Original Article

RegII Is a β-Cell Protein and Autoantigen in Diabetes of NOD Mice

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The Reg family of proteins has been studied in the context of growth and regeneration in several organs including pancreatic islets. We previously suggested that Reg proteins act as autoantigens in type 1 diabetes, based on evidence that a member of the Reg family (hepatocellular carcinoma intestine pancreas [HIP]/pancreatitis-associated protein [PAP]) was overexpressed in the islets of a patient who died after sudden onset of type 1 diabetes, and that, in NOD mice, Reg-specific T-cells adoptively transferred diabetes. In the current study, we developed antisera to detect individual Reg members in mouse islets and found that RegIIIα was present in the non–β-cell portion of the islets, while RegII was predominantly expressed in β-cells. Vaccination of NOD mice with the separately expressed N-terminal (NtfrII) or C-terminal (CtfrII) portion of RegII revealed a dichotomy: NtfrII vaccination accelerated and CtfrII vaccination delayed type 1 diabetes. Vaccination with CtfrII was more effective when given at later stages in the pathogenesis of type 1 diabetes, a time dependency different from that seen with other antigen-dependent vaccine strategies in NOD mice, which might have therapeutic implications. In conclusion, RegII is a novel β-cell–derived autoantigen in NOD mice. The autoimmune response against this protein may convert a regenerative into an islet-destructive process accelerating development of type 1 diabetes. *Diabetes* 56:34–40, 2007

The Reg family of proteins received its name because cDNA encoding a first member of this family was isolated from a library derived from a rat model of pancreas regeneration (1). Reg proteins consist of a carbohydrate recognition domain found in C-type lectins and a short N-terminal tail (2). They have been studied mostly in the context of regeneration/survival and growth, not only in endocrine and exocrine pancreas (3–6), but also in a number of other organs and tissues (7–12). We have shown that one of the human Reg members (hepatocellular carcinona intestine pancreas [HIP]/pancreatitis-associated protein [PAP]) was overexpressed in islets from a patient who had died as a consequence of sudden onset of type 1 diabetes after a viral infection. We argued that overexpression of this islet protein could accelerate the pathogenesis of type 1 diabetes if it acted as an autoantigen. To test this possibility, we established a $CD4^+$ T-cell line derived from islet-infiltrating cells of NOD mice, which recognized HIP/PAP and cross-reacted with islets. This line induced diabetes in NOD-SCID mice when co-injected with a small, nondiabetogenic dose of a cytotoxic insulin-specific T-cell clone (13). A role for Reg as an autoantigen was also suggested by recent studies (14) reporting that patients with type 1 diabetes develop autoantibodies to RegI. These findings raised the possibility that Reg proteins may play a dual role as β-cell trophic factors and autoantigens in type 1 diabetes. Direct trophic effects on islets have been implicated to explain the beneficial effects of Reg treatment previously seen in the NOD mouse model (6). However, our studies suggested that the clinical effects observed after Reg treatment might be the result of two different processes acting via distinct mechanisms.

To demonstrate immune-mediated effects, we vaccinated NOD mice with Reg proteins and an N- and a C-terminal fragment (NtfrII and CtfrII) of RegII, a Reg member found to be expressed in β-cells. The intact protein was cleaved to reduce or eliminate potential direct effects, while maintaining immune-mediated disease-protective mechanisms. Subsequently, we investigated if the effects induced by Reg vaccination were transferable by lymphocytes, characterized the alteration of the immune system in the vaccinated mice, and attempted to identify the mechanism underlying the clinical effects. Taken together, the results of these experiments clearly indicate that immune-mediated mechanisms must be taken into account to explain the clinical effects seen after Reg treatment and support a role for Reg as a new autoantigen in type 1 diabetes.

RESEARCH DESIGN AND METHODS

RT-PCR and TA cloning. Islets from 7- to 9-week-old NOD mice were isolated by collagenase digestion. Islets were cultured for 6 h in serum-free Iscove's modified Dulbecco's medium. Interleukin (IL)-6 was added at 0, 1, and 10 units/ml. RNA was extracted from cultured islets and total pancreas with TRIZOL reagent (Gibco/Invitrogen). Reverse transcription was performed with oligo dT primers and the Moloney Murine Leukemia Virus reverse transcriptase (Gibco/Invitrogen). cDNA was amplified for 35 cycles using specific primers for the six Reg members. Amplified DNA was purified and TA cloned into the pCR 2.1 TOPO vector (Invitrogen). Inserts were sequenced to confirm their identity.

Development and characterization of rabbit sera specific for RegII, RegIIIα, and RegIIIδ. Peptides to be used as haptens and the corresponding peptides of the other Reg members were synthesized and purified at the Yale Small Scale Peptide Synthesis facility. The following peptides were used as haptens: RegII, 127-LFKSWATGAPSTANRGYC-144; RegIIIα, 26-GEDFQKEVP-

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CtfrII, C-terminal fragment of RegII; HIP, hepatocellular carcinoma intestine pancreas; IL, interleukin; NtfrII, N-terminal fragment of RegII; PAP, pancreatitis-associated protein; TGF, transforming growth factor; TNF, tumor necrosis factor.

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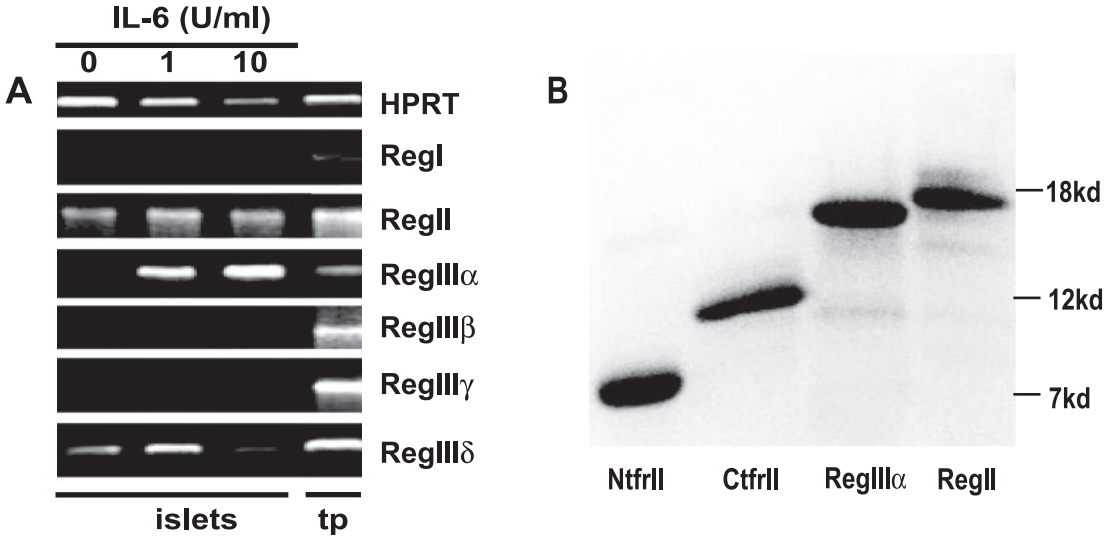

FIG. 1. ***A*****: RT-PCR with primers specific for individual Reg members and hypoxanthine guanine phosphoribosyl transferase (HPRT). cDNA was derived from untreated or IL-6–treated islets or total pancreas (tp).** ***B*****: Coomassie-stained gel of recombinant Reg proteins and fragments used to immunize mice.**

SPRTSC-40; and RegIIIδ, 93-KNSLTTFPYC-100 (the C-terminal cysteine was added to allow immobilization of this peptide).

Rabbits were immunized by Yale Veterinary Clinical Services. RegII and RegIIIδ immune sera and affinity-purified antibodies specific for RegIIIα were tested for cross-reactivity with the corresponding peptides of the other Reg members by enzyme-linked immunosorbent assay using Maleimide-activated plates (Pierce) to immobilize peptides via their C-terminal sulfhydryl group.

Immunohistochemistry. Pancreata and salivary glands were fixed for 6–8 h in 10% buffered formalin solution (Sigma) at 4°C. Sections were stained with the immune sera for RegII and RegIIIδ and with affinity-purified antibodies for RegIIIα. Secondary antibody was a monoclonal anti-rabbit IgG coupled to Texas Red (Invitrogen) for immunflourescence and to horseradish peroxidase (Sigma) for light microscopy. Red color development for light microscopy was obtained with 3-amino–9-ethylcarbazole. Double staining for Reg and insulin/glucagon was obtained by first staining for Reg members followed by staining for the islet hormones (guinea pig anti-insulin [Zymed] and anti-glucagon [Linco Research] with an fluorescein isothiocyanate–labeled secondary antibody [Chemicon]).

Expression and purification of RegIIIα, RegII, NtfrII, and CtfrII. Appropriately tailed primers were used to amplify templates excised from the TOPO PCR vector. The PCR products were cloned into the pQE30 series of vectors (Qiagen), allowing protein expression with an N-terminal hexahistidine tag. Proteins were purified from bacterial lysates by metal-chelate affinity followed by ion exchange chromatography. Fractions were analyzed by SDS-PAGE, and the purest fractions were pooled (Fig. 1). The peptide corresponding to the histidine-tag [Met-Arg-Gly-Ser-$(His)_6$] served as control for T-cell proliferation experiments. Proteins were expressed as follows: RegIIIα from Gly 26 to Gln 175, RegII from Gly 22 to Ala 173, NtfrII from Gly 22 to Asn 75, and CtfrII from Ala 76 to Ala 173.

Animals and vaccination. Female NOD mice were obtained from The Jackson Laboratories. They were kept under specific pathogen-free conditions in filter cages on a 12-h light-dark cycle and fed with autoclaved standard chow. Experimental protocols were approved by the Yale University Institutional Animal Care and Use Committee.

Immunizations were carried out in female NOD mice. Animals were intraperitoneally immunized at a dose of 1.5 mg/kg. The volume injected was 150–250 μl consisting of 50% alum adjuvant (Pierce) and 50% antigen solution. Control animals received a 250-μl injection consisting of 50% alum adjuvant and 50% buffer solution (100 mmol/l monobasic sodium phosphate, 10 mmol/l Tris, and 4 mol/l urea, pH 7.5). Mice were monitored for diabetes by testing for glucosuria (Diastix; Bayer) and were considered diabetic when a positive reaction to full-color development (>2,000 mg/dl) was obtained.

Three separate vaccination experiments were performed. For statistical analysis and data plots, results of the following experimental groups showing closely resembling treatment effects were pooled: control groups 1e, 2f, and 3b ($n = 24$); RegII groups 1b and 2a ($n = 15$); CtfrII groups 1d, 2b, and 2d ($n = 18$); and NtfrII groups 1c and 2e ($n = 12$) (Table 1). A log-rank test was used to assess the statistical significance between groups.

Cell enrichment and adoptive transfer. $CD4^+$ or $CD8^+$ cells were depleted by labeling with a rat anti-mouse CD4– or rat anti-mouse CD8–specific antibody (BD Biosciences), followed by incubation with anti-rat IgG antibodies coupled to magnetic beads (Dynal biotech). Depletion of B-cells was obtained with magnetic beads coupled to anti-mouse IgG and IgM. Cells were transferred to female NOD-SCID recipients via the retro-orbital route under anesthesia by xylazine and ketamine (20 and 100 mg/kg, respectively).

NOD-SCID mice received either spleen cells from diabetic NOD mice (inducer cells) alone or a co-injection of inducer cells and lymphocytes from pancreatic lymph nodes of CtfrII-immunized mice (test cells). Inducer cells were injected at 4×10^6/mouse. Test cells depleted of $CD8^+$ T-cells and B-cells were injected at 4×10^6 or 1×10^6/mouse. Test cells depleted of $CD4^+$ T-cells and B-cells were injected at 4×10^6/mouse.

Spleen cells from NtfrII vaccinated mice, collected after onset of diabetes, were depleted of $CD4^+$ or $CD8^+$ T-cells and B-cells and injected into NOD-SCID recipients at a dose of 6×10^6/mouse. Spleen cells from nonimmunized mice collected after the onset of diabetes were depleted as above and injected at 6×10^6/mouse.

Cytokine measurements. Commercially available cytokine arrays (Schleicher & Schuell) were used to measure the concentration of the following cytokines in the tissue culture supernatant of spleen cells from vaccinated mice: IL-1β, IL-2, IL-4, IL-6, IL-10, IL-12p70, IFN-γ, and tumor necrosis factor (TNF)-α. Concentrations of transforming growth factor (TGF)-β were measured by a commercially available enzyme-linked immunosorbent assay kit (R&D Systems). Mice were given two vaccinations, and the measurements were taken at 8, 12, and 22 days after the second immunization.

TABLE 1
Vaccination experiments

Group	Antigen	Age at vaccination (weeks)
1a ($n = 5$)	RegIIIα	6 and 9
1b ($n = 6$)	RegII	6 and 9
1c ($n = 6$)	NtfrII	6 and 9
1d ($n = 6$)	CtfrII	6 and 9
1e ($n = 10$)	Control	6 and 9
2a ($n = 9$)	RegII	6 and 9
2b ($n = 6$)	CtfrII	4, 7, and 11
2c ($n = 6$)	CtfrII	4
2d ($n = 6$)	CtfrII	11
2e ($n = 6$)	NtfrII	4, 7, and 11
2f ($n = 6$)	Control	4, 7, and 11
3a ($n = 6$)	CtfrII	4, 7, and 11
3b ($n = 8$)	Control	4, 7, and 11

Mice in groups 1 and 2 were monitored to 22 weeks of age, whereas mice in group 3 to 32 weeks.

RESULTS

The members of the mouse Reg family are differentially expressed in pancreatic islets and salivary gland. To determine which of the six murine Reg members was expressed in pancreatic islets, we identified the members whose cDNA could be derived from islets. Since the Reg genes carry an IL-6–response element in their promoter (15), we also incubated islets with this cytokine before mRNA extraction. While cDNA for all Reg members could be derived from total pancreas, only cDNA for RegII, RegIIIα, and RegIIIδ could be derived from islets. Addition of IL-6 strongly upregulated transcription of RegIIIα and slightly that of RegIIIδ but had no effect on the mRNA levels of RegII (Fig. 1*A*).

Based on these data, we developed rabbit antisera for RegII, RegIIIα, and RegIIIδ and characterized their specificities. The affinity-purified RegIIIα-specific antibodies and the RegIIIδ-specific immune serum minimally cross-reacted with the corresponding peptides of the remaining Reg members. The RegII-specific serum showed 30% cross-reactivity with the corresponding peptide of RegI. However, given that RegI cDNA could not be derived from islets, it is highly likely that the Reg member detected in islets by this reagent is RegII.

Immunohistochemical analysis of mouse islets obtained from 7-week-old female NOD mice using the newly developed reagents showed that RegIIIα-specific antibodies strongly stained the periphery of the islets, whereas RegII-specific antibodies predominantly bound to the central part of the islet (Fig. 2*A* and *B*). RegII-specific antibodies also gave a weak signal in the exocrine pancreas. Staining for both RegII and RegIIIα could be blocked by preincubation with the hapten-peptide and the respective recombinant full-length protein. The observed staining pattern suggested that RegII was predominantly expressed in β-cells, whereas RegIIIα expression was restricted to non-β-cells in the islets. We confirmed this hypothesis by double staining for the islet hormones insulin/glucagon and RegII/RegIIIα. Costained cells were absent on sections double stained for insulin and RegIIIα (Fig. 2*C*), whereas some costained cells occurred in the glucagon RegII double stain (Fig. 2*D*). Staining for glucagon/RegIIIα revealed some non–glucagon expressing cells that stained for RegIIIα. These might represent δ- or pancreatic polypeptide–cells or α-cells that contain little or no glucagon (Fig. 2*E*). An identical staining pattern was found in the islets of IL-6 knockout mice indicating that, although IL-6 enhances expression of RegIIIα, it is not necessary for its constitutive expression. Staining for RegIIIδ gave no signal in islets from 4- and 7-week-old mice, but a signal could be detected in islets from mice with recent-onset diabetes (data not shown).

RegII expression was predominantly found in β-cells; only a small number of non–insulin expressing islet cells showed staining for RegII. RegII and insulin expression were independently regulated in each β-cell, as demonstrated by the wide range of different intermediate colors between green and red (Fig. 2*F*). The observed distribution pattern for RegIIIα and RegII was not specific for NOD mice but was also found in pancreas sections from C57bL/6 mice.

Since insulitis in type 1 diabetes of NOD mice is accompanied by sialitis, we examined whether Reg proteins were also expressed in salivary glands. We found

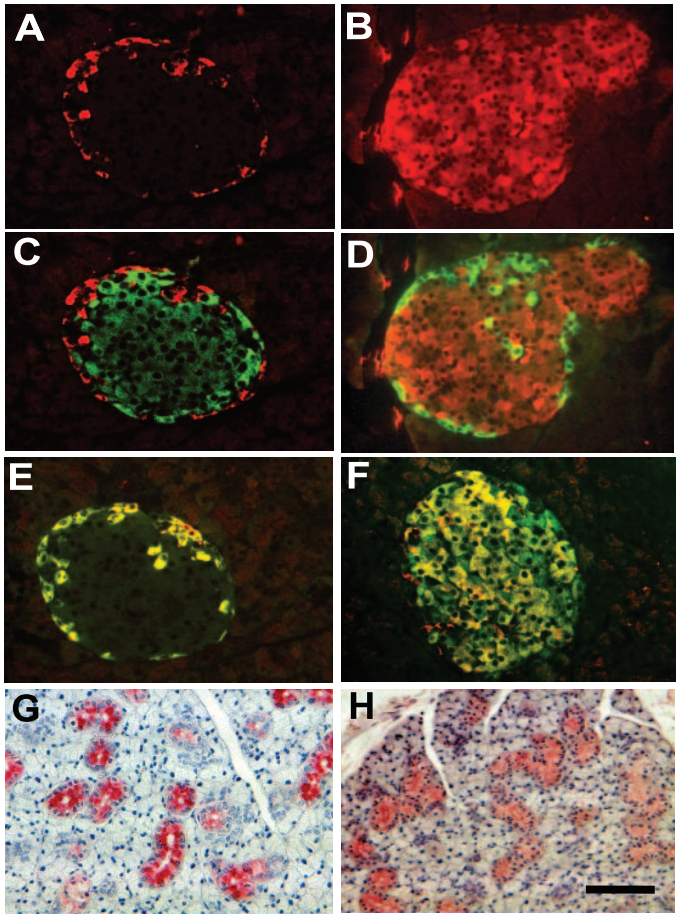

FIG. 2. Reg protein expression in mouse pancreatic islets and salivary gland. Staining for Reg was in red and for insulin or glucagon in green. Scale bar is 25 μm. Single staining for RegIIIα (*A*) and RegII (*B*). Double staining for insulin and RegIIIα (*C*) and glucagon and RegII (*D*). Double staining for glucagon and RegIIIα (*E*) and insulin and RegII (*F*). Salivary gland: RegIIIδ (*G*) and RegII (*H*) (scale bar 50 μm).

both RegIIIδ (Fig. 2*G*) and RegII (Fig. 2*H*) expressed in the salivary gland ductal cells. Staining for RegIIIα was absent.

Vaccination with RegII (a β-cell Reg) delays onset of diabetes, whereas vaccination with RegIIIα (a non–β-cell Reg) has no effect on the course of disease. Based on the localization studies, we chose RegIIIα and RegII as vaccine antigens. Although RegIIIα was not expressed in β-cells, we nevertheless included this antigen in our experiments because RegIIIα and RegII share sequence homologies (40% identity), and both are secreted proteins. A RegIIIα-directed immune reaction might therefore also target RegII. We obtained a 70% incidence of diabetes in the control group by age 19 weeks. Contrary to our expectation, immunization with RegIIIα had no effect on disease development. However, immunization with full-length RegII led to a delay in the onset of diabetes with only 14.2% (2 of 15 mice) having developed overt disease by 20 weeks of age ($P = 0.023$). Thereafter, the diabetes incidence in this group quickly climbed to 40% (6 of 15 mice) (Fig. 3*A*).

The response to vaccination with RegII is characterized by a dichotomy: NtfrII vaccination accelerates, whereas vaccination with CtfrII delays diabetes. CtfrII vaccination is more effective when given at a late stage in the pathogenesis of type 1 diabetes. Surprisingly, a considerable difference was observed in the effects induced by vaccination with the two fragments of

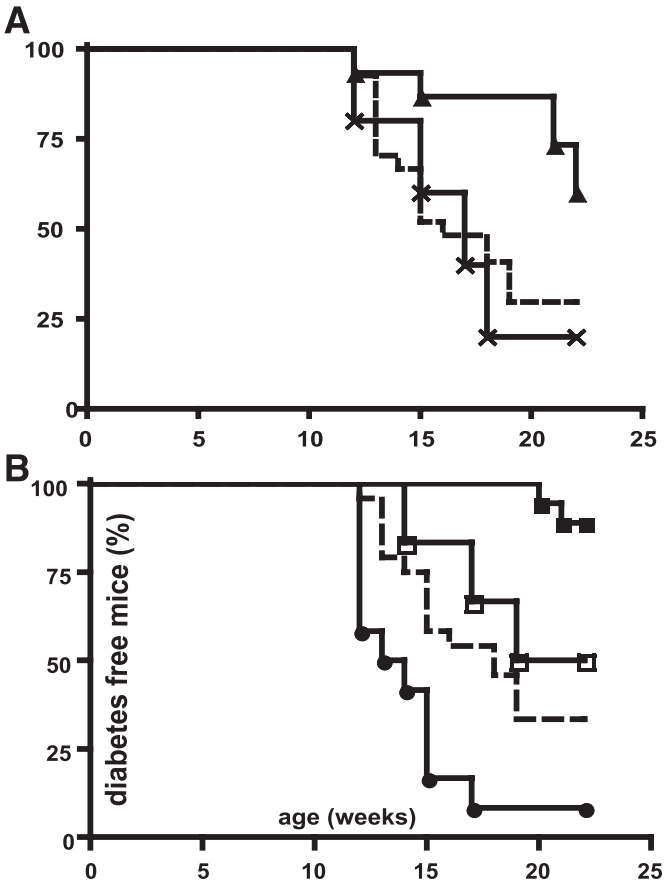

FIG. 3. Diabetes development after Reg vaccination followed up to 22 weeks of age. *A*: Vaccination with RegIIIα (×) (n = 5). Vaccination with RegII (▲) (n = 15). *B*: CtfrII vaccine schedules containing a late-stage vaccination (■) (n = 18). CtfrII vaccination at 4 weeks of age (□) (n = 6). NtfrII vaccination (●) (n = 12). Control group (dashed lines) (n = 24).

RegII. Immunization with NtfrII led to an acceleration of the disease with 41% (5 of 12) of the animals being already diabetic at age 12 weeks climbing to 91% (11 of 12) by age 17 weeks. Compared with control animals, the effects induced by NtfrII vaccination caused an accelerated conversion to overt disease (P = 0.0068) (Fig. 3*B*).

A very different effect was obtained by immunization with CtfrII, which led to suppression of disease with only 11% (2 of 18) of the vaccinated mice having developed diabetes by the end of the study at 22 weeks (P = 0.0002). This effect was more pronounced than the delay induced by vaccination with the full-length RegII protein. The following CtfrII vaccination schedules induced a delay: triple vaccinations at 4, 7, and 11 weeks (one of six with diabetes at 21 weeks of age); double vaccinations at 6 and 9 weeks of age (zero of six); and a single vaccination at a time point when the first mouse in this group had developed diabetes (between 11 and 12 weeks of age; one of six with diabetes at 20 weeks of age). All three schedules included a vaccination at a late stage in the pathogenesis of type 1 diabetes. Omitting vaccination at this late stage greatly reduced the effectivity of the treatment. As shown in Fig. 3*B*, a single vaccination with CtfrII given at 4 weeks of age had no, or only a minimal, effect on the course of the disease.

To expand the window of observation, we performed a third experiment. A schedule with CtfrII vaccinations at 4, 7, and 11 weeks of age was able to significantly delay diabetes up to 32 weeks of age (P = 0.034) (Fig. 4).

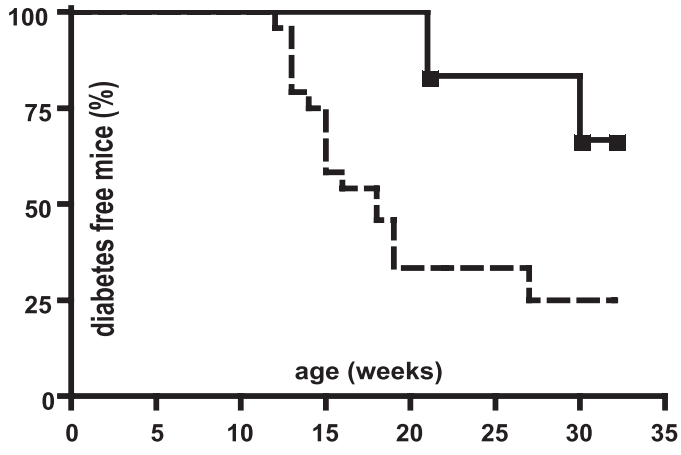

FIG. 4. Diabetes development after vaccination with CtfrII followed up to 32 weeks of age. ■, test group (n = 8); dashed line, control group (n = 24).

Histological examination of pancreata collected at 22 weeks of age from mice treated with late-stage CtfrII vaccination revealed the following degree of islet inflammation: of 74 islets assessed, 20% showed intra-islet infiltrates, 7% had peri-islet infiltrates, and the remaining 73% were free of lymphocyte infiltrates. Pancreata from mice immunized with RegIIIα collected at the onset of diabetes or on termination of the experiment revealed the following degree of islet inflammation: of 53 islets assessed, 71% had intra-islet infiltrates, 19% had peri-insulitis, and 10% were free of lymphocyte infiltrates.

CtfrII versus NtfrII vaccination induces different cytokine profiles. Spleen cells from mice vaccinated with either NtfrII or CtfrII responded to both stimulation with the specific vaccine antigen and to stimulation with the full-length RegII (data not shown). No response against the peptide representing the histidine-tag could be detected. IL-6, IL-10, TGF-β, and TNF-α were detectable in the supernatant of antigen-stimulated spleen cells derived from vaccinated mice. TNF-α release from spleen cells of CtfrII-vaccinated mice was higher than the release from spleen cells of NtfrII-vaccinated mice at all measured time points (Fig. 5*A*). The main source of the TNF-α released from the spleen cells of CtfrII-vaccinated mice were $CD4^+$ T-cells. Removal of these cells caused a >70% decrease in the TNF-α level in the supernatant. The cytokine release values of IL-6, IL-10, and TGF-β did not consistently (i.e., at all measured time points) correlate with the antigen that was used for vaccination.

Elevated IL-12 production by macrophages of NOD mice may predispose and contribute to the Th1-mediated pathogenesis of type 1 diabetes (16,17). Analysis by ELISPOT demonstrated that vaccination with CtfrII had reduced the number of IL-12–producing cells compared with control-immunized mice (Fig. 5*B*).

CtfrII vaccination increases Foxp3 expression levels in pancreatic lymph nodes. Given the consistently elevated TNF-α response from spleen cells of CtfrII-vaccinated mice, we searched for evidence implicating this cytokine as a mediator of the clinical effects induced by vaccination with CtfrII. Studies in the NOD mouse model of type 1 diabetes have revealed that the effects of TNF-α are dependent on the age at which treatment is began (18–20). The type of age-dependent differential clinical effects of CtfrII vaccination might fit a model that relies on TNF-α as a potential intermediate in the disease-delaying

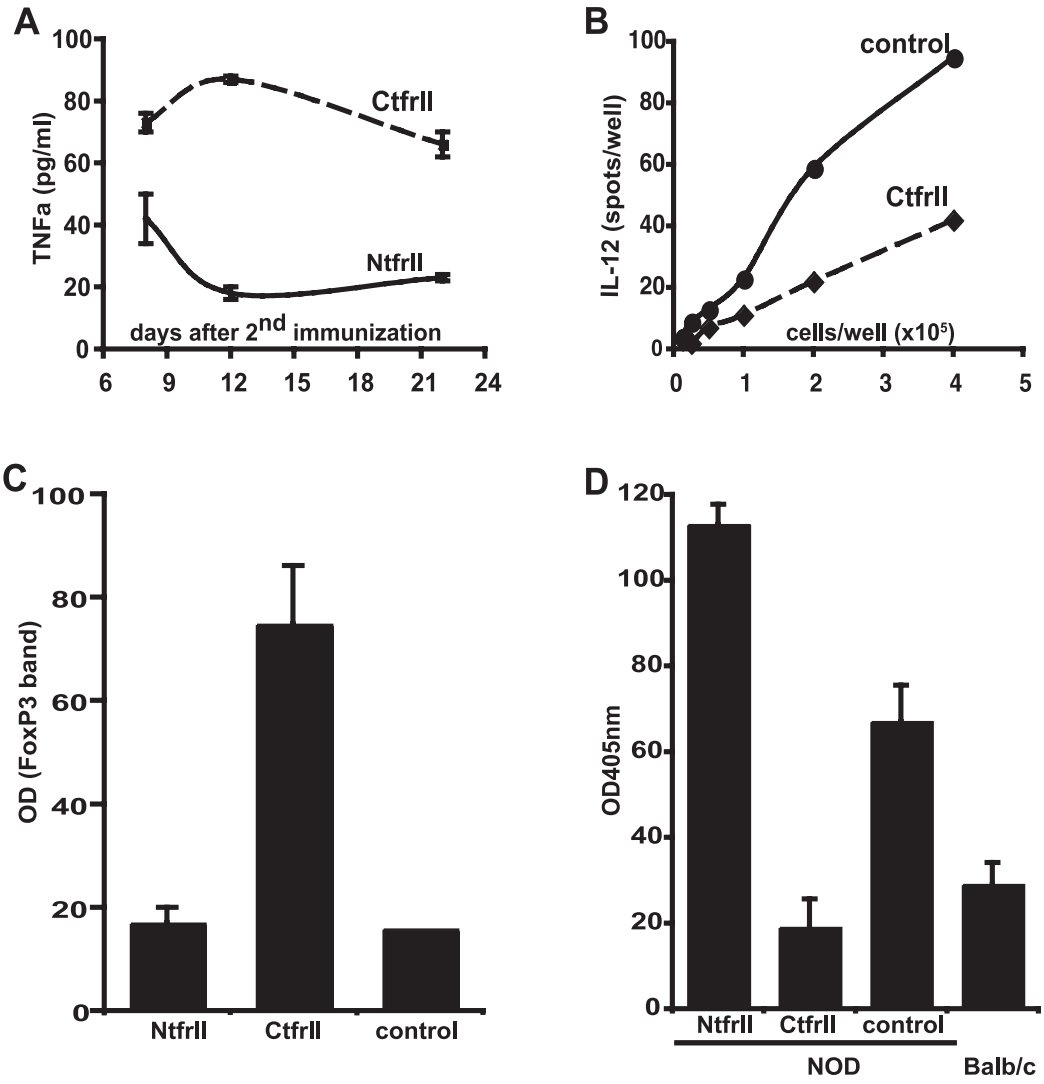

FIG. 5. Immune parameter after NtfrII and CtfrII vaccination. *A*: TNF-α release from spleen cells of NOD mice vaccinated with NtfrII versus CtfrII measured at 8, 12, and 22 days after the second of two vaccinations given at 6 and 9 weeks of age. *B*: Number of IL-12–releasing cells in spleen cell suspensions of control- or CtfrII-vaccinated mice measured 8 days after the second of two vaccinations. *C*: Relative optical density (OD) of the Foxp3 band on Western blots of $CD4^+$ T-cell lysates from pancreatic lymph nodes of NtfrII-, CtfrII-, and control-immunized mice. CD4 and Foxp3 were analyzed on different blots. The optical density for the CD4 band was set to 100 for each set of blots. For NtfrII- and CtfrII-immunized mice, there were three independent experiments; for control mice, there were two independent experiments. *D*: Anti-RegII IgG antibody titers in serum of NtfrII-, CtfrII-, and control-immunized NOD mice ($n = 6$ for each group). Balb/c serum ($n = 4$). Sera from mice within each group were pooled, and antibodies were affinity purified on immobilized RegII.

process. Since it is known that this cytokine influences the $CD4^+CD25^+$ T–regulatory cell levels in the NOD mouse (21), we tested whether quantitative differences in Foxp3 (a transcription factor associated with $CD4^+CD25^+$ T–regulatory cells [22,23]) were detectable in vaccinated mice. Analysis by Western blot of $CD4^+$ T-cell lysates derived from pancreatic lymph nodes showed that CtfrII-immunized mice had higher expression levels of Foxp3 than NtfrII- or control-immunized mice (Fig. 5*C*).

$CD4^+$/$Foxp3^+$ T–regulatory cells can directly suppress the B-cell response (24,25). In accordance with these reports, we showed that CtfrII vaccination led to a suppression of the anti-RegII IgG antibody titer to a level below that found in control-vaccinated mice. Vaccination with NtfrII, in contrast, increased the anti-RegII IgG antibody titer (Fig. 5*D*).

$CD4^+$ T-cells are necessary to delay diabetes in CtfrII-immunized mice. Inducer and test cells were adoptively cotransferred to NOD-SCID recipients. Test cells depleted of $CD8^+$ T-cells delayed the onset of disease in the recipients. The length of the delay was dependent on the ratio between test and inducer cells. A ratio of 1 caused a delay of 23 days, whereas a ratio of 0.25 delayed disease for 10 days beyond the disease onset observed in recipients of inducer cells alone. Test cells depleted of $CD4^+$ T-cells were unable to delay disease. Thus, $CD4^+$ T-cells are necessary to mediate the effects observed after CtfrII vaccination (Fig. 6*A*).

Both $CD4^+$ and $CD8^+$ T-cells from NtfrII-immunized mice transfer diabetes to NOD-SCID recipients. Spleen cells from NtfrII-immunized mice were collected after the onset of diabetes and were depleted of $CD8^+$ or $CD4^+$ T-cells, as well as B-cells. These cell preparations were transferred to two groups of NOD-SCID mice: group 1 received $CD4^+$- and B-cell–depleted spleen cells, whereas group 2 received $CD8^+$- and B-cell–depleted spleen cells. Both recipient groups developed diabetes, albeit with different kinetics. Mice in group 1 showed a

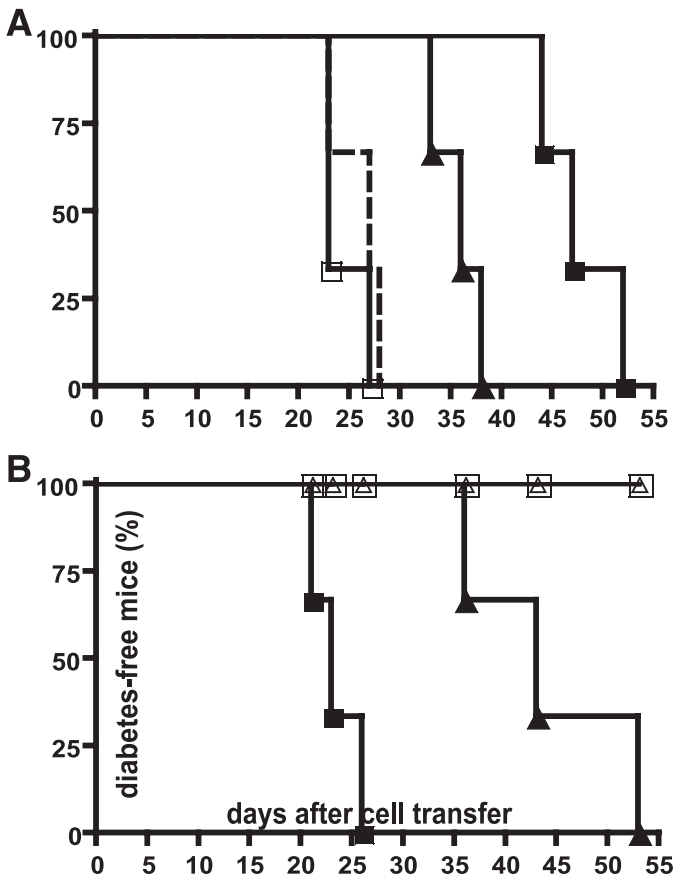

FIG. 6. Adoptive transfer experiments. *A*: NOD-SCID mice received spleen cells from diabetic mice (inducer cells) and lymphocytes derived from CtfrII-vaccinated mice (test cells). Cotransfer of test cells depleted of $CD8^+$ T-cells and inducer cells at a ratio of 1 (■, solid line) or 0.25 (▲, solid line) delayed diabetes compared with recipients of inducer cells alone (dashed line). Cotransfer of test cells depleted of $CD4^+$ T-cells and inducer cells at a ratio of 1 had no effect on disease (□, solid line) (n = 3 for each group). *B*: Development of diabetes in NOD-SCID recipients of $CD8^+$ (■) or CD4+ T-cells (▲) from NtfrII-immunized mice with recent onset of diabetes. NOD-SCID recipients of $CD8^+$ (□) or $CD4^+$ T-cells (△) from nonimmunized mice with recent onset of diabetes did not develop diabetes (n = 3 for each group).

rapid onset of diabetes with three of three diabetic mice 23 days after transfer. Mice in group 2 developed diabetes at a much slower rate, with the first mouse developing diabetes at 36 days after transfer, rising to three of three at 53 days after transfer.

Lymphocytes from nonimmunized NOD mice with recent onset of diabetes, depleted according to group 1 or 2 above, were unable to transfer diabetes to NOD-SCID recipients by day 55 after transfer (Fig. 6*B*).

DISCUSSION

We have expanded and refined our investigation on the role of the Reg proteins as autoantigens in type 1 diabetes. The experiments were designed to isolate and separate immune-mediated effects caused by Reg vaccination from the direct trophic effects these proteins might have on pancreatic islets. It was previously reported that delivery of Reg proteins reduces the development of type 1 diabetes in the NOD mouse. These studies assumed direct trophic/protecting effects induced by recombinant human RegI on pancreatic islets as the mechanism to explain the positive clinical outcome of Reg treatment in type 1 diabetes (6). Our data, in contrast, confirm that autoimmune responses both to accelerate or to suppress type 1 diabetes can be triggered by vaccination with RegII and its fragments and support the classification of the Reg protein family as a new group of potential autoantigens in type 1 diabetes. It remains to be seen if a Reg-based therapy can be designed that combines its putative direct trophic with its protective immune-mediated effects, particularly in the later pre-diabetic stages of disease.

If the disease delay observed after RegII vaccination was caused by a T-cell–mediated mechanism, it should be possible to identify the portion of the molecule containing the epitope(s) of the T-cells responsible for the initiation of the regulatory process. A step toward this goal was to cleave RegII into two parts. The cleavage site was placed between Asn75 and Ala76, thus disrupting the carbohydrate recognition domain of RegII. We argued that destroying this feature would minimize possible trophic effects, while still permitting the induction of immune-mediated effects. Our results demonstrated that vaccination with CtfrII still delayed disease. This observation, taken together with the data from the adoptive transfer experiments, strongly supports the hypothesis that the clinical effects seen after Reg treatment are immune mediated. The possibility that even a truncated form of Reg might still exert some trophic effects cannot solely account for the results obtained here.

Surprisingly, vaccination with NtfrII accelerated the disease. This observation and the adoptive transfer studies imply that within NtfrII, there must exist T-cell epitopes able to stimulate autoagressive $CD8^+$ and $CD4^+$ lymphocytes that target pancreatic islets and contribute to the destruction of β-cells. This arguably constitutes stronger support for the classification of RegII as an autoantigen in the NOD mouse than the observed disease delay induced by CtfrII vaccination, although the latter might be clinically more important.

It is noteworthy that T-cells from mice immunized with either NtfrII or CtfrII still recognize the intact protein; therefore, it is conceivable that vaccination with the full-length RegII triggers two opposing processes: one with a disease-delaying regulatory outcome and the other with a disease-accelerating outcome. Vaccination with either NtfrII or CtfrII alone could activate each of these processes separately. To explain the clinical data obtained after RegII vaccination, it could be argued that initially the processes induced by the C-terminal fragment dominate. They are able to suppress the autoaggressive T-cells that have been simultaneously activated by peptides in the N-terminal portion of RegII. However, since the suppressive process may wane, the disease-accelerating processes induced by the N-terminal fragment eventually prevail.

Our data suggest that CtfrII vaccination may exert preventive effects on diabetes development via the induction of regulatory cells. Consistent with this view, we found higher levels of Foxp3 expression in $CD4^+$ T-cells isolated from pancreatic lymph nodes of CtfrII- versus NtfrII-vaccinated mice. An indication of the potential involvement of $CD4^+$/$Foxp3^+$ T–regulatory cells in the processes initiated by vaccination with CtfrII was the observed reduction of the anti-RegII B-cell response in the treated mice. The reduction to levels below those seen in control-immunized mice suggests the presence of an active mechanism of suppression and not simply the lack of antigenicity in CtfrII. The latter explanation is also unlikely to be correct because mice vaccinated with intact RegII protein do develop anti-CtfrII–specific antibodies.

Late-stage vaccination with CtfrII proved to be more

effective than vaccination given at an earlier time point. This time-dependent change is clinically important, as a vaccination protocol that can be applied at late stages in the pathogenesis of type 1 diabetes is more relevant for humans where susceptibility to type 1 diabetes is determined at a much later stage in the disease process.

Although Reg proteins share high-sequence homologies and all display a single carbohydrate recognition domain found in C-type lectins, it has become clear that the individual family members possess different structural properties when studied at a finer level. This might indicate that members of this family generate specific responses to different ligands (26,27). Their highly specific tissue distribution illustrated in this study and potential involvement in islet growth and regeneration would warrant a more intense investigation of the functional properties and ligand(s) of individual Reg family members in this process. It has been suggested that anti-Reg autoantibodies might block the trophic functions of Reg (14). Clearly, to understand the role of these proteins in type 1 diabetes, results from both (auto)immune and functional studies will have to be combined.

We conclude that RegII, a member of the Reg family of proteins, can act as an autoantigen in type 1 diabetes. Vaccination with RegII can result in the generation of two separate processes: one triggered by the C-terminal portion of the molecule involving a mechanism possibly mediated by TNF-α and acting on $CD4^+/Foxp3^+$ T–regulatory cells and another triggered by vaccination with the N-terminal portion of the molecule leading to the activation of autoagressive T-cells. Thus, autoimmunity directed at RegII in the NOD mouse may accelerate the development of diabetes by creating a vicious circle preventing compensatory islet regeneration.

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