

Kinetics of TNF-alpha and IFN-gamma mRNA expression in islets and spleen of NOD mice

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Abstract

Insulin-dependent diabetes mellitus is caused by autoimmune destruction of pancreatic β cells. Non-obese diabetic (NOD) mice spontaneously develop diabetes similar to the human disease. Cytokines produced by islet-infiltrating mononuclear cells may be directly cytotoxic and can be involved in islet destruction coordinated by CD4⁺ and CD8⁺ cells. We utilized a semiquantitative RT-PCR assay to analyze *in vitro* the mRNA expression of TNF- α and IFN- γ cytokine genes in isolated islets (N = 100) and spleen cells (5×10^5 cells) from female NOD mice during the development of diabetes and from female CBA-j mice as a related control strain that does not develop diabetes. Cytokine mRNAs were measured at 2, 4, 8, 14 and 28 weeks of age from the onset of insulinitis to the development of overt diabetes. An increase in IFN- γ expression in islets was observed for females aged 28 weeks (149 ± 29 arbitrary units (AU), $P < 0.05$, Student *t*-test) with advanced destructive insulinitis when compared with CBA-j mice, while TNF- α was expressed in both NOD and CBA-j female islets at the same level at all ages studied. In contrast, TNF- α in spleen was expressed at higher levels in NOD females at 14 weeks (99 ± 8 AU, $P < 0.05$) and 28 weeks (144 ± 17 AU, $P < 0.05$) of age when compared to CBA-j mice. The data suggest that IFN- γ and TNF- α expression in pancreatic islets of female NOD mice is associated with β cell destruction and overt diabetes.

Key words

- Diabetes
- NOD mice
- Cytokines
- RT-PCR
- Pancreatic islets
- Time course

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Introduction

Type I diabetes mellitus is a T cell-dependent autoimmune disease resulting in selective destruction of β cells of the islet of Langerhans (1). Non-obese diabetic (NOD) mice spontaneously develop type I diabetes mellitus and serve as an animal model for human type I diabetes mellitus (2-4). The

occurrence of a mixed lymphocytic population in pancreatic β -islets (insulinitis) can result in progressive β cell destruction, insulin deficiency and hyperglycemia. Cells such as macrophages and dendritic cells appear early, followed by CD4⁺ and CD8⁺ T cells (5-7).

The first stage of insulinitis occurs when the lymphocytic infiltrate is located around the islet (peri-islet) without significant β cell

destruction and usually begins at 4 weeks of age in both female and male NOD mice. The second stage, that occurs between 12 and 25 weeks of age (median at 15 weeks of life), is characterized by intra-islet infiltration and an aggressive attack upon the insulin-producing cells, leading to a loss of β cell mass and overt diabetes. Interestingly, in most NOD mouse colonies (8) a clear sexual dimorphism is present showing high incidence of diabetes among females (60-90%) and low incidence among males (10-20%). Both CD4⁺ and CD8⁺ T cell subsets are required for development of diabetes in NOD mice (9-12). However, the precise mechanisms by which T cells destroy β cells are unclear. Studies on the correlation between cytokines expressed in islets and autoimmune diabetes in NOD mice have demonstrated that β cell destructive insulinitis is associated with increased levels of proinflammatory cytokines (IL-1, TNF- α and IFN- γ) (13-20). It has now been well documented *in vitro* that certain cytokines are cytotoxic to pancreatic islets. IL-1, TNF- α and IFN- γ impair insulin secretion and when they are added to synergic cytokines their phlogistic effect is amplified, leading to β cell destruction *in vitro* (14,20), although *in vivo* the direct cytotoxicity of cytokines to β cells remains to be demonstrated. On the other hand, the cytokines can be cytotoxic to β cells by inducing nitric oxide and oxygen free radicals (21-23).

Expression of cytokine mRNA in islet lesion has been characterized by the reverse transcriptase-polymerase chain reaction (RT-PCR) assay. In NOD mice, the numbers of infiltrating cells increase with age and there is elevated expression of IL-2 and IFN- γ after 13 weeks of age in NOD females (24). Furthermore, there is evidence suggesting that IFN- γ may be directly toxic to β cells, decreasing the secretion of insulin *in vitro* (14,25). TNF- α mRNA expression is detected in islets of NOD mice; however, the direct effect of TNF- α on β cells upon the

progression to diabetes remains unclear; it is possible that TNF- α can lead to insulinitis but not to diabetes (21,26). Nevertheless, taken as a whole, the observations suggest that the presence of these cytokines, IFN- γ and TNF- α , may be playing an important role in β cell destruction and development of diabetes (27). However, the relation between the expression of these cytokines in islets and in spleen cells and the progression of autoimmune diabetes in NOD mice aged 2 to 28 weeks remains to be studied. The purpose of the present study was to analyze the mRNA expression of IFN- γ and TNF- α in islets and spleen of female NOD mice at five times (2, 4, 8, 14 and 28 weeks) from the onset of insulinitis to the development of overt diabetes, using a semiquantitative RT-PCR assay. Furthermore, we compared the degree of insulinitis severity detected by morphological studies, and IFN- γ /TNF- α expression at the same time points.

Material and Methods

Animals and experimental groups

NOD/Uni mice originating from the colony at INSERM U-25, Hospital Necker (Paris, France) and maintained at the center of animal breeding of the State University of Campinas, São Paulo, Brazil (28) under germ-free conditions were transferred to a specific pathogen-free animal facility in our laboratory. The incidence of spontaneous diabetes in our colony was 60% in females and 5% in males by 25 weeks of age. The animals were maintained on an autoclaved free water and food regimen under 12-h cycles of darkness and light. The animals used in this study included female NOD and CBA-j mice as a related strain that does not develop diabetes, at 2, 4, 8, 14 and 28 weeks of age, and diabetic NOD mice a few days after the onset of the disease. Mice were monitored for the onset of diabetes by measurements of urine glucose using Test-strips (Eli Lilly do Brasil

Ltda., São Paulo, SP, Brazil) twice a week. The glucose concentration in blood obtained from a tail vein was measured using Prestige LX Smart System Test-strips (Home Diagnostic, Inc., Fort Lauderdale, FL, USA). Consecutive readings of blood glucose levels ≥ 300 mg/dl (12 mmol/l) accompanied by glycosuria on two consecutive days were considered to be diagnostic of diabetes onset.

In the first set of experiments, 42 female NOD mice divided into six groups of 7 animals each at 2, 4, 8, 14 and 28 weeks of age, 10 diabetic mice (aged 17 to 28 weeks), and 25 CBA-j mice, as a non-correlated strain, were divided into five groups of 5 animals each at same age as NOD mice. The mice were killed by a sodium pentobarbital overdose and the pancreas was removed for morphological classification of islet inflammation stages (insulinitis). In the second set of experiments, 35 female NOD mice and 35 female CBA-j mice divided into five groups of 7 animals each at 2, 4, 8, 14 and 28 weeks of age were used. The pancreas was removed at each time and islets were isolated and separately processed. Total RNA was extracted from a pool of 100 islets obtained from 7 animals for semiquantitative study of cytokine mRNA expression by RT-PCR.

Histological analysis

To evaluate the severity of insulinitis in non-diabetic females and diabetic female NOD mice the pancreas from each animal was snap-frozen in Tissue-Tek OTC embedding compound (Miles Laboratories Inc., Clifton, NJ, USA) on liquid nitrogen. Cryostat sections were prepared with a cryostat model CM1850 (Leica Instruments, Nußloch, Germany) as follows: nine consecutive 5- μ m sections were cut and placed on different slides coated with γ -methacryloxypropyl-methoxysilane (Sigma, St. Louis, MO, USA) with a cryostatic distance of 300 μ m between sections. Eighteen slides with three

sections were obtained for each specimen. The histological sections were stained with hematoxylin and eosin and examined by light microscopy (Zeiss, Axioscop, Jena, Germany). The severity of insulinitis was classified by the magnitude of mononuclear cell infiltration: grade 0, intact islet, with no infiltration by mononuclear cells; 1, peri- and intra-islet infiltration of $<25\%$; 2, intra-islet infiltration of 25-80%; 3, invasive insulinitis of $>80\%$; 4, destructive insulinitis, with total islet invasion by mononuclear cells. A mean insulinitis score was calculated. The number of total islets was determined and the mean was estimated considering the number of sections examined and animals studied.

Islet isolation

Pancreatic islets were isolated from mice by stationary collagenase digestion of the pancreas using previously described methods (29,30) with minor modifications. Mice were anesthetized by intraperitoneal injection of 3% sodium pentobarbital (20 mg/kg). The pancreas was excised, minced and digested with collagenase V (Sigma) for 15 min. Islets were isolated and collected under a dissection microscope with a micropipette. The number of islets (50-100/mice) recovered depended on age. After isolation, the cell number was counted and viability was checked by Trypan blue exclusion. Only a batch of islets with more than 90% viability was used for RT-PCR procedure.

Spleen cell isolation

Mice were anesthetized by intraperitoneal injection of 3% sodium pentobarbital (20 mg/kg). Spleen cells were excised, passed through a stainless-steel mesh screen and washed three times in Hank's (Hyclone, CA, USA) balanced salt solution. Isolated spleen cells were diluted to 5×10^6 cells in a solution containing 4 M guanidinium thiocyanate, 0.1 M 2- β -mercaptoethanol, 25 mM

sodium citrate, pH 7.0, and 0.5% sarkosyl (N-lauroylsarcosine, sodium salt; Sigma), frozen in liquid nitrogen and maintained at -80°C until use.

Total RNA extraction

Total RNA was prepared by the guanidinium thiocyanate method (31) from pooled islets isolated from 7 female NOD mice (100 islets/mouse) and CBA-j mice (non-diabetic animal control) at different ages (2, 4, 8, 14 and 28 weeks of age). Pooled islets were immediately transferred to Rnase-free plastic tubes containing 4 M guanidinium thiocyanate, 0.1 M 2- β -mercaptoethanol, 25 mM sodium citrate, pH 7.0, and 0.5% sarkosyl, frozen in liquid nitrogen and maintained at -80°C until the time for use. All aliquots were analyzed by spectrophotometry (Spectra Max 190, Molecular Devices, Sunnyvale, CA, USA) before use. The ratio of readings at 260/280 nm was determined and only samples presenting a ratio between 1.6 and 1.8 were used.

RT-PCR

cDNA was synthesized from 5 μg of total RNA using 0.5 μl of Oligo d(pt) and ultrapure water to complete 31 μl . Tubes were heated to 65°C for 10 min and then refrigerated at 4°C for 5 min when 18 μl of reaction solution (10 μl super RT buffer, 2 μl 0.5 mM dNTP mix, 5 μl 0.1 M DTT and 1 μl RNAsin; GibcoBRL, Life Technologies, Gaithersburg, MD, USA) was added. The temperature was then set at 42°C for 2 min and 1 μl SuperscriptTM RT (GibcoBRL) was added to each tube. The reaction was developed at 42°C for 50 min followed by 15 min at 70°C and then 4°C at the end. cDNA samples were stored at -20°C until the time for use.

PCR was performed in 200- μl tubes containing 2 μl cDNA, 100 ng sense primer, 100 ng antisense primer, and 43 μl reaction solution (5 μl PCR buffer, 5 μl 0.5 mM dNTP

mix, 1.5 μl 50 mM MgCl_2 , and 31.5 μl ultrapure water). Each sample was overlaid with mineral oil (Sigma) and incubated in a thermocycler (GeneAmp 9700, Perkin-Elmer, Foster City, CA, USA) using one cycle at 94°C for 3 min followed by 40 cycles of 94°C for 60 s, 58°C for 45 s and 72°C for 90 s. PCR fragments were visualized by agarose gel electrophoresis and ethidium bromide staining. Sample contamination by genomic DNA was verified by submitting the RNA sample to PCR amplification omitting the RT step. Cyclophilin (housekeeping gene) was co-amplified as an internal control. The following primers were used: TNF- α (254 bp), sense 5'-CTTAGACTTTGCGGAGTCCG-3', antisense 5'-CCCTGTCACTGGACCTGACA-3'; IFN- γ (428 bp), sense 5'-CGCTACACACTGCATCTTGG, antisense 3'-GGCTGGATTCCGGGCAACA; cyclophilin (276 bp), sense 5'-GACAGCAGAAAACCTTTCGTGC-3', antisense 5'-GGTTCTGACTCACCGACCT-3'; insulin (257 bp), sense 5'-GCTATAATCAGAGACATC-3', antisense 5'-GTTGCAGTAGTCTCCAGCTG-3'.

Semiquantitative analysis of RT-PCR products

PCR products were submitted to electrophoretic analysis in 1.5% agarose gel with ethidium bromide (2/50 μl). Samples contained 8 μl cDNA, 1.5 μl PCR loading buffer and 5.5 μl ultrapure water and migrated in the presence of TBE buffer containing 2 μl /100 ml ethidium bromide for 45 min (70 V, 150 mA). PCR products were visualized by excitation of ethidium bromide under ultraviolet light and digitally recorded using the Nucleovision[®] system (NucleoTech, San Mateo, CA, USA) and their molecular weight and band pixel area were calculated using the Gel Expert[®] Software (NucleoTech).

Semiquantitative expression (SE) of apoptosis components was calculated for each sample using the following formula and ex-

pressed as arbitrary absorbance units (AU): SE (AU) = pixel area of the product to be analyzed/pixel area of cyclophilin x 100.

Statistical analysis

All results are reported as means \pm SD. Comparisons of semiquantitative measurements of cytokine expression and score severity were made using ANOVA or the unpaired Student *t*-test.

Results

Insulinitis kinetics in female NOD mice

Pancreatic islet infiltration by mononuclear cells in female NOD mice was progressive from 4 to 28 weeks of age for both diabetic and non-diabetic animals. These results are described below as mean \pm SD insulinitis score and are summarized in Table 1.

At 8 weeks of life an increase of infiltrative cells surrounding the islets (peri-islet, grade 1, 39 ± 23 , $P < 0.05$) was observed and the severity score increased at age 14, with most islets being invaded by infiltrating cells (grade 2, 44 ± 24 , grade 3, 32 ± 15 and grade 4, 35 ± 15 , $P < 0.01$). The severity score at age 14 compared to 28 weeks showed grade 0, 39 ± 30 ($P < 0.05$) and grade 1, 52 ± 9 ($P < 0.01$). The values for grades 2, 3 and 4 were not statistically different ($P < 0.1$).

In 28-week-old CBA-j animals and in diabetic animals, insulinitis scores between grades 0 and 3 were the same for both groups. Nevertheless, in 28-week-old animals the grade 4 score (51 ± 23 , $P < 0.02$) was significantly higher than in the diabetic group probably due to the smaller amount of islets than in the non-diabetic animals.

Mean numbers of pancreatic islets per animal did not differ significantly among non-diabetic animals, in contrast to diabetic animals whose total number of islets (96 ± 31 , $P < 0.05$) was significantly lower than in the other animals.

Expression of IFN- γ and TNF- α mRNA

In order to investigate the expression of TNF- α and IFN- γ mRNA in the autoimmune diabetes process, multiple time point analyses were performed to identify the association between cytokine gene expression and the infiltration of immune cells during disease development. All cDNA samples used for the study expressed the product of the housekeeping gene cyclophilin (276 bp) and insulin (257 bp), confirming their quality and the source. The expected products for the genes of TNF- α (254 bp) and IFN- γ (428 bp) were expressed in all animals studied.

Semiquantitative analysis of RT-PCR products

A cross-sectional study involving 7 female NOD mice at 2, 4, 8, 14 and 28 weeks of age and CBA-j mice was performed. Two cytokines, TNF- α and IFN- γ , were exam-

Table 1. Insulinitis scores as a function of age.

Week of life	Grade				
	0	1	2	3	4
2	212 \pm 42*	0	0	0	0
4	144 \pm 35	17 \pm 9*	0	0	0
4	144 \pm 35	17 \pm 9	0	0	0
8	142 \pm 46	39 \pm 23*	6 \pm 5	5 \pm 4	0
8	142 \pm 46*	39 \pm 23	6 \pm 5	5 \pm 4	0
14	39 \pm 29	52 \pm 9	44 \pm 24*	32 \pm 15*	35 \pm 15*
14	39 \pm 29*	52 \pm 9*	44 \pm 24	32 \pm 15	35 \pm 15
28	15 \pm 15	19 \pm 8	32 \pm 13	31 \pm 16	51 \pm 23
28	15 \pm 15	19 \pm 8	32 \pm 13	31 \pm 16	51 \pm 23*
Diabetic rats	5 \pm 3	21 \pm 11	22 \pm 12	22 \pm 11	27 \pm 12

The pancreases were removed from 7 female NOD mice at the age of 2, 4, 8, 14 and 28 weeks. Three tissue sections from each diabetic animal were analyzed and pancreatic islet insulinitis was graded from 0 to 4 according to severity. The analysis was performed comparing ages 2-4, 4-8, 8-14, 14-28, and 28 weeks and diabetic rats. A mean insulinitis score was calculated and the total number of islets was estimated considering the number of sections examined and the animals studied. Data concerning grades of insulinitis and severity of the islet inflammation process were assessed by the unpaired Student *t*-test.

* $P < 0.05$ for paired comparison among age groups. N = 55 female NOD mice.

ined for these analyses. Total RNA samples were prepared from the islets of NOD mice at different ages, i.e., 2, 4, 8, 14 and 28 weeks, representing different stages of disease. From 2 to 14 weeks there was a nonsignificant increase in IFN- γ mRNA expression, which then increased significantly (149 ± 29 AU, $P < 0.05$) in female NOD mice at 28 weeks compared to both youngest groups of animals and CBA-j mice at 14 and 28 weeks of life (Figure 1A). However, in the spleen of these animals the level of IFN- γ expression was not significantly increased ($P < 0.1$) (Figure 1B).

In contrast, the expression of TNF- α in islets of NOD and CBA-j mice progressively increased from 2 weeks of age, being statistically significant at 14 (99 ± 8 AU, $P < 0.05$) and 28 weeks of age (144 ± 17 AU, $P < 0.05$) and being related to the increase of infiltrating cells in pancreatic islets (Figure 2A). In comparison with CBA-j mice, TNF- α mRNA levels in spleens from NOD females started to increase significantly (168 ± 14 AU,

$P < 0.05$) at 8 weeks of age (Figure 2B). Thus, TNF- α mRNA levels in islets and spleens correlated with the inflammatory process and diabetes risk in older NOD mice. In contrast, islets of CBA-j animals expressed low and constant levels at all ages studied.

Discussion

Several studies have correlated cytokine expression by islets with the development of autoimmune diabetes in NOD mice and have demonstrated that islet destruction is associated with increased expression of cytokines such as TNF- α and IFN- γ (13-20). However, the present study is the first to focus on mRNA expression and the morphological features of pancreatic islets in NOD mice aged 2 to 28 weeks. Our results are consistent with published data concerning the expression of TNF- α and IFN- γ as a candidate for the final effector of autoimmune diabetes (20). The expression of a particular group of

Figure 1. Kinetics of IFN- γ mRNA expression in islets (A) and spleen (B) assessed by semi-quantitative RT-PCR in female NOD and CBA-j mice in a time-course study (2, 4, 8, 14 and 28 weeks of age) representing different stages of the disease process. * $P < 0.05$ compared to CBA-j mice of the same age (Student *t*-test). N = 55 female NOD and 25 female CBA-j mice.

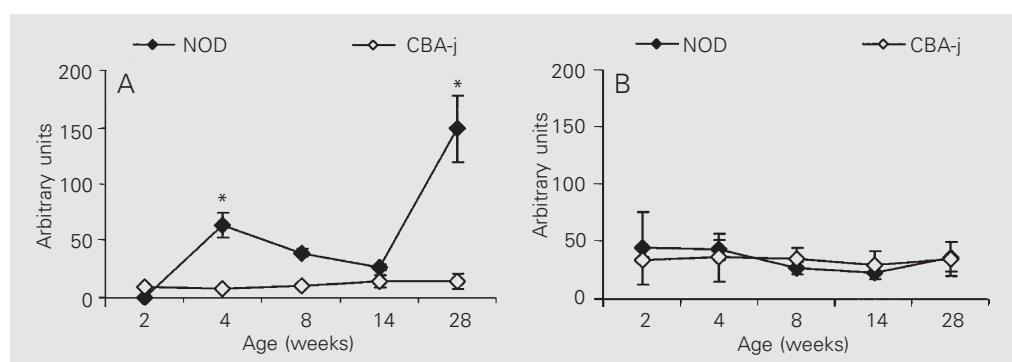
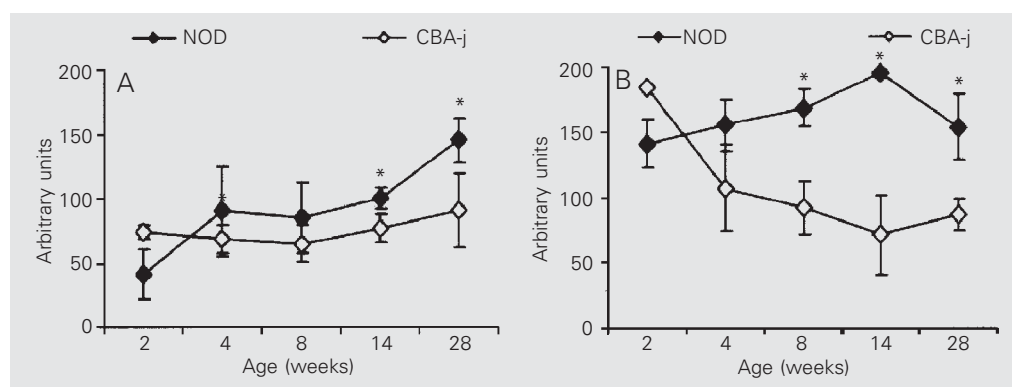


Figure 2. Kinetics of TNF- α mRNA expression in islets (A) and spleen (B) assessed by semi-quantitative RT-PCR in female NOD and CBA-j mice in a time-course study (2, 4, 8, 14 and 28 weeks of age) representing different stages of the disease process. * $P < 0.05$ compared to CBA-j mice of the same age (Student *t*-test). N = 55 female NOD and 25 female CBA-j mice.



cytokines in islets can modulate the inflammatory process leading to β cell destruction or suppressing inflammation. In addition, cytokines such as IL-1, TNF- α or IFN- γ impair insulin secretion and, when added in combination, are destructive to islets (13,20). In the present study, we examined the relations between TNF- α and IFN- γ mRNA expression in pancreatic islets of NOD mice and the progression of insulinitis, its severity, islet destruction and overt diabetes. Furthermore, the expression of these cytokine genes was examined in spleen isolated at the same time as the islets, as a central lymphoid organ. IFN- γ is a product of the Th1 subset of T lymphocytes, cells involved in cell-mediated immune responses and related to modulation of islet β cell destruction (11,18,32). Indeed, previous studies showed that diabetes could be transferred to neonatal NOD mice, suggesting that both CD4+ and CD8+ are required for the initiation of insulinitis, contributing to the development of diabetes (9,33). Furthermore, the morphological findings of the presence of macrophages in islet infiltrating cells suggest their participation in early events of the inflammatory reaction which lead to the recruitment of T cells (5). In the early stages of insulinitis, at 2 and 4 weeks of age, the mild IFN- γ mRNA expression in islets and spleen coincides with the low number of activated T cells in pancreatic islets (24,26,34). However, with the increase of infiltrating T cells in islets the level of IFN- γ expression increases in older mice after 14 weeks of age, associated with destructive insulinitis (21,24,32). The cytotoxic action of IFN- γ against islet β cells has been described in *in vitro* experiments (13,20). In transgenic mice, the expression of IFN- γ in β cells can lead to insulinitis and diabetes (35-37). Thus, our results concur with studies showing that IFN- γ mRNA expression correlates with islet destructive insulinitis and diabetes development in NOD mice and emphasizes the importance of IFN- γ in the pathogenesis of autoimmune diabetes. How-

ever, in the present study, the group of female NOD mice studied at 14 weeks of age (pre-diabetic stages) showed a decreased expression of IFN- γ despite the presence of advanced insulinitis (grades 3 and 4). In contrast, the genetic absence of IFN- γ did not prevent diabetes in NOD mice, but delayed the onset of diabetes (38). Moreover, a previous study dissociated class I major histocompatibility complex upward regulation from progression to diabetes due to local IFN- γ action, suggesting that β cells are not the direct targets of IFN- γ in autoimmune diabetes (37). This apparent contradiction suggests that the levels of IFN- γ expression at this age could determine, together with other factors, the rate of development of overt diabetes. On the other hand, the expression of IFN- γ mRNA levels in spleen did not show a correlation with severity of insulinitis progression, suggesting a compartmental IFN- γ response.

The levels of TNF- α gene expression progressively increased with age, with overexpression being observed at the 28th week. In some cases, TNF- α expression did not correlate with the progression of diabetes, suggesting that TNF- α can be more important for the development and maintenance of insulinitis than diabetes (39,40).

On the other hand, TNF- α was present in spleen at all ages and was overexpressed at 14 weeks of life. Analysis of TNF- α expression in NOD mice showed higher levels of mRNA in spleen than in pancreatic islets, probably due to the high number of mononuclear cells present in the spleen. In addition, comparative analysis of TNF- α expression in spleen showed higher levels in islets and spleen of NOD mice than CBA-j mice.

Finally, in the present study we show the association between IFN- γ and TNF- α expression and insulinitis severity and its age-dependent property. Taken together with other reports, our results emphasize the relevance of the participation and possible synergism of IFN- γ /TNF- α in the pathophysiology of

insulinitis and diabetes (20). Furthermore, our findings suggest that spleen TNF- α and IFN- γ were not correlated with severe insulinitis and overt diabetes and that increased islet expression may reflect a local modulation of cytokines by the inflammatory environment.

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