

# Leptin Accelerates Autoimmune Diabetes in Female NOD Mice

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**We have recently shown that leptin, the product of the *obese* gene, can directly influence T-cell function. In the work presented here, we explored the role of leptin in the development of spontaneous autoimmunity in the nonobese diabetic (NOD) mouse, an animal model for the study of human insulin-dependent diabetes mellitus (type 1 diabetes). We found that expression of serum leptin increased soon before the onset of hyperglycemia and diabetes in susceptible females. A pathogenetic role of leptin was assessed by administering recombinant leptin to young female and male NOD mice. Intraperitoneal injections of leptin accelerated autoimmune destruction of insulin-producing  $\beta$ -cells and significantly increased interferon- $\gamma$  production in peripheral T-cells. These findings indicate that leptin can favor proinflammatory cell responses and directly influence development of autoimmune disease mediated by Th1 responses. *Diabetes* 51: 1356–1361, 2002**

**T**ype 1 diabetes is an organ-specific autoimmune disease that results from inflammatory destruction of insulin-producing pancreatic  $\beta$ -cells. The pathways and the molecules involved in the initiation and amplification of the autoimmune injury to  $\beta$ -cells remain largely unknown (1,2).

The nonobese diabetic (NOD) mouse is a well-characterized animal model for the study of the immunopathogenetic events that lead to type 1 diabetes (3). In the NOD mouse, spontaneous development of autoimmune diabetes is associated with lymphomonocytic infiltration of pancreatic islets and local release of proinflammatory cytokines such as tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interferon- $\gamma$  (IFN- $\gamma$ ), and interleukin (IL)-1 $\beta$  (4). These cytokines, secreted by antigen (Ag)-presenting cells and T-cells, can favor initiation and progression of autoimmune responses by influencing Ag

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Received for publication 31 May 2001 and accepted in revised form 28 January 2002.

Ab, antibody; Ag, antigen; BG, blood glucose; ELISA, enzyme-linked immunosorbent assay; IFN- $\gamma$ , interferon- $\gamma$ ; IL, interleukin; i.p., intraperitoneal; TNF- $\alpha$ , tumor necrosis factor- $\alpha$ .

processing and presentation (5), by modulating adhesion molecule expression on Ag-presenting cells (6), and by inducing proliferation and differentiation of autoreactive T-cells (7). Furthermore, these cytokines can be finely regulated by other cytokines and chemokines in a complex network of reciprocal interactions (8).

Leptin, the product of the *obese* gene, is a 16-kDa protein that structurally belongs to the family of the long-chain helical cytokines (that also includes IL-2, IL-15, and IL-12) (9). Initial evidence suggested that the main function of leptin was to regulate body weight, energy balance, and endocrine functions (9,10). However, it was shown recently that leptin can also influence immune responses (11,12). In particular, leptin can enhance delayed-type hypersensitivity, modulate cognate T-cell interactions, favor proliferation of naïve T-cells and IL-2 secretion, and sustain proinflammatory responses (11,12). The proinflammatory properties of leptin prompted us to ask whether this cytokine could influence organ-specific autoimmune diseases associated with strong Th1 responses, such as type 1 diabetes. In particular, we examined the role of leptin during the development of spontaneous type 1 diabetes in the NOD mouse model.

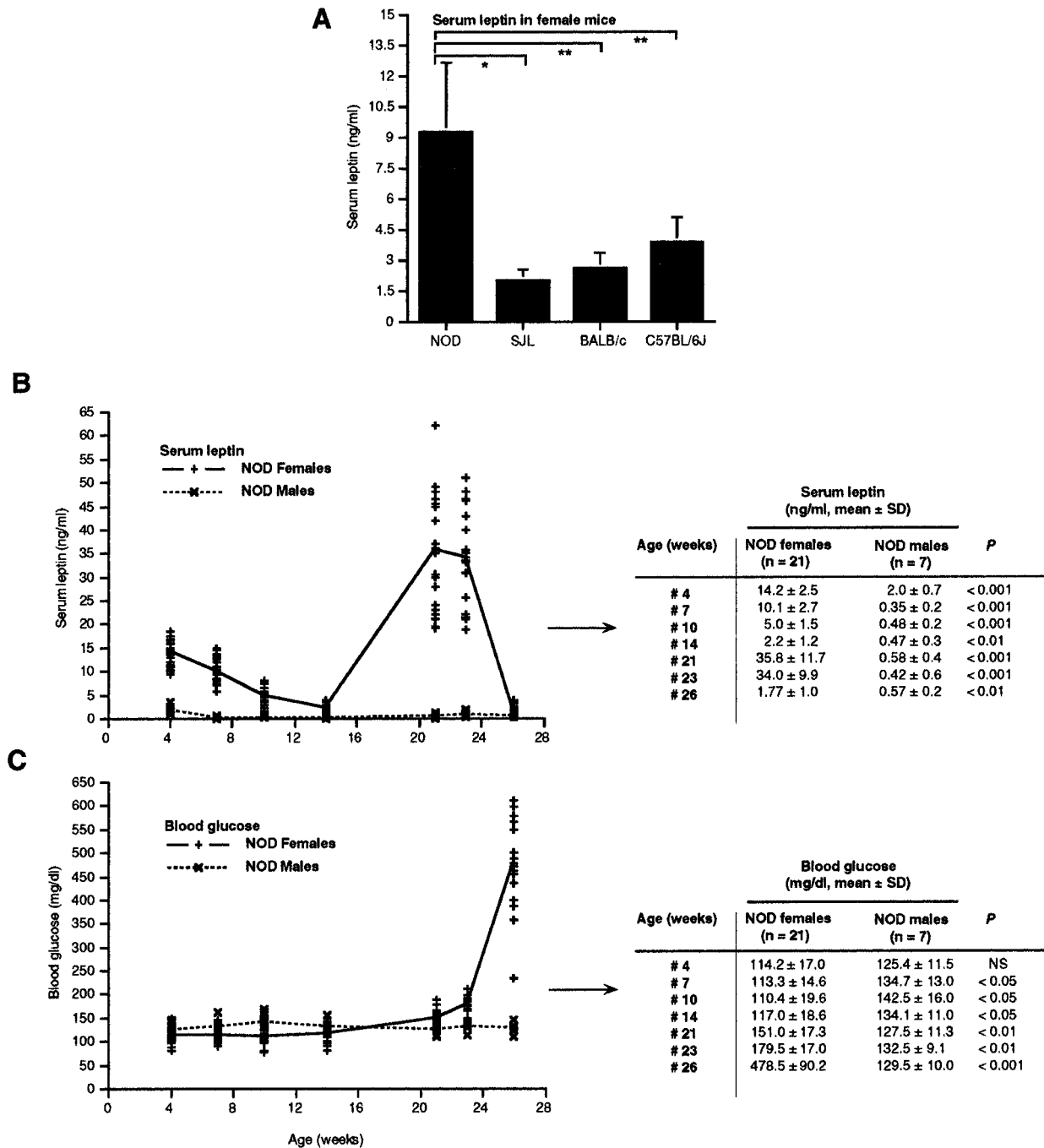
## RESEARCH DESIGN AND METHODS

**Mice.** NOD/LtJ mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and isolator-reared at the Charles River Laboratories (Calco, Italy) before transfer to our animal facility, where they were kept under specific pathogen-free conditions. Mice had free access to autoclaved food and water. The cumulative incidence of type 1 diabetes in untreated female mice was ~85% by 6 months of age, whereas in males it was ~40% by 12 months of age. All of the experiments were performed under an approved protocol in accordance with the animal use guidelines of the Istituto Superiore di Sanità, Rome, Italy.

**Protocol of recombinant leptin administration.** Young NOD/LtJ mice were treated with intraperitoneal (i.p.) injections of mouse recombinant leptin, purchased from R&D Systems Europe (Oxon, U.K.); purity was >97%, as determined by SDS-PAGE and visualized by silver staining analysis. The endotoxin level was <0.1 ng/ $\mu$ g leptin, as determined by the *Limulus* amoebocyte lysate method. NOD mice received an injection if 1  $\mu$ g of leptin/g body wt or PBS. Treatment was started at the 1st week of age, and injections were administered thereafter at weekly intervals until the 4th week of age. From the 4th week of age, i.p. injections were repeated every 36 h for 2 consecutive weeks.

**Assessment of diabetes.** Mice were monitored for diabetes by weekly measurement of blood glucose (BG) levels with a one-step glucometer (Bayer, Newbury, U.K.). Animals were considered diabetic when BG levels were >300 mg/dl after two sequential measurements. Ketoacidosis was assessed by measuring the concentration of urinary acetoacetate using the Ketostix kit (Bayer) according to the manufacturer's instructions. The detection limit for the assay was <5 mg/dl.

**Histological analysis.** Lymphocytic infiltration of pancreatic islets was evaluated on hematoxylin and eosin-stained 6- $\mu$ m paraffin-embedded sections. Histological scoring for insulinitis was performed on hematoxylin and eosin-stained pancreatic sections of 5-week-old leptin-treated or PBS-treated



**FIG. 1.** NOD female mice have higher serum leptin levels than NOD males and other nonsusceptible strains of mice. **A:** Serum leptin at 6 weeks of age in female NOD mice is significantly higher than age- and sex-matched SJL ( $*P < 0.001$ ), BALB/c ( $**P < 0.01$ ), and C57BL/6J ( $**P < 0.01$ ) mice. **B:** Serum leptin surge precedes appearance of clinical type 1 diabetes in female NOD mice but not in males. ELISA for serum leptin (**B**) and blood glucose measurement (**C**) in female and male NOD mice at different ages. Single mice are represented at each time point, and the horizontal line represents the mean value. For females, data are accumulated and averaged from four independent experiments that gave similar results. For males, data are accumulated and averaged from two independent experiments with similar results.

control mice. Scoring was assessed blind by two investigators according to the following scale of 0–3: 0, no insulinitis; 1, peri-insulinitis; 2, insulinitis in <50% of the islet; 3, insulinitis in >50% of the islet (13). A mean score was derived from a number of 10 mice per group and 30–40 islets per individual pancreas from sections taken throughout the organ. Islets were also analyzed for insulin content by immunohistochemistry performed with anti-insulin antibodies (Dako, Carpinteria, CA).

**In situ hybridization.** In situ hybridization for IFN- $\gamma$  and IL-4 mRNAs was performed according to the technique of Kikuchi et al. (14) with the following modification. Briefly, 6- $\mu$ m paraffin-embedded sections of spleens from leptin- and PBS-treated mice were incubated with 50  $\mu$ g/ml proteinase K (Sigma-Aldrich, Milan, Italy), washed, and hybridized overnight at 42°C with biotin-

ylated antisense or control sense probes for IFN- $\gamma$  and IL-4 (Primm s.r.l., Milan, Italy). Probe sequences corresponded to amino acids 885–920 for IFN- $\gamma$  (accession no. M28621) and to amino acids 140–185 for IL-4 (accession no. M25892), respectively. After hybridization, samples were treated with RNase A (Sigma-Aldrich), incubated with 3,3'-diaminobenzidine (Sigma-Aldrich) in the presence of H<sub>2</sub>O<sub>2</sub>, and then counterstained with hematoxylin.

**Enzyme-linked immunosorbent assay.** Serum leptin levels and cytokine concentrations in cell culture supernatants were determined by colorimetric sandwich enzyme-linked immunosorbent assay (ELISA) with R&D Quantikine kits (Oxon, U.K.) following the manufacturer's instructions. Briefly, samples were titrated in test solution and incubated overnight at 4°C in plates coated with capture antibody (Ab). Plates were then incubated with biotinylated

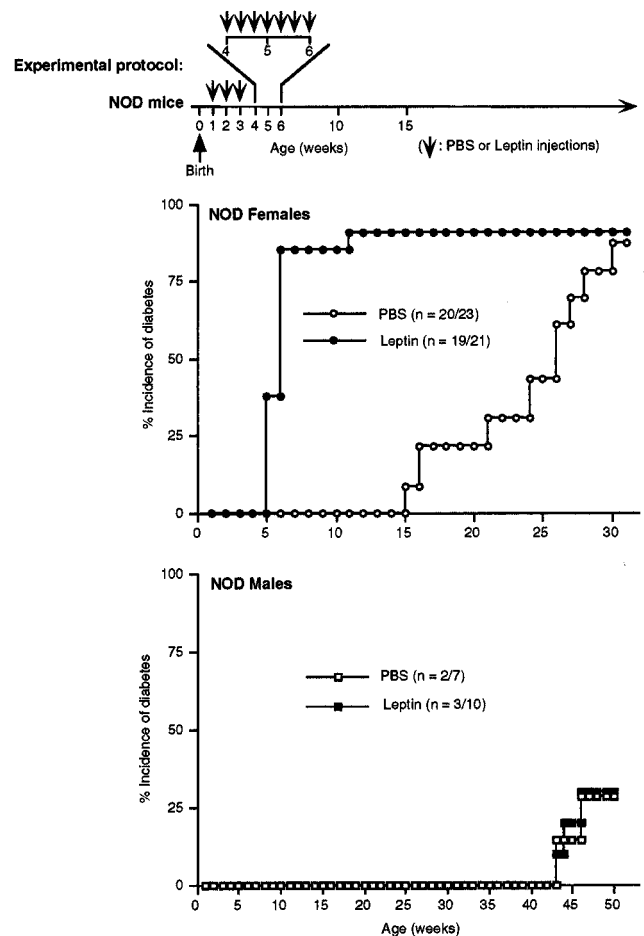
detection Ab diluted in PBS/0.1% Tween 20/1% BSA. After washing and incubation for 30 min with peroxidase-conjugated avidin Ab, secondary Ab was detected by addition of substrate. Absorbance was read at 450 nm, and quantification was obtained from two to three titration points using standard curves of purified recombinant molecules. Detection limits were 25 pg/ml for leptin, 10 pg/ml for IL-4, and 20 pg/ml for IFN- $\gamma$ .

**Data analysis.** Analyses were performed using the Mann-Whitney *U* test (for unpaired two-group analyses). Results are expressed as means  $\pm$  SD;  $P < 0.05$  was considered to be statistically significant.

## RESULTS

**Serum leptin concentrations in NOD mice compared with other mouse strains.** NOD females show increased type 1 diabetes susceptibility compared with males (1,3). We first compared basal serum leptin levels (at 6 weeks of age) of NOD female mice with normal age- and sex-matched females from other strains of mice that are not susceptible to type 1 diabetes (Fig. 1A). It is interesting that NOD females showed a statistically significant increase of serum leptin compared with all other strains of mice tested (NOD females serum leptin:  $9.3 \pm 3.3$  vs.  $1.96 \pm 0.58$  ng/ml in SJL females,  $P < 0.001$ ;  $2.6 \pm 0.71$  ng/ml in BALB/c females,  $P < 0.01$ ; and  $3.84 \pm 1.2$  ng/ml in C57BL/6J females,  $P < 0.01$ ; Fig. 1A). Subsequently, we examined the serum leptin levels in (type 1 diabetes susceptible) female NOD mice at different time points corresponding to preclinical (where no animals exhibited disease signs), subclinical (where asymptomatic insulinitis was present), and clinical diabetes (as assessed by hyperglycemia  $>300$  mg/dl). Parallel measurement was also performed in diabetes-resistant NOD male mice. The results shown in Fig. 1B and C indicate that a surge of serum leptin occurred only in preclinical female mice before the onset of hyperglycemia, increasing 5- to 10-fold to a peak level of  $35.8 \pm 11.7$  ng/ml in 21-week-old mice and  $34.0 \pm 9.9$  in 23-week-old mice. After this surge, a drop of serum leptin accompanied the establishment of clinical diabetes (Fig. 1B). Conversely, leptin levels were significantly lower in male NOD mice (Fig. 1B) in which there were no significant changes of serum leptin and blood glucose (Fig. 1B and C).

**Administration of leptin to young female NOD mice accelerates type 1 diabetes onset and mortality.** To understand whether the changes observed in the expression of serum leptin were related to diabetogenesis, we treated early in life young female and male NOD mice from the first week of age with i.p. injections of recombinant leptin (details of the protocol are indicated in RESEARCH DESIGN AND METHODS). Leptin administration led to rapid acceleration of diabetes, and by the 6th week of age  $\sim 90\%$  of the leptin-treated female mice had developed diabetes, whereas all age- and sex-matched PBS-treated controls were free from diabetes (Fig. 2). More specific, we observed higher severity of disease in leptin-treated female mice (median time of onset,  $6.5 \pm 0.5$  weeks of age in leptin-treated vs.  $25.5 \pm 0.5$  in PBS-treated females;  $P < 0.0001$ ) and a rapid evolution to ketoacidosis (Table 1), this complication being responsible for a mortality rate of approximately one third in leptin-treated animals ( $n = 7$  of 21 [33.3%]) within  $\sim 2$ –3 days of the onset of hyperglycemia (Table 1). This phenomenon did not occur in any of the PBS-treated controls and in leptin-treated males (Table 1). We also tested NOD male mice in parallel with females using the same protocol of leptin injection (see RESEARCH



**FIG. 2.** Early administration of leptin accelerates onset and progression of type 1 diabetes in female but not in male NOD mice. Cumulative incidence of diabetes in NOD mice treated with leptin or PBS. For females, the median time of onset was  $6.5 \pm 0.5$  weeks of age in leptin-treated vs.  $25.5 \pm 0.5$  in PBS-treated females ( $P < 0.0001$ ). In males, nonsignificant differences were observed between the two groups (median time to onset was  $45.6 \pm 1.0$  weeks of age for both groups). Injections were performed at times indicated by arrows (see RESEARCH DESIGN AND METHODS). BG was determined weekly, and mice were considered diabetic after two consecutive measurements of BG above 300 mg/dl. Data are representative of two experiments with similar results.

DESIGN AND METHODS). Administration of leptin did not result in an increase in incidence of either diabetes or mortality (Table 1, Fig. 2).

Finally, accelerated diabetes occurred in leptin-treated female mice only when leptin administration was started early in life. Indeed, the treatment regimen that caused diabetes in young female mice (Fig. 2) did not significantly influence development of type 1 diabetes in 10-week-old adult female NOD mice ( $n = 10$ ) in comparison with age-matched PBS-treated controls ( $n = 8$ ; not shown).

**Histological analysis.** We also examined the pancreata of 5-week-old mice from both the leptin-treated and control groups. Although perivascular lymphomonocytic infiltration was sporadically observed in the islets of PBS-treated controls, morphological integrity of the islets was generally maintained in these mice (Fig. 3A). Conversely, leukocytic infiltrates invaded the islets of leptin-treated mice, with the result that widespread insulinitis occurred, leading to erosion of the  $\beta$ -cell mass as leukocytes penetrated into the islet core (Fig. 3B).



TABLE 1  
Effect of leptin treatment on female and male NOD mice

	PBS-treated	Leptin-treated*	<i>P</i> †
<b>Females</b>			
<i>n</i>	23	21	
Body weight (g)	19.1 ± 1.16	15.9 ± 1.1	<0.001
BG (mg/dl)	110.0 ± 16.1	327.0 ± 130.6	<0.01
Urinary acetoacetate (mg/dl)	0.0 ± 0.0	23.0 ± 15.1	<0.001
Mortality	0.0%	33.3%	<0.01
<b>Males</b>			
<i>n</i>	7	10	
Body weight (g)	24.2 ± 3.32	23.39 ± 2.63	NS
BG (mg/dl)	121.7 ± 13.1	112.4 ± 33.2	NS
Urinary acetoacetate (mg/dl)	0.0 ± 0.0	0.0 ± 0.0	NS
Mortality	0.0%	0.0%	NS

Data are means ± SD. \*NOD mice were injected intraperitoneally with 1 µg/g body wt of mouse recombinant leptin or PBS starting from 1 week of age, weekly for the first 4 weeks of life. Beginning in the 4th week of age, injections were repeated every 36 h for 2 consecutive weeks until week 6. Body weight, BG, urinary acetoacetate, and mortality were scored weekly. The table shows these parameters at 6 weeks of age; data were accumulated and averaged from two separate experiments. NS, not significant. †Mann-Whitney *U* test.

Scoring for insulinitis confirmed severe pancreatic damage in leptin-treated mice ( $2.25 \pm 0.45$  vs.  $0.65 \pm 0.15$  in matched controls;  $P < 0.001$ ; Fig. 3C). Finally, immunohistochemical analysis for insulin revealed insulin depletion in pancreata of leptin-treated mice but normal insulin content in the age-matched control group (not shown).

**Leptin-treated female NOD mice have increased Th1 responses.** We next compared the cytokine expression profile of splenic T-cells from leptin-treated female NOD mice ( $n = 4$ ) and PBS-treated age- and sex-matched controls ( $n = 4$ ) stimulated in vitro with anti-CD3 Ab (2C11 hybridoma, ATCC). Nonsignificant differences were observed between these two groups of mice for the secretion of IFN- $\gamma$  ( $200 \pm 49$  vs.  $190 \pm 77$  pg/ml, NS, respectively) or IL-4 (below the detection limit of the assay). However, in situ hybridization for the IFN- $\gamma$  mRNA expression in the spleen revealed that the periarteriolar sheaths of leptin-treated mice were rich in IFN- $\gamma$  mRNA-expressing cells (Fig. 4C and D), a phenomenon lacking in PBS-treated controls (Fig. 4A and B).

## DISCUSSION

In the present study, we have shown that leptin can exert profound influence on the development of spontaneous autoimmune diabetes in the NOD mouse. Indeed, we first observed a serum leptin surge at preclinical stage, before the onset of hyperglycemia, but after T-cell infiltration into the Langerhans islets had started. This was consistent with the possibility that serum leptin could sustain  $\beta$ -cell damage and favor hyperglycemia; this phenomenon was not observed in males. Furthermore, it has long been known that plasma leptin levels correlate with fat stores and changes in energy balance (15). However, we did not observe any significant association between plasma leptin surge and changes in animal body weight or food intake (not shown). Therefore, our findings raised the possibility that changes of serum leptin may associate not only with energy balance (16) but possibly also with certain immune functions associated with the autoimmune attack against pancreatic  $\beta$ -cells. In relation to possible influence of the leptin gene or its receptor on autoimmune diabetes sus-

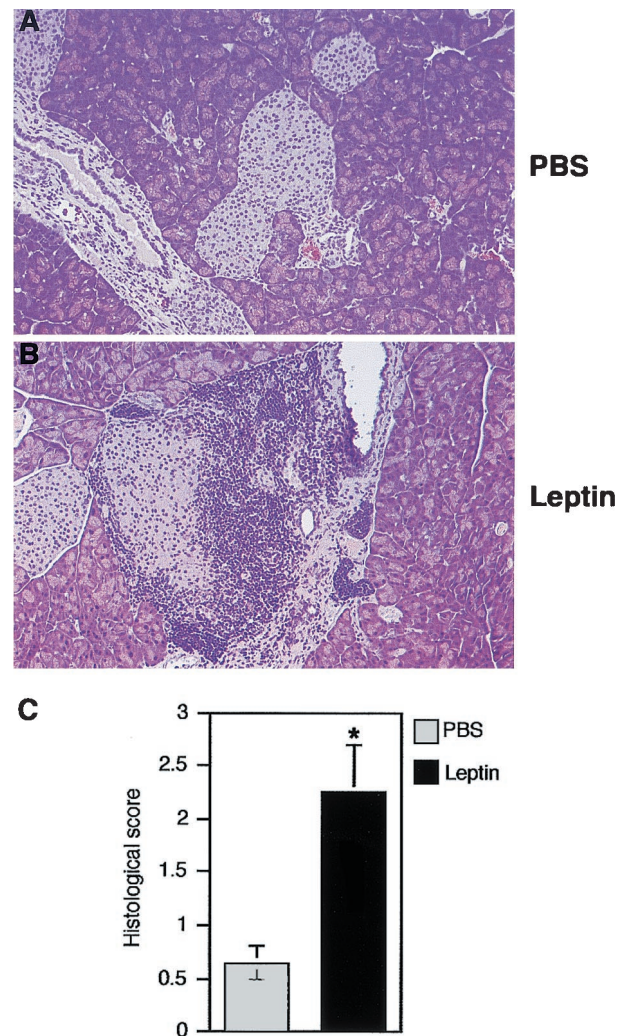
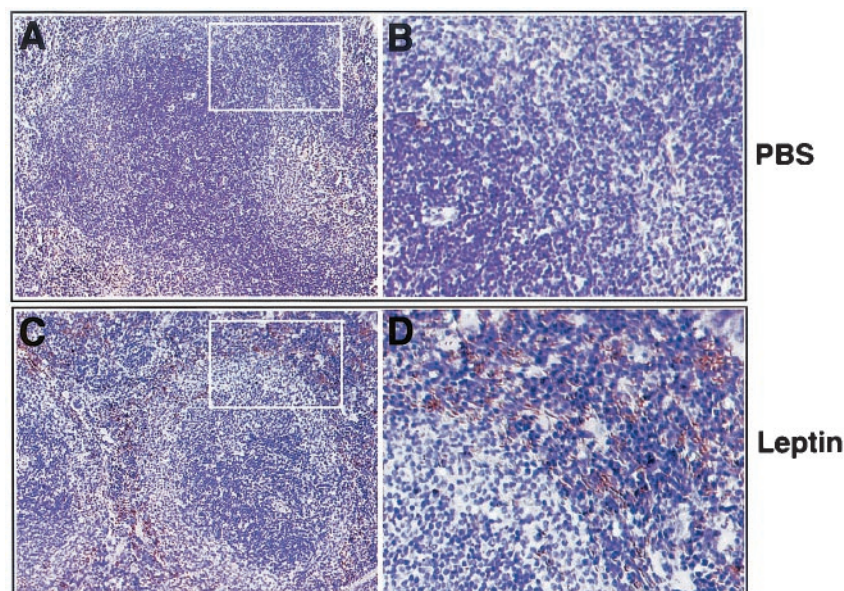


FIG. 3. Leptin administration leads to early islet inflammatory infiltration in NOD mice. Hematoxylin and eosin staining of pancreata from control NOD mice (A) and leptin-treated mice (B) at 5 weeks of age. Magnification: 200 $\times$ . C: Insulinitis score of leptin- and PBS-treated control mice (\* $P < 0.001$ , Mann-Whitney *U* test) is expressed as mean  $\pm$  SD. Pancreatic islets of leptin-treated mice are heavily infiltrated (B and C), whereas matched controls mostly have normal islets (A and C).



**FIG. 4.** Early administration of leptin increases T-cell-mediated IFN- $\gamma$  mRNA expression in NOD mice. In situ hybridization for the presence of IFN- $\gamma$  in spleens of PBS-treated controls (A, B) and leptin-treated mice (C, D). More IFN- $\gamma$  mRNA expression (in brown) is found within the periarteriolar sheaths (T-cell areas) of leptin-treated mice (C, D) than controls (A, B). Magnification: 200 $\times$  (A, C) and 800 $\times$  (B, D). The white squared areas in A and C represent the zone of higher magnification shown in B and D, respectively. Representative experiment of three.

ceptibility, there was no association between *Idd19*, *Idd6*, *Idd11*, and *Idd9* and leptin or leptin-receptor genes (17).

Leptin administration favored early inflammatory infiltration of pancreatic islets and rapidly accelerated type 1 diabetes. These effects were associated with polarization toward Th1 responses, suggesting that leptin could exert at least part of its pathogenic influence by favoring proinflammatory pathways. Because there were no differences in IL-4 mRNA expression between leptin- and PBS-treated controls in these same T-cell areas (not shown), our findings suggested that leptin had affected the kinetics of IFN- $\gamma$  mRNA expression by tilting the balance between Th1 and Th2 responses toward Th1 responses. However, these effects only occurred early in life, as leptin administration in adult mice resulted in lack of clonal expansion and/or Th1 commitment of diabetogenic T-cells. The different outcomes of leptin administration at different ages in the NOD mouse might be related to lack of operating counterregulatory immune mechanisms to the poised status in younger animals. Nonetheless, the influence of leptin and possibly the closely related nutritional status of the host (9,18) should be profound enough to affect susceptibility to autoimmune diabetes, possibly by increasing the priming of autoreactive T-cells. Taken together, these results suggested that during early life, leptin could bias the autoreactive repertoire of prediabetic NOD mice toward a proinflammatory phenotype resulting in accelerated diabetes. Conversely, lack of pathogenicity of leptin later in life may reflect that clonal expansion and Th1 commitment of diabetogenic T-cells have already occurred. According to these findings, recent reports have also shown that leptin-deficient C57BL/6J-*ob/ob* mice are resistant to induction of Th1-mediated diseases such as experimental autoimmune encephalomyelitis (19) and hepatitis (20). Conversely, administration of leptin in NOD male mice did not result in increased susceptibility or mortality for diabetes. Possibly, the amount of leptin required in male NOD mice to trigger diabetes is higher than what was

observed for females, because in the males the leptin levels are 5- to 10-fold lower than in females (9). Additional investigation is in progress to address this point. We also observed a trend in the increase of leptin levels preceding onset of type 1 diabetes in males by 60 weeks of age, although it did not reach statistical significance (not shown). Additional studies with larger number of mice followed over time are needed to address this point. Of note, SJL male mice displayed reduced disease susceptibility when compared with females in the experimental autoimmune encephalomyelitis model of autoimmunity, and leptin administration to males reversed disease resistance to susceptibility, thus suggesting a role for leptin in sex-related susceptibility to autoimmune disease (21).

Recently, Serreze et al. (22) reported that IFN- $\gamma$ -deficient mice also become diabetic and that prevention of diabetes by bacillus Calmette-Guérin was dependent on IFN- $\gamma$  and due to activation-induced cell death of pathogenic Th1 cells. We suggest that susceptibility to type 1 diabetes in IFN- $\gamma$ -deficient mice may be due to the redundancy of the proinflammatory cytokine network (5,8,9) that could sustain and promote type 1 diabetes in the absence of IFN- $\gamma$ . Conversely, leptin also reduces the apoptotic rate of T-cells by upregulating bcl-2 expression (12,23), thus suggesting the possibility that leptin could have interfered with apoptosis of Th1 pathogenic cells by promoting their survival with the result of increased disease severity.

Our data are also in agreement with and may support the findings by Bruining (24), who described increased incidence of type 1 diabetes at younger ages in affluent countries, where affluence is associated with increased postnatal growth and abundant nutrition. More specific, children developing diabetes had increased early BMI gain in the first year of life as compared with healthy siblings and early presence of autoantibodies against IA-2 (pancreatic islets tyrosine phosphatase) (24). Because serum



leptin levels directly correlate with fat stores and BMI (10), our findings suggest an explanation of the observations by Bruining with a possible involvement of leptin in human type 1 diabetes. Furthermore, because leptin displays pleiotropic functions (9), it might have influenced components of the immunologic synapse, such as adhesion and/or co-stimulatory molecules (9,11,25), endocrine pathways, and apoptosis (10,26–28), through its intracellular signaling pathways mediated by the Janus-activated kinase/signal transducers activator of transcription and mitogen-activated protein kinase proteins (29).

The majority of proinflammatory cytokines, such as IL-12, IFN- $\gamma$ , and TNF- $\alpha$ , has been shown to play an important function in the pathogenesis of animal and human autoimmune diseases (30). In vivo neutralization of these cytokines often improves the clinical score as well as the disease progression (31). We suggest that modulation of circulating leptin levels may be a possible strategy to consider for prevention and/or treatment of type 1 diabetes. However, future studies targeting leptin with such pharmacological antagonists and/or nutritional intervention coupled to a better understanding of sex-gender bias and NOD mouse biology are needed to address such possibility.

#### ACKNOWLEDGMENTS

This work was partly supported by Consiglio Nazionale delle Ricerche (CNR-CEOS) and by Università di Napoli “Federico II.” G.M. is a Fondo Sociale Europeo Fellow, Università di Napoli “Federico II,” and V.S. is a Consiglio Nazionale delle Ricerche Fellow. A.L.C. is a recipient of a Juvenile Diabetes Foundation International Fellowship.

We thank S. Sequino and G. Sequino for expert animal care.

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