stimulation indices of 5.3 and 8.5, respectively, at an antigen concentration of 25 µg/ml. No response was observed with GST, a control recombinant antigen, which was produced in our laboratory in an identical manner to mouse and rat phogrin and, thus, is likely to have a similar spectrum of contaminants (Fig. 2).

Proliferative response of lymph node cells from phogrin-immunized NOD mice. Draining lymph nodes of

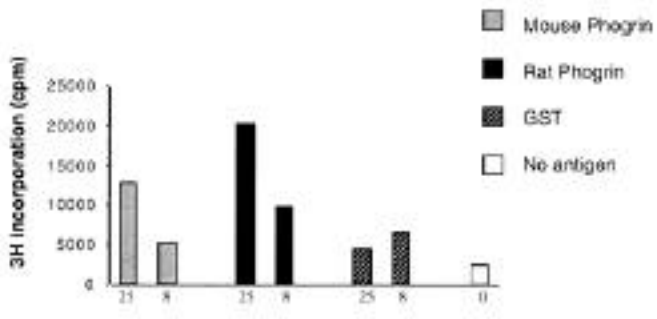

FIG. 2. Spontaneous proliferative response of mesenteric lymph node cells from a 12-week-old NOD female mouse to phogrin and GST. Lymph node cell proliferation assay was performed as described in METHODS. Briefly, 1×10^6 freshly harvested cells were cultured in 0.2 ml CMC for 6 days with threefold serial dilutions of phogrin or GST. Cultures were pulsed with 0.5 µCi [^{3}H]thymidine 24 h before harvest.

female NOD mice immunized with phogrin/CFA exhibited a robust proliferative response to recombinant phogrin with maximal responses around 25 µg/ml (stimulation index [SI] = 64) and detectable proliferation with around 0.3 µg/ml (SI = 3.8) (Fig. 3*A*). A weak response was observed to GST above 8 µg/ml (SI = 5.1), which could reflect either residual GST contamination of the immunogen or bacterial mitogen contamination. The GST was produced in the same strain of bacteria as the GST phogrin hybrid and was similarly purified by two cycles of glutathione agarose affinity chromatography, and thus was likely to have a similar spectrum of contaminants. It follows that the dominant response of the animals was to the phogrin molecule itself.

Proliferative response of phogrin-specific lines and clones. A number of phogrin-specific T-cell lines were established from a series of five separate immunizations and tested for proliferative responses to phogrin and GST. As shown in Fig. 3*B*, a typical line after four restimulation cycles retained a robust proliferative response to phogrin similar to the lymph node cells (Fig. 3*A*), whereas the response to GST did not differ from background levels even at the highest concentration of antigen used.

A panel of 10 phogrin-specific T-cell clones was generated by limiting dilution cloning of a phogrin-specific T-cell line. All of the clones responded to phogrin (Fig. 4*A*) to varying extents and exhibited negligible responses in the absence of antigen. Three of the clones (12, 13, and 15) proliferated in response to DA rat islet cells (50,000 cells/ml) (Fig. 4*B*). The latter reaction was consistent with a response to an endogenous native antigen and is unlikely to be a xenoreaction because these clones fail to respond to DA rat lymph node cells in either the presence or the absence of NOD antigen-presenting cells (APCs) (results not shown).

Phenotypic characteristics of the phogrin-specific T-cell clones. Flow cytometric analysis of the 10 clones for

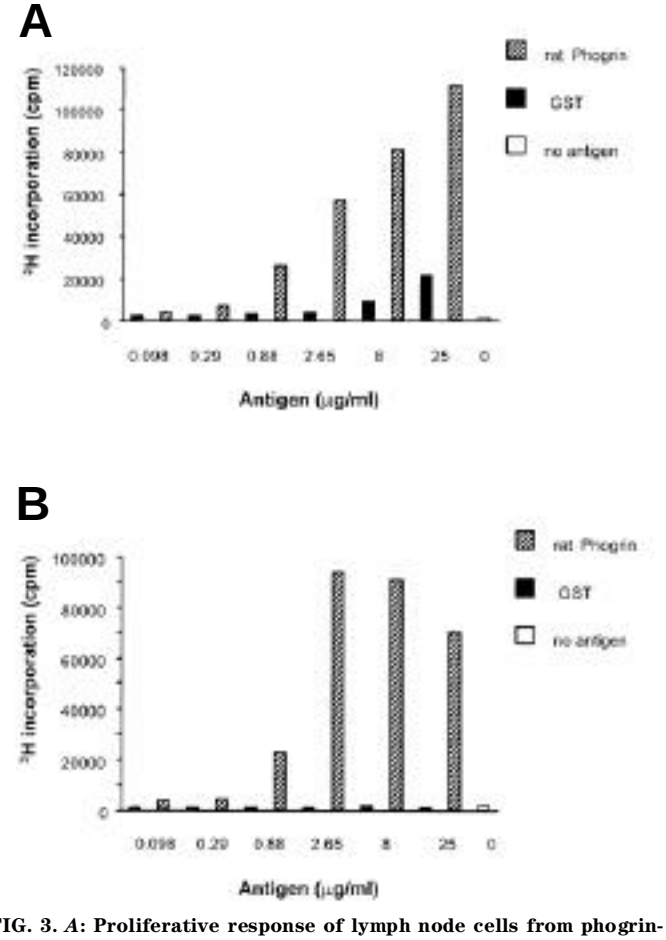

FIG. 3. *A*: Proliferative response of lymph node cells from phogrin-immunized NOD mice to phogrin and GST. *B*: Response of a phogrin-specific line after four rounds of phogrin/IL2 stimulation in vitro. Proliferation assays were performed as described in METHODS.

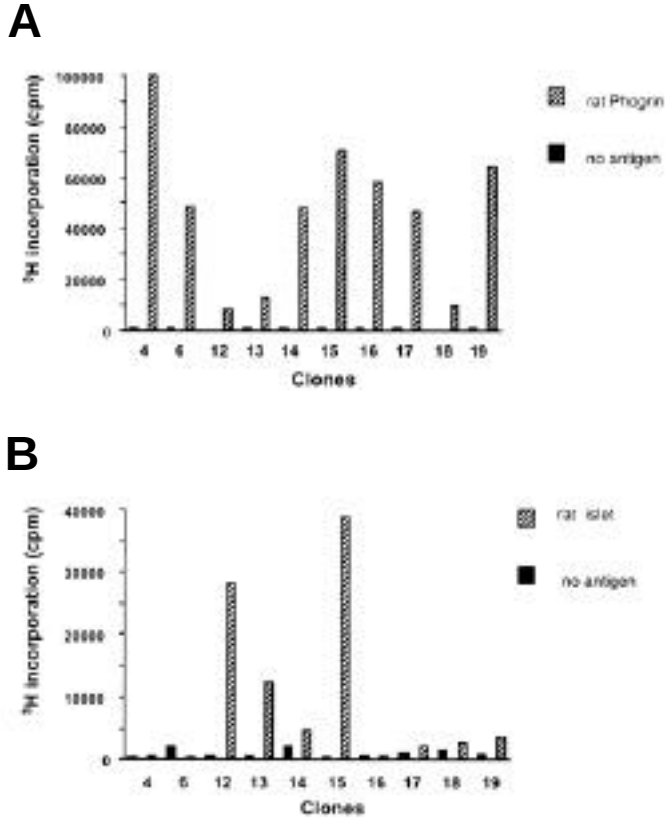

FIG. 4. Proliferative response of 10 phogrin-specific T-cell clones to either phogrin (*A*) or DA rat islet cells (*B*). Proliferation assays were performed as described in METHODS.

TABLE 1
Cytokine secretion by phogrin-specific T-cell clones

Clone	IFN-γ (ng/ml)		IL-4 (ng/ml)		IL-10 (ng/ml)	
	No Ag	+Phogrin	No Ag	+Phogrin	No Ag	+Phogrin
4	<0.10	0.27	<0.18	<0.18	<0.18	<0.18
6	<0.10	0.30	<0.18	<0.18	<0.18	<0.18
12	<0.10	4.33	<0.18	<0.18	<0.18	<0.18
13	<0.10	0.45	<0.18	<0.18	<0.18	<0.18
14	<0.10	0.27	<0.18	<0.18	<0.18	<0.18
15	<0.10	>25	<0.18	<0.18	<0.18	<0.18
16	<0.10	>25	<0.18	<0.18	<0.18	<0.18
17	<0.10	1.23	<0.18	<0.18	<0.18	<0.18
18	<0.10	0.54	<0.18	<0.18	<0.18	<0.18
19	<0.10	0.15	<0.18	<0.18	<0.18	<0.18

Ag, antigen; +Phogrin, with phogrin.

CD4/CD8 expression showed them all to be $CD4^+/CD8^-$. Analysis of the islet-responsive clones 12, 13, and 15 revealed that the TCRs used Vβ 5, an unknown variant, and Vβ 8.3, respectively. This demonstrated that the clones were truly independent and that the T-cell response to phogrin in NOD mice is probably diverse with regard to TCR usage. The cytokine profile of the phogrin-specific T-cell clones determined by ELISA assay (Table 1) showed that all clones produced IFN-γ in the presence of antigen and APC, whereas IL-4 or IL-10 production was below the detection limit of the assay. On this basis, the clones may be classified as being of a TH-1 phenotype, consistent with the fact that the mice were immunized with phogrin in CFA.

Pathogenicity of phogrin-specific T-cell clones in vivo. Two islet-reactive phogrin-reactive T-cell clones of known Vβ usage (clones 12 and 15) were tested for their respective ability to target islet transplant destruction in vivo. Since these clones were generated against rat phogrin, a rat islet transplant model was used to asses in vivo pathogenicity of the clones. NOD/*scid* male mice were rendered diabetic by streptozotocin, and then transplanted with DA rat islets and thyroid grafts beneath opposite poles of the recipient kidney capsule. After restoration of euglycemia, graft-bearing NOD/*scid* recipients were challenged with phogrin-reactive T-cell clones intravenously. Three of the four mice challenged with clone 12 and five of the seven animals receiving clone 15 reverted to the diabetic state within the 6-week course of the experiment (Table 2).

Histological analysis of the islet engraftment sites of animals receiving phogrin-reactive T-cells showed extensive islet destruction and fibrosis in all animals receiving clone 15 and in three of four animals receiving clone 12 (Fig. 5*A*).

TABLE 2
Adoptive transfer of diabetes by phogrin-specific T-cell clones

Clone	Diabetes incidence	Histopathology	Mean destruction time (days)
15	5/7 (71)	7/7 (100)	23
12	3/4 (75)	3/4 (75)	28
Unchallenged	0/7 (0)	0/7 (0)	—

Data are *n* (%) or means.

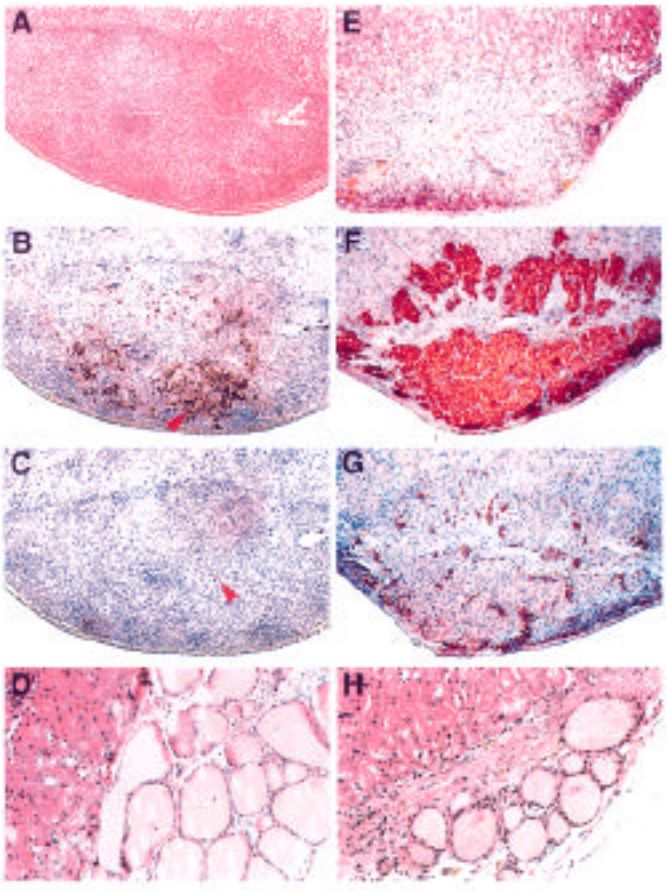

FIG. 5. Immunocytochemical analysis of engraftment site of streptozotocin-treated NOD *scid* mice transplanted with rat islets (*A–C* and *E–G*) or thyroid tissue (*D* and *H*) and then either challenged intravenously with 1×10^7 cells from T-cell clone 15 (*A–D*) or left untreated (*E–H*). Grafts were evaluated by staining with hematoxylin-eosin (*A*, *D*, *E*, and *H*), anti-insulin antibodies (*B* and *F*), or glucagon antibodies (*C* and *G*). Residual immunoreactive cells in challenged islets are marked with arrows.

These changes were accompanied by the marked loss of both insulin- and glucagon-producing cells (Fig. 5*B* and *C*) even though the animals were not overtly diabetic. In most of our clone-challenged animals, no recognizable islet structure remained at the appearance of clinical diabetes. Phogrin-reactive cells that were also confined to the islets were similarly lost upon challenge with phogrin-reactive T-cells (data not shown). Islet graft in non-challenged control animals showed intact morphology and function after six weeks (Fig. 5*E–G*). Engrafted rat thyroid tissue, on the other hand, retained its characteristic morphology with little sign of cellular infiltration or fibrosis in the challenged animals, and was indistinguishable from that in control animals (Fig. 5*D* and *H*). These results indicated that phogrin-reactive T-cells were pathogenic for mediating islet injury in vivo. Islet graft damage was not the result of inadvertent rat xenoreactivity either by the donor T-cell clones or by host-derived components, as shown by the fact that the DA rat thyroid grafts adjacent to the islet grafts were not damaged.

DISCUSSION

IA-2 and phogrin have been extensively studied as serological markers in type 1 diabetes in human subjects, and the

major conformational and linear epitopes have been mapped (20,21). Autoantibodies to them appear to emerge coincidentally or soon after the appearance of insulin and GAD65 autoantibodies during the natural history of the disease and persist after onset of clinical disease. Inter- and intramolecular spreading of antigenic determinant specificity appears to occur within a single individual during the course of disease progression, although not in a consistent way between individuals. Little is known, however, about the human T-cell response to these antigens, and even less is known about this phenomenon in the NOD mouse, a remarkable circumstance given the utility of this animal model to evaluate the pathogenicity of T-cell responses directed at specific antigens. The weak humoral responses of the NOD mouse and BB rat to IA-2 has led to the suggestion that these PTP family members do not play a role in autoimmune diabetes in rodents. The same could be said, however, for GAD65 (33), based on autoantibody responses, and the importance of IA-2 and phogrin to disease pathogenesis needs to be evaluated directly by determination of the cell-mediated immune response to these molecules in this animal model.

In the present study, we chose to focus on the cytosolic domain of phogrin, which bears the major autoantibody reactive epitopes in humans. This portion of the molecule is highly conserved between rodents and humans (92% identity) and shows the greatest homology (80% identity) with IA-2, which displays overlapping humoral reactivity (21). It also contains putative DQ8 binding motifs, which share some structural similarity to antigenic epitopes of Rotavirus and which are postulated to be a source of viral mimicry in autoimmune diabetes (34). The fact that the cytoplasmic region of phogrin constitutes a domain and folds tightly, as evidenced by its resistance to proteolytic cleavage (14), facilitated both its production as a soluble fusion protein with GST and its purification by affinity chromatography. Nevertheless, further manipulations were required to remove the potentially antigenic GST moiety and to reduce contaminating bacterial antigens and mitogens to acceptable levels. Lymph node cells of immunized NOD mice showed marginal responses to GST that disappeared after four rounds of in vitro antigen stimulation, while a robust phogrin-specific response was retained.

The T-cell response to phogrin in unprimed NOD mice was addressed using mesenteric lymph node cells from a series of 12-week-old NOD female mice and demonstrated that phogrin-specific T-cells are present during a period when marked insulitis occurs. Since the incidence of diabetes in our colony is 90%, it is assumed that the animals were prediabetic. The antigen used for this and subsequent experiments was the complete cytosolic tail of rat phogrin, which differs from the mouse sequence in nine positions within the 376–amino acid cytosolic tail. The T-cell clones developed from primed animals, like the spontaneously responsive lymph node cells derived from non-primed animals, were also shown to proliferate in response to mouse phogrin fusion protein, suggesting either that the epitopes recognized do not span variant residues or that these sequence differences do not substantially affect major histocompatibility complex binding or TCR recognition of the antigen. The results illustrate that the NOD mouse can mount a robust primed cellular immune response to what is essentially a self antigen, and demonstrate the feasibility of establishing stable IA^{g7} restriction of phogrin-specific T-cell lines and clones from these animals. The 10 clones that were obtained all were $CD4^+$, produced IFN-γ but not IL-4 or IL-10, and showed variation in their Vβ usage indicative of variation in their TCR usage and probably variation in epitope specificity. Most importantly, 3 of the 10 phogrin-reactive clones responded to rat islet cells in in vitro proliferation assays, and at least two of these were capable of destruction of rat islet grafts placed into NOD/*scid* recipients. We have previously demonstrated that an IA^{g7}-restricted $CD4^+$ T-cell clone reactive with keyhole limpet hemocyanin (KLH), which was generated by CFA immunization of NOD mice with KLH, did not transfer diabetes (35). It has been previously observed that immunization with CFA alone prevents the development and adoptive transfer of diabetes in NOD mice (36), as does a single injection with mycobacterium tuberculosis alone (37). Reactivity of the clones to rat xenoantigens was excluded by the lack of a proliferative response to rat spleen cells in vitro, either with or without NOD APC present. Adoptive transfer of phogrin-reactive T-cells to NOD/*scid* mice transplanted with either rat thyroid or islet tissue likewise ruled out a more general xenoresponse to rat cells. Histological analysis of the transplant sites revealed the loss of both insulin- and glucagon-producing cells consistent with the expression of phogrin in pancreatic endocrine cells and its absence in thyroid follicular tissue. The responsiveness to islet tissue in vitro and the pathogenicity of these clones are remarkable since phogrin and IA-2 are only minor islet constituents amounting to 0.01–0.05% of the cellular protein (around 2–10 ng/well in the proliferation assay) (Fig. 3*B*). In a broader technical context, the finding that T-cells with diabetogenic activity can be generated by CFA immunization of NOD mice should be applicable to other autoantigens for which generation of pathogenic autoreactive T-cells has proven difficult.

The conclusion, based on serological studies, that granule PTP members are not important antigens in rodent models of type 1 diabetes requires reevaluation in light of the present findings. It remains to be shown that T-cells specific to the endogenous mouse forms of phogrin and IA-2 are present in monocytic infiltrates within islets of spontaneously diabetic animals; however, their presence in mesenteric lymph nodes is suggestive of this. Further analysis of the epitopes in phogrin and IA-2 and their overlap appear warranted by the present findings and should provide insight into the phenomenon of determinant spreading within these autoantigens that figure prominently in the humoral autoimmune response in human type 1 diabetes.

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