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Early Th1 Response in Unprimed Nonobese Diabetic Mice to the Tyrosine Phosphatase-Like Insulinoma-Associated Protein 2, an Autoantigen in Type 1 Diabetes

Sylvie Trembleau,* Giuseppe Penna,* Silvia Gregori,* Giovanni Magistrelli,† Antonella Isacchi,† and Luciano Adorini2*

The insulinoma-associated protein 2 (IA-2) is a phosphatase-like autoantigen inducing T and B cell responses associated with human insulin-dependent diabetes mellitus (IDDM). We now report that T cell responses to IA-2 can also be detected in the nonobese diabetic (NOD) mouse, a model of human IDDM. Cytokine secretion in response to purified mouse rIA-2, characterized by high IFN-γ and relatively low IL-10 and IL-6 secretion, was elicited in spleen cells from unprimed NOD mice. Conversely, no response to IA-2 was induced in spleen cells from BALB/c, C57BL/6, or Biozzi AB/H mice that express, like NOD, the I-A<sup>b</sup> class II molecule, but are not susceptible to spontaneous IDDM. The IA-2-induced IFN-γ response in NOD spleen cells could already be detected at 3 wk and peaked at 8 wk of age, whereas the IL-10 secretion was maximal at 4 wk of age and then waned. IA-2-dependent IFN-γ secretion was induced in CD4<sup>+</sup> cells from spleen as well as pancreatic and mesenteric lymph nodes. It required Ag presentation by I-A<sup>b</sup> molecules and engagement of the CD4 coreceptor. Interestingly, cytokines were produced in the absence of cell proliferation and IL-2 secretion. The biological relevance of the response to IA-2 is indicated by the enhanced IDDM following a single injection of the recombinant protein emulsified in IFA into 18-day-old NOD mice. In addition, IFN-γ production in response to IA-2 and IDDM acceleration could be induced by IL-12 administration to 12-day-old NOD mice. These results identify IA-2 as an early T cell-inducing autoantigen in the NOD mouse and indicate a role for the IA-2-induced Th1 cell response in IDDM pathogenesis. The Journal of Immunology, 2000, 165: 6748–6755.

Insulin-dependent diabetes mellitus (IDDM)<sup>3</sup> is an autoimmune disease characterized by a chronic progressive inflammation of the pancreatic islets. The islet cellular infiltrate is composed of different types of mononuclear cells with a predominance of T cells, which play an important role in the destruction of insulin-secreting pancreatic β cells (1).

The nonobese diabetic (NOD) mouse, which spontaneously develops IDDM with a pathogenesis similar to human type I diabetes, represents a useful model for the disease (1, 2). T cells from diabetic NOD can transfer IDDM to non diabetic NOD mice, demonstrating that autoreactive T cells mediate diabetic NOD can transfer IDDM to nondiabetic NOD mice, demonstrating that autoreactive T cells mediate diabetes. T cell reactivity to other Ags, like heat-shock protein 60, heat-shock protein 65, peripherin, carboxypeptidase H, and insulin, was initially described to arise later in the disease process (4, 8). However, Wong et al. (11) recently described, using tetrameric H-2K<sup>d</sup>-peptide complexes, that about 85% of T cells infiltrating the islets of 4-wk-old NOD mice recognize insulin β-chain residues 15–23, suggesting that insulin-specific CD8<sup>+</sup> cells may provoke the initial insulitic attack. The role of insulin in the development of IDDM is also indicated by the prevention of IDDM in NOD mice expressing transgenic proinsulin under the MHC class II promoter (12). Therefore, autoantigens other than GAD may also play a crucial role in the initial events leading to IDDM in NOD mice.

The putative tyrosine phosphatase protein insulinoma-associated protein 2 (IA-2), expressed in human brain and pancreatic islets, is an autoantigen associated with IDDM (13, 14). Abs to IA-2 react almost exclusively with the intracellular domain of this molecule and are an important marker of IDDM (15, 16). In addition, anti-IA-2 Abs have been found in children at risk (17) and are more prevalent in patients with early and aggressive disease onset (18). Low levels of IA-2-specific Abs have been detected in the sera of NOD mice (19), but these results are still controversial (20, 21).

To investigate the immune responsiveness to IA-2 in NOD mice, we analyzed T cell responses to the cytoplasmic portion of mouse rIA-2 in unprimed mice. We report in this study that T cell
responses to IA-2 are already detectable in 3-wk-old unprimed NOD mice, suggesting that this self.Ag may be critical in IDDM initiation.

Materials and Methods

Mice
NOD/Lt, BALB/c, and C57BL/6 female mice were purchased from Charles River Laboratories (Calco, Italy) and Biozzi AB/H female mice from Harlan (Bicester, U.K.). In a few experiments, as described in the text, NOD male mice were also used. All mice were kept under specific pathogen-free conditions. Glucose levels in the tail venous blood were quantified using a Glucometer Elite (Bayer, Wuppertal, Germany). A diagnosis of diabetes was made after two sequential glucose measurements higher than 200 mg/dl.

Antigens
The hen egg white lysozyme (HEL) peptide 10–22 was synthesized as described previously (22). The GAD peptide 524–543 was synthesized by F-moc/tBu chemistry using a 431A automated synthesizer (Applied Biosystems, Foster City, CA). The crude peptide was purified by reverse-phase HPLC and the sequence was confirmed by amino acid analysis and fast atom bombardment mass spectrometry. A BamHI-EcoRI fragment encoding the protein tyrosine phosphatase 35 intracellular domain (aa 601–979) was amplified by PCR on the protein tyrosine phosphatase 35 cDNA (23) and subcloned into the expression vector pGEX-2T (Pharmacia, Uppsala, Sweden). Mouse rIA-2 was expressed in Escherichia coli as a GST-fusion protein and purified by affinity chromatography on glutathione-Sepharose, followed by thrombin cleavage to recover >98% pure mouse rIA-2 (rmIA-2), as described (24). The endotoxin content was 5 ng/mg rmIA-2, as determined by the Limulus amebocyte lysate test (Sigma, St. Louis, MO). The fragment corresponding to residues 302–506 of the human tyrosine/threonine phosphatase cyclin-dependent kinase complex (CDC) 25B was obtained by PCR from the cd25B cDNA (25), expressed in E. coli as a GST-fusion protein, and purified under exactly the same conditions used for rmIA-2.

Injections
Mouse rIA-2, HEL (Sigma), or horse myoglobin (Mb; Sigma) emulsified in IFA (Difco, Detroit, MI), was injected once i.p. (100 μg/mouse) into 18-day-old NOD mice. Eight-week-old NOD mice were immunized s.c. into the hind footpads with IA-2 (100 μg/mouse) emulsified in CFA containing H37Ra mycobacteria (Difco). Mouse rL2,12, obtained as previously described (26), was diluted in PBS containing 100 μg/ml mouse serum albumin (Sigma), and injected daily i.p. (7.5 μg/kg) in NOD mice starting at 12 days of age.

Cell cultures
Total spleen cells (10^6 cells/well) from unprimed mice were cultured for 24–48 h, as specified in the figure legends, in flat-bottom 96-well plates (Costar 3595; Costar, Cambridge, MA) with the indicated Ag concentrations. Total spleen cells (3 × 10^5 cells/well) were also cultured for 48 h in round-bottom 96-well plates (Costar) precoated with 3 μg/ml purified anti-I-TCR mAb (ATCC HB 218; American Type Culture Collection, Manassas, VA). Alternatively, CD4+ cells were positively selected from pancreatic or mesenteric lymph nodes or spleen of NOD mice, using anti-CD4-coated magnetic MicroBeads and Mini-MACS separation columns (Miltenyi Biotech, Auburn, CA). Purified CD4+ cells (2 × 10^5/well) were purified for 72 h with rmIA-2 and 10^5 T cell-depleted spleen cells as APCs. T cells were depleted by cyotoxic treatment with HO-13-4 anti-Thy-1 mAb (Low-Tox M; Cedarlane Laboratories, Hornby, Ontario, Canada). In some experiments, the few remaining T cells were removed using anti-CD4- and anti-CD8-coated MicroBeads and Mini-MACS separation columns. Blocking assays were performed using the following mAbs: GK1.5 anti-CD4 (ATCC TIB 207), KT1.5 anti-CD8 (27), and 10.3.62 anti-I-AP0 and 14.4.4S anti-I-Eu (PharMingen, San Diego, CA). Culture medium was RPMI 1640 (Life Technologies, Rockville, MD) supplemented with 2 mM l-glutamine and 50 μg/ml gentamicin (Sigma). For culture of cells from primed mice, polypeptil lymph nodes were removed and 4 × 10^6 cells/well were cultured in 96-well culture plates (Costar 3595) in synthetic HL-1 medium (Ventrex Laboratories, Portland, ME) supplemented with 2 mM l-glutamine and 50 μg/ml gentamicin (Sigma) with the indicated Ag concentrations. To measure cell proliferation, cultures were incubated for 3 days in a humidified atmosphere of 5% CO2 in air and pulsed 8 h before harvesting with 1 μCi [3H]TdR (40 Ci/nmol; Radiochemical Center, Amersham, U.K.). Incorporation of [3H]TdR was measured by liquid scintillation spectrometry.

Quantification of secreted cytokines
Cytokines were quantified by two-site ELISA. To detect IFN-γ, polynvinyl microtiter plates (Falcon 3012; Becton Dickinson, Mountain View, CA) were coated with 100 μl of AN.18.17.24 mAb (28) in carbonate buffer. After blocking, samples (50 μl/well) diluted in test solution (PBS containing 5% FCS and 1 g/L phenol) were incubated along with 50 μl peroxi- dase-conjugated XMGI2 mAb (29). After overnight incubation at room temperature, bound peroxidase was detected by 3,3’5,5’-tetramethylben- zidine (Fluka Chemical, Ronkonkoma, NY), and absorbance was read at 450 nm with an automated microplate ELISA reader (MR5000; Dynatech Laboratories, Chantilly, VA). IL-2, IL-4, IL-6, and IL-10 were determined using paired mAb from PharMingen. For capture, the mAb JES6-1A12 (anti-IL-2), 11B11 (anti-IL-4), MP5-20F3 (anti IL-6), or JESS-2A5 (anti-IL-10) were used diluted in PBS. Samples were titrated in test solution and incubated overnight at 4°C. To detect bound cytokines, plates were then incubated for 1 h with the biotinylated mAb JES6-5H4 (anti-IL-2), BVD6- 24G2 (anti-IL-4), MP5-32C11 (anti-IL-6), or JESS-16E3 (anti-IL-10) in test solution. After washing, the bound biotinylated Abs were revealed by a 30-min incubation with alkaline phosphatase-conjugated streptavidin (Jackson ImmunoResearch Laboratories, Avondale, PA) diluted 1/5000. The plates were washed again and incubated with the developing substrate p-nitrophenylphosphate disodium (Sigma) in diethanolamine buffer, pH 9.6 (100 μl/well). The reaction was stopped by adding 50 μl/well 3 N NaOH and absorbance was read at 405 nm. Cytokines were quantified from two to three titration points using standard curves generated by purified recombinant mouse cytokines, and results expressed as cytokine concentration in pg or ng/ml. Detection limits were 15 pg/ml for all cytokines.

Statistical analysis
IFN-γ levels secreted in response to IA-2 by spleocytes from female and male NOD mice were compared using a two-tailed Mann-Whitney U test. The proportions of mice that eventually became diabetic were compared by Fisher’s exact test. Differences were considered to be statistically significant at p < 0.05.

Results
Reactivity to GAD and IA-2 Ags in spleen cells from unprimed NOD mice
GAD65-specific IFN-γ-producing Th1 cells develop spontaneously in NOD mice and were initially revealed by spleen cell proliferation or IFN-γ production in response to GAD65, or its immunodominant peptides 247–266, 509–528, and 524–543 (4). To assess whether IA-2-specific type 1 responses also develop spontaneously in NOD mice, spleocytes from 7- to 8-wk-old unprimed mice were cultured with different concentrations of intracytoplasmic rmIA-2 (aa 601–979) and control Ags. IFN-γ secretion was measured in culture supernatants (Fig. 1), although cell proliferation was never observed (Fig. 2). NOD spleen cells secreted dose-dependently IFN-γ in response to rmIA-2 and GAD 524–543, but failed to respond to HEL 10–22, an IA-8-binding peptide not associated with IDDM (Fig. 1). In addition, NOD spleen cells did not respond to the tyrosine/threonine phosphatase CDC25B (aa 302–506), an irrelevant phosphatase produced and purified under the same conditions as rmIA-2 (Fig. 1). Spleocytes from Biozzi AB/H, BALB/c, and C57BL/6 were also cultured with rmIA-2 to assess whether this response was specific of the NOD mouse. Biozzi AB/H express the same IA-8 molecule as NOD mice, but fail to develop IDDM (30). Spleocytes from neither Biozzi AB/H mice, nor BALB/c or C57BL/6 mice produced IFN-γ when stimulated with rmIA-2 or with the GAD immunodominant peptide 524–543. In contrast, IA-2 and GAD 523–543 both induced IFN-γ production by NOD spleen cells (Fig. 1). Thus, a response to IA-2 is specifically elicited in spleen cells from unprimed NOD, but not other mouse strains, including Biozzi AB/H sharing with NOD mice the IA-8 molecule.
Response to IA-2 by lymph node cells from primed NOD mice

To further confirm the capacity of NOD mice to respond to IA-2, 8-wk-old NOD females were primed with 100 μg/mouse rmIA-2 emulsified in CFA. Nine days later, lymph node cells were restimulated in vitro with different concentrations of IA-2, and cell proliferation as well as IFN-γ production were measured. Results in Fig. 2 show Ag dose-dependent proliferation and IFN-γ secretion in response to IA-2 by lymph node cells from primed NOD mice. Conversely, no proliferation was observed after culture of spleen cells from unprimed NOD mice with IA-2 but, as already shown in Fig. 1, IFN-γ was secreted. These results demonstrate the capacity of IA-2 to prime NOD mice, indicating that the intracytoplasmic portion of IA-2 can be processed and presented in vivo, and show that a proliferative response to IA-2 can be induced by Ag priming. Interestingly, spleen cells from unprimed NOD mice respond to IA-2 by secreting IFN-γ in the absence of detectable proliferation.

Cytokine profile in response to IA-2 by spleen cells from unprimed NOD mice

In addition to IFN-γ, spleen cells from unprimed 8-wk-old NOD mice incubated in vitro with rmIA-2 produced IL-6 and little IL-10, but no IL-4 or IL-2 (Fig. 3). The absence of IL-2 production was consistent with the absence of proliferation to IA-2 (Fig. 2). Spleen cells from 8-wk-old Biozzi AB/H mice, cultured in the same conditions, did not secrete any of the cytokines tested (Fig. 3), nor did they proliferate (data not shown). Conversely, when stimulated with insolubilized anti-TCR mAb, splenocytes from NOD and Biozzi AB/H mice secreted similar levels of IL-2, IL-6, IL-10, and IFN-γ (Fig. 3). Interestingly, NOD cells did not produce IL-4 neither in response to IA-2 nor after TCR ligation, reflecting a defective production of this cytokine (Fig. 3). In conclusion, these data indicate that spleen cells from unprimed NOD mice, but not other I-Ag7-expressing mouse strains, can be stimulated by IA-2 to mount a response dominated by IFN-γ secretion.

Age-dependent cytokine response to IA-2 in spleen cells from unprimed NOD mice

We next tested the cytokine secretion by NOD splenocytes at various time points to determine whether reactivity to IA-2, like GAD, was present at the beginning of insulitis. Indeed, GAD-reactive T cells were considered to play a crucial role because they were detected at 4 wk of age in unprimed NOD mice (4, 8). We found that spleen cells from 3-wk-old NOD female mice did respond to IA-2 by secreting IFN-γ, IL-10, and IL-6 (Fig. 4). The cytokine response varied with the age of NOD mice, indicating a dynamic process particularly during the first week of age. A peak of IA-2-induced IFN-γ production, about 20 ng/ml, was seen at 8 wk of age (Fig. 4). IL-6 production by spleen cells in response to IA-2 did not change substantially between 3 and 15 wk of age, and IL-4 was always undetectable (Fig. 4). Intriguingly, IL-10 secretion induced by IA-2 peaked sharply at 4 wk and was almost undetectable by 8 wk of age. Splenocytes from 20-wk-old recently diabetic mice secreted very low amounts of IFN-γ and no IL-10 or IL-4. IL-6 was still secreted in response to rmIA-2, but at lower levels compared with 3- to 15-wk-old normoglycemic NOD mice (Fig. 4). A comparison of IFN-γ levels (pg/ml ± SD) secreted in response to IA-2 by splenocytes from female and male NOD mice revealed both in 4- and 8-wk-old mice a trend toward a reduced response in spleen cells from male compared with female mice, but no statistical difference (4-wk-old mice, n = 11: females, 247 ± 189 vs males, 142 ± 105; 8-wk-old mice, n = 5: females, 34,276 ± 17,294 vs males, 22,402 ± 6,465). Collectively, these results show that a response to IA-2, like GAD65, can be detected as early as 3 wk of age in unprimed NOD mice. In addition, IA-2-specific IFN-γ production, characteristic of a type 1 T cell response, increases progressively with a peak at about 8 wk of age, a few weeks before IDDM onset.
CD4+ cells from different lymphoid organs of unprimed NOD mice secrete IFN-γ in response to IA-2

We also determined whether IFN-γ was secreted by CD4+ cells in response to IA-2 and whether these cells were present in different tissues of the NOD mouse. Positively selected CD4+ cells from mesenteric or pancreatic lymph nodes, or from the spleen of unprimed NOD female mice were cultured with rmIA-2 and T cell-depleted splenocytes as a source of APCs. As shown in Fig. 5, IA-2 induced dose dependently IFN-γ secretion in CD4+ cells from all tissues examined, more abundant in CD4+ cells from spleen than from lymph nodes at the highest IA-2 concentration tested. As with spleen cells, IFN-γ secretion in response to IA-2 was observed in the absence of detectable cell proliferation.

IFN-γ secretion by IA-2-specific CD4+ cells requires Ag presentation by I-Aγ7 molecules and CD4 engagement

To define the basic requirements for IA-2-induced IFN-γ secretion by CD4+ cells, neutralizing mAbs specific for coreceptors and class II MHC molecules were added to cultures of positively selected CD4+ cells, rmIA-2, and T cell-depleted splenocytes as a source of APCs. As shown in Fig. 6, a near-complete inhibition of IA-2-specific IFN-γ secretion was induced by an anti-CD4, but not an anti-CD8 mAb with the same isotype. This result, obviously, does not imply lack of IA-2-specific CD8+ responses in the NOD mouse. Similarly, the IFN-γ response induced by IA-2 in CD4+ cells was inhibited by an anti-I-Aβγ7, but not an isotype-matched anti-I-Eα mAb. These results demonstrate that CD4+ T cell activation by IA-2 requires Ag presentation by I-Aγ7 and engagement of the CD4 coreceptor molecules.

IA-2 administration to NOD mice exacerbates IDDM

Oral, i.v., intranasal, intrathymic, and s.c. administration of insulin, GAD, or their peptides have been shown to modulate IDDM in NOD mice (4, 8, 10, 31, 32). To determine whether IDDM development could be affected by IA-2 administration, the recombinant mouse protein emulsified in IFA was injected once (100 μg/mouse) i.p. into 18-day-old NOD mice. The cumulative incidence of IDDM was significantly higher in IA-2-treated mice compared with controls injected with HEL, an immunogenic protein in NOD mice (33), or Mb in IFA (p = 0.043 by two-tailed Fisher’s exact test), indicating the importance of IA-2 responsiveness in IDDM progression (Fig. 7).

Early administration of IL-12 into NOD mice provokes IDDM and induces IFN-γ secretion to IA-2

Because the IA-2-specific Th1-like response is low in 3-wk-old NOD mice (Fig. 4), we thought to exacerbate it by administering IL-12 to NOD mice before this age. We have previously shown
that daily IL-12 administration to adult NOD mice induces Th1 cells and accelerates IDDM (26). Daily IL-12 administration from 12 to 44 days of age provoked IDDM onset in about 75% of NOD mice before 7 wk of age, whereas none of the vehicle-injected mice developed IDDM (Fig. 8). Usually, spontaneous IDDM onset in our NOD colony starts at about 11–15 wk of age (34). We quantified the IA-2-induced IFN-γ secretion by splenocytes from vehicle- and IL-12-treated mice after six injections. IFN-γ was not secreted in response to IA-2 by spleen cells from 18-day-old vehicle-treated NOD mice (Fig. 8B), indicating that a T cell response to IA-2 can be first detected only after 18 days of age (Fig. 4). In contrast to cells from vehicle-treated littermates, splenocytes from 18-day-old NOD mice, injected with IL-12 daily from 12 to 17 days of age, secreted IFN-γ in response to IA-2 (Fig. 8B). There was no response to CDC25B, a protein produced under the same conditions as IA-2. IL-10 was undetectable in these cultures, and IL-6 secretion in response to IA-2, already induced at low levels in vehicle-treated mice, was up-regulated by IL-12 administration (data not shown). Interestingly, cells from vehicle- and IL-12-treated 18-day-old NOD mice did not respond to GAD 524–543, suggesting that a response to this epitope occurs later in the disease process (Fig. 8B). In conclusion, early administration of IL-12 to NOD mice induces IA-2-specific IFN-γ production and provokes IDDM, suggesting a link between the two events.

Discussion

Results in the present work demonstrate that the tyrosine phosphatase-like protein IA-2 is an autoantigen able to induce a T cell response in unprimed NOD mice and to exacerbate IDDM development. IA-2, corresponding to the islet cell Ag 512 (35, 36), and IA-2β, also known as phogrin (37–39), are autoantigens associated with human IDDM and were originally identified by Abs immunoprecipitating 40- and 37-kDa proteolytic islet polypeptides, respectively (13–15). These 40- and 37-kDa fragments encompass most of the cytoplasmic domain of IA-2 and IA-2β, respectively (15, 16, 36). Although both molecules have extracellular, transmembrane, and intracellular domains, autoantibodies from IDDM patients were found to react almost exclusively with the cytoplasmic domain. Thus, we have focused our study on the intracellular portion of mouse IA-2, and found that it can induce a response in CD4+ cells from unprimed NOD mice.

Several self-Ags have been described to be associated with human IDDM (40), but three appear to be most relevant: GAD, insulin, and IA-2. The presence of Abs to these autoantigens is now routinely used for screening, diagnosis, and prognosis of human IDDM (41). The combined detection of Abs to insulin, GAD, and IA-2 identifies a large proportion of individuals at risk and about 90% of patients with IDDM (41, 42). Autoantibodies to IA-2β, although less common than anti-IA-2 Abs (16, 36), are also associated with human IDDM. Conversely, autoantibodies to IDDM-associated Ags are not easily detectable in NOD mice. Anti-GAD Abs were detected (8, 43), but their presence was not confirmed (44–46). Similarly, a low anti-IA-2 Ab level has occasionally been reported in NOD mice (19), but this finding has not been confirmed (20, 21). However, anti-insulin Abs have been observed in the unprimed NOD mouse (31), and early expression of anti-insulin Abs, at 8 wk, has been found to correlate with early IDDM onset, at 16–18 wk of age (47).

IDDM-associated human autoantibodies to GAD, insulin, and IA-2 are mostly of IgG1 isotype (48), indicating that autoantigen-specific CD4+ T cells have been induced. PBMC from prediabetic patients or from patients with newly diagnosed IDDM proliferate in vitro to a number of β cell proteins, including insulin (49), GAD65 (50), GAD67 (51), and IA-2 (52). Although characterization of the human T cell response using PBMC is still problematic (53), T cell epitopes from GAD, insulin, and IA-2 presented by IDDM-associated HLA class II alleles have been identified by testing overlapping synthetic peptides (54–56), analyzing HLA class II transgenic mice (57–59), and eluting peptides from the IDDM-associated HLA-DR4 molecule (60). T cell responses to GAD (4, 8) and insulin (61) have also been documented in the unprimed NOD mouse. The identification of T cell responses against the intracytoplasmic portion of IA-2 in the unprimed NOD mouse described in the present study now demonstrates that IA-2 is also a relevant autoantigen in NOD IDDM, further strengthening the similarities between human and NOD mouse type 1 diabetes.

We found IA-2-specific CD4+ T cells in different lymphoid organs of the NOD mouse. However, pancreatic and mesenteric CD4+ T cells from 8-wk-old mice responded less strongly to IA-2 than splenic CD4+ cells, suggesting a different frequency of autoantigen-specific cells in these organs. This result parallels the observation that lymph node T cells from NOD mice transfer diabetes less efficiently than T cells isolated from the spleen (62).

The T cell response to IA-2 in unprimed NOD mice could not be detected by simply measuring Ag-induced T cell proliferation, consistent with the lack of detectable IL-2 secretion. Defective IL-2 production was also observed in the syngeneic MLR (63). In addition, splenic T cells from NOD mice were found defective in their capacity to proliferate to TCR ligation, most likely due to a

FIGURE 6. IFN-γ secretion by IA-2-specific CD4+ cells requires Ag presentation by I-Aβ7 molecules and CD4 engagement. CD4+ cells (2 × 10^6/well) were cultured for 72 h with 0.3 μM rmIA-2, with or without 3 μg/ml of the indicated mAbs, and 10^5 T cell-depleted splenocytes as a source of APCs. All cells were isolated from pooled splenocytes from five 11-wk-old NOD mice. The mAbs used were anti-CD4 (rat IgG2b mAb GK1.5), anti-CD8 (rat IgG2b mAb KT1.5), anti-IA-2β7 (mouse IgG2a mAb 10.3.6.2), and anti-I-Eα (mouse IgG2a mAb 14.4.4S). The amounts of IFN-γ secreted by CD4+ cells in a representative experiment of three performed are shown.

FIGURE 7. Administration of IA-2 IFA to 3-wk-old NOD mice exacerbates IDDM. rmIA-2, HEL, or Mb emulsified in IFA was injected i.p. once into 18-day-old NOD female mice (10 mice/group). The proportion of mice that became diabetic was significantly higher in IA-2-treated mice compared with the control group, HEL−, or Mb-treated mice (p = 0.043, two-tailed Fisher’s exact test).
poor secretion of both IL-2 and IL-4 (64). We found that NOD compared with Biozzi AB/H spleen cells were defective in IL-4, but not IL-2, production following TCR ligation. The addition of exogenous IL-4 could restore the proliferative responsiveness of NOD T cells by enhancing their IL-2 secretion (64). Intriguingly, the IDDM susceptibility locus idd 3 has been mapped to a 0.35-cM interval containing the il-2 gene (65). The NOD il-2 gene appears to be an allelic variant resulting in a serine-to-proline substitution at position 6 of the mature IL-2 protein, which is, however, functional (65). Thus, IFN-γ, IL-10, and IL-6 secretion by spleen cells from unprimed prediabetic NOD mice to the intracytoplasmic domain of IA-2 is neither associated with cell proliferation nor IL-2 production, indicating that proliferation assays are not suitable for the detection of T cell responses to IA-2 in the unprimed NOD mouse.

IFN-γ was the dominant cytokine produced by NOD T cells in response to IA-2, and IL-4 remained always undetectable, concordant with the pathogenic role of TH1 cells in IDDM development (26). A predominant IFN-γ response has also been observed in NOD mice in response to GAD peptides (4), and this cytokine may represent an important marker of T cell reactivity in IDDM development. A time-course analysis revealed that IFN-γ is already secreted in response to IA-2 by spleen cells from 3-wk-old NOD mice, although it is not detectable at 18 days of age. Its production, peaking at 8 wk, remained high from 6 to 12 wk of age, when Th1-type cells progressively infiltrate the pancreatic islets (34), but before IDDM onset. Indeed, by 15 wk of age, the IFN-γ response to IA-2 was very low, although still detectable in most NOD mice, and it also remained low in recently diabetic mice tested at 20 wk of age. This indicates that the IFN-γ response to IA-2 arises early in IDDM development and it appears to be concomitant with the anti-GAD (4, 8) and anti-insulin (66) T cell response. A low proliferative response to IA-2β has also been detected in unprimed 12-wk-old NOD mice, but its kinetics were not analyzed (67). The early IFN-γ response to IA-2 is consistent with its possible role in IDDM pathogenesis. This is further supported by the IL-12-induced IDDM provocation accompanied by IFN-γ secretion in response to IA-2. Moreover, IL-12 antagonist administration from 3 wk of age reduced IDDM incidence and IFN-γ secretion in response to IA-2 (34).

We could also detect IL-10 in response to IA-2, although at lower levels compared with IFN-γ secretion. IL-10 production peaked sharply at 4 wk of age, when insulitis is first observed in NOD mice. Given the protective role of IL-10 in IDDM, when administered to adult NOD mice (68, 69), its reduction concomitant with the rise of IFN-γ secreted in response to IA-2 and the initiation of islet infiltration suggests an aborted regulatory mechanism. Conversely, the IL-6 secretion in response to IA-2 was relatively stable from 4 to 15 wk of age and declined only slightly in recently diabetic NOD mice. Consistent with this result, persisting IL-6 production has been detected between 6 wk and 6 mo of age in the pancreas of NOD mice, and this cytokine appears to be secreted by various cell types, including T cells, APCs, and even islet β cells (70, 71). IL-6 seems important for the development of IDDM, as demonstrated by the significant reduction of diabetes incidence following treatment of NOD mice with neutralizing anti-IL-6 mAb (72). Moreover, transgenic expression of IL-6 in β cells leads to the development of insulitis in mouse strains with some NOD genetic components (73). Undoubtedly, IL-6 plays a pivotal role in inflammatory responses (74), also by amplifying leukocyte recruitment (75). However, IL-6-transgenic NOD mice present marked insulitis, but a decreased IDDM incidence, suggesting that IL-6 is not directly involved in the effector mechanisms leading to β cell destruction (73).

Autoantigen administration to NOD mice has usually been found to prevent IDDM development. For instance, i.v. or intrathymic administration of GAD to young NOD mice, soon after weaning, can inhibit IDDM development (4, 8). Protection has also been reported after intranasal administration of a combination of GAD peptides (10). Likewise, aerosol administration of insulin (31) and intranasal or s.c. administration of the insulin β-chain peptide 9-23 (32) to NOD mice have a beneficial effect on IDDM. Nevertheless, in some cases, autoimmune administration may accelerate rather than retard diabetogenesis (76). Thus, when injected into the thymus of 4-wk-old NOD mice, GAD65 significantly retards IDDM development, whereas GAD peptides 509–528 or 524–543 accelerate diabetes onset (77). In addition, s.c. or i.p. immunizations with GAD65 in IFA protect NOD mice from IDDM (9, 21). However, s.c. and i.p. injections of the insulin β-chain in IFA have opposite effects, the former being protective and the latter having a diabetogenic effect (21, 78). Studies of IDDM modulation by mouse IA-2 administration to NOD mice have not yet been reported, but human IA-2 injected s.c. in IFA at 4, 8, and 12 wk of age in NOD mice does not affect IDDM development (21). Our results now show that i.p. injection of mouse IA-2 in IFA into 18-day-old NOD mice significantly enhances IDDM development, indicating that a response to IA-2 can exert a pathogenic role. The enhancement of IDDM development by a

FIGURE 8. Early administration of IL-12 into NOD mice provokes IDDM and induces IFN-γ secretion to IA-2. A, rmIL-12 (7.5 μg/kg) or vehicle (PBS containing 100 μg/ml mouse serum albumin) was injected i.p. into NOD female mice daily from 12 to 44 days of age. A diagnosis of diabetes was made after two sequential glucose measurements higher than 200 mg/dl. B, rmIL-12 or vehicle was administered i.p. into NOD female mice daily for 6 days, starting at 12 days of age. Total spleen cells (10⁶ cells/well) from IL-12- or vehicle-treated individual NOD mice (four mice per group) were cultured without Ag or with 30 μM GAD 524–543, 0.3 μM CDC25B, or 0.3 μM rmIA-2. After 48 h, IFN-γ secretion was quantified in culture supernatants by two-site ELISA.
single administration of IA-2 is consistent with the observation that Th1-type cells with diabetogenic activity can be generated by immunizing NOD mice with GAD65 (7) or with IA-2B (67). A pathogenic role of the T cell response to IA-2 is further supported by the observation that IL-12 administration to 12-day-old NOD mice provokes IDDM and induces IFN-γ secretion in response to IA-2, but not to GAD 524–543. Thus, a Th1-type response to IA-2 appears earlier than the response to the GAD peptide 524–543, a determinant from GAD65 able to induce an early T cell response in NOD spleen cells (4).

In conclusion, the T cell response to IA-2 represents an early event in IDDM pathogenesis, both in humans and in the NOD mouse. This implies that the autoantibodies to IA-2 seen in human IDDM not only reflect destruction of pancreatic β cells, releasing the intracytoplasmic IA-2 Ag that in turn may induce an immune response, but also, and perhaps primarily, the helper activity of pathogenic T cells.

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