Journal of Cellular Biochemistry 71:479–490 (1998)

Macrophages and Antioxidant Status in the NOD Mouse Pancreas

Gianpaolo Papaccio,[1]* Bruno De Luca,[2] and Francesco Aurelio Pisanti[3]

[1]Institute of Anatomy and Histology, School of Medicine, Second University of Naples, 5–80138 Naples, Italy
[2]Institute of Topographical Anatomy, School of Medicine, Second University of Naples, 5–80138 Naples, Italy
[3]Department of Clinical Pathology, ASL 1, 80044 Ottaviano, Naples, Italy

Abstract This study showed that citiolone (CIT), a free radical scavenger, significantly increased superoxide dismutase ($P < 0.001$ vs. untreated NOD, NMMA-treated, and silica-treated animals), catalase ($P < 0.01$ vs. untreated NOD), and glutathione peroxidase ($P < 0.001$ vs. untreated NOD and C57BL6/J) values. Silica treatment was capable of counteracting the plasma antioxidant capacity (TRAP) decrease observed in untreated NOD mice, although it did not block the blood glucose rise and insulitis progression in type 1 diabetes significantly. Conversely, early silica administration was able to deplete macrophages (as demonstrated by immunocytochemistry) and to block the rise in blood glucose levels and insulitis progression significantly. Silica-treated animals in this study showed the highest TRAP levels, demonstrating that depletion of macrophages also was able to improve the antioxidant status. This study suggested that macrophages are essential for type 1 diabetes development and showed that they also are involved when the antioxidant status is affected. The reported findings are significant in view of previous studies indicating that oxygen and/or nitrogen free radicals contribute to the islet β-cell destruction in type 1 diabetes animal models. J. Cell. Biochem. 71:479–490, 1998.

Key words: macrophages; antioxidant status; NOD mice; immunocytochemistry; type 1 diabetes

The onset of type 1 diabetes is preceded by a long, clinically silent period, which is characterized by mononuclear infiltration of pancreatic islets of Langerhans. In many animal models of this disease (including the nonobese diabetic (NOD) mouse), a widely distributed infiltration involving pancreatic ducts [Papaccio et al., 1993, 1995a], pancreatic exocrine parenchyma and vessels [Linn et al., 1994], and several other organs such as thyroid [Asamoto et al., 1984], adrenals [Asamoto et al., 1984], submandibular [Miyagawa et al., 1986], lachrymal [Asamoto et al., 1984], and Harderian [Papaccio et al., 1996] glands also are found. These changes, which result in a polyendocrinopathy, actually lead to a selective destruction of the islet β cells (with the exception of the Harderian glands in NOD mice). The majority of mononuclear cells in the pancreas of these animals are $CD8^+$ (cytotoxic/suppressor) T lymphocytes [Signore et al., 1989; Hayakawa et al., 1991] and to a lesser extent $CD4^+$ lymphocytes and natural killer cells [Anderson et al., 1993]. Islet β cells with major histocompatibility complex (MHC) class II and intercellular adhesion molecule-1 (ICAM-1) expression have been found, which lends support to the hypothesis that islet β cells could present surface antigens to the immune system [Papaccio et al., 1991a; Yagi et al., 1995]. In addition, epithelial cells of pancreatic ducts [Papaccio et al., 1995a] and endothelia [Linn et al., 1994; Hänninen et al., 1993] also express those antigens. The latter also have been found to be "hypertrophic" [Papaccio et al., 1998] and to lyse syngeneic islet cells like cytotoxic effector cells in culture [Steiner et al., 1997].

Ordinarily it is the macrophage which strongly expresses MHC class II molecules and which is active in antigen presentation. The occurrence of macrophages, together with $CD8^+$ elements, has been confirmed in human IDDM [Hänninen et al., 1992]. In addition, it has been

Contract grant sponsor: Campania Region, C.N.R.

*Correspondence to: Gianpaolo Papaccio, 21 via Giuseppe Bonito, 80129 Naples, Italy. E-mail:gpapacc@tin.it

Received April 18, 1998; Accepted June 1, 1998

reported that macrophages are the first immune system cells to be activated in low-dose streptozocin-induced (LDS) diabetes [Kolb-Bachofen et al., 1988]. An involvement of these cells also has been described in the development of insulitis in the NOD mouse [Lee et al., 1988], but their role is limited to antigen-presenting cells rather than their being major effector cells [Ihm and Yoon, 1990]. Interest in macrophages derives from the fact that they can liberate cytokines, and they can participate actively in the mechanisms involved in generating other toxic radicals such as nitric oxide (NO), whose role in type 1 diabetes is still under debate [Welsh et al., 1994]. Macrophages (and lymphocytes) also seem to possess impaired antioxidant abilities [Pereira et al., 1994], and the levels of superoxide dismutase, the first cellular defense against toxic free radicals, are very low in the NOD mouse pancreas [Papaccio et al., 1995b]. Glutathione peroxidase (GPX), catalase (CAT) activities, and total tissue lipid peroxide content also have been found altered in experimental diabetes [Werms and Lucchesi, 1990].

It has been found recently that both oxygen and nitrogen free radicals contribute to islet β-cell destruction via peroxynitrite formation in NOD mice. A marked nitrotyrosine increase in islet cells from diabetic NOD mice has found [Suarez-Pinzon et al., 1997]. It also has been found that nitrotyrosine, a marker of peroxynitrite, is highly expressed by monocyte/macrophages infiltrating the islets.

The aims of the present study are to investigate the role of macrophages in the infiltrating process in relationship with antioxidant enzyme activities and to compare diabetes development after macrophage depletion with silica particles and after treatment with free radical scavenger drugs in the NOD mouse.

MATERIALS AND METHODS

Animals

Female diabetes-prone NOD mice purchased from Bommice (Bomholtgarten, Denmark) were used. They were bred in our laboratory, were fed ad libitum, were free from viral infections, and weighed 18–30 g. Approximately 90% of females in this colony become diabetic. They showed a perivasculitis at 5 weeks of age and a periinsulitis with an intraislet infiltration around 10 weeks of age. Clinical signs of diabetes were detectable about 12–15 weeks later. Only a small fraction (10–15%) of male NOD mice become diabetic.

Experimental Procedure

Five-week-old female NOD mice (n = 16, group 1, silica-treated) were treated intraperitoneally (i.p.) with silica (200 mg/Kg body weight) every fifth day (Sigma, St. Louis, MO) for 30 days and were sacrificed in either week 10 (n = 8) or week 25 (n = 8). Silica treatment clears all phagocytes, including macrophages [Oschilewski et al., 1985; Charlton et al., 1988; Papaccio et al., 1991b). Thirty-two 5-week-old NOD mice (groups 2 and 3, n = 16 each) were treated, respectively, with a free radical scavenger, namely citiolone (acetyl-homocysteine-thiolactone; Sigma, Milan, Italy) (group 2, CIT-treated group) at a daily i.p. dose of 50 mg/Kg body weight for 30 days and with an L-arginine analogue (group 3, NMMA-treated group), which inhibits NO-synthase, namely the N^G-monomethyl-L-arginine (acetate salt) (Sigma, Milan, Italy), given subcutaneously [Wu, 1995] at 15 mg/Kg body weight in sterile phosphate-buffered saline (pH 7.4) in daily doses from week 5 to week 10 (n = 8) or 25 (n = 8). Group 2 was sacrificed on week 10, and group 3 on week 25.

Sixteen vehicle-only treated 5-week-old NOD mice (control NOD group) and 16 C57Bl6/J (eight males and eight females) mice of the same age were used as controls. Animals were sacrificed by decapitation after ether anesthesia. Whole pancreatic glands were excised and processed for biochemical and morphological examinations.

Glycemia

Blood glucose levels were tested weekly using the hexokinase method (Boehringer, Mannheim, Germany). Animals were considered hyperglycemic when their nonfasting blood glucose levels were higher than 8 mMol/L but lower than 12 mMol/L in two successive determinations. Mice were considered to be diabetic when their blood glucose levels exceeded 12 mMol/L.

Enzyme Assays

The extraction medium for the measurements of superoxide dismutase (SOD), CAT, and GPX activities was 0.1 M sodium-phosphate buffer at pH 7.0. For the SOD assay, we used the method previously described by us

[Papaccio et al., 1986 1995b]. Briefly, tissue samples were removed and suspended in buffered Hank's solution at 4°C, dissected free from extraneous fat, and minced with scissors. The tissue was incubated in 5 ml of Hank's solution containing 1 mg/ml of type V collagenase at 37°C with vigorous shaking for 15–20 min. After centrifugation (500*g*, 15 min.), the pellet was washed twice with Hank's solution and was resuspended in 0.5 ml Hank's solution in a Petri dish. Pancreatic islets were transferred with a Pasteur pipette with the aid of a stereomicroscope. Isolated islets were prepared according to Grankvist et al. [1981]. When insulitis occurs, the infiltrating mononuclear cells form a translucent capsule around the islet which can easily be distinguished and microdissected away. Isolated islets, spleen, or lymph nodes were homogenized (Ultra Turrax mechanical blender, Milan, Italy) in 100 vol of 10 mM potassium phosphate buffer (pH 7.4) supplemented with 30 mM KCl. The homogenates then were sonicated (Sonifier Branson B12, 1 min at 4°C) and left for 30 min at 4°C to dissolve both CuZn-SOD and Mn-SOD from the tissue. After centrifugation at 20,000*g* for 30 min at 4°C, the supernatant was removed and stored at -70°C. The Harboe [1979] method for determining the hemoglobin content of tissue homogenates was used. Because of the absence of hemoglobin, there was no need to correct for the enzyme's activity. The concentration of proteins was determined by Lowry's method [Lowry et al., 1951]. The measurement of SOD activity (made in triplicate with an intravariation coefficient of 2.7%) used in this study was based on the ability of the enzyme to inhibit the autooxidation of pyrogallol, as described by Marklund and Marklund [1974]. Results are given as units per milligram of protein. The detection limit was found to be 2 U/ml [Oellerich et al., 1980].

For catalase and GPX assays, the procedures were those described by Cornelius et al. [1993]. Spectrophotometric measurements were carried out at 25°C. CAT and GPX activities are expressed as units per milligram of protein [Cornelius et al., 1993].

Measurement of Plasma Antioxidant Capacity (TRAP)

The assay is based on the quenching of the 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) radical cation ($ABTS^{\cdot+}$) by antioxidants. In the method, originally described by Rice-Evans and Miller [1994], $ABTS^{\cdot+}$ is produced by the interaction of ABTS with a ferrylmyoglobin radical species, generated by activating metmyoglobin with H_2O_2. ABTS (150 µmol/l), metmyoglobin (2.5 µmol/l), and plasma (25 µl) were mixed, and the reaction was started by adding H_2O_2 (75 µmol/l). $ABTS^{\cdot+}$ formation was monitored continuously by absorbance increase at 734 nm at 20°C. The delay or inhibition-time between the addition of H_2O_2 (time zero) and the onset of absorbance increase ($ABTS^{\cdot+}$ formation) was measured. All reagents were dissolved in phosphate buffer treated with Cheelex-100 which contained DTPA 0.1 mmol/l to prevent any metal-catalyzed oxidation. The assay was standardized using Trolox, a water-soluble vitamin E analog. Intra- and interassay coefficients of variation were 7.5% and 9.1%, respectively.

Islet Culture and Nitrite Assay

Animals aged 10 or 25 weeks were used for isolation of pancreatic islets and islet culture. Pancreatic islets were isolated using a collagenase digestion method [Sandler et al., 1987]. Groups of 50–100 islets were cultured for 6 days while free-floating in RPMI-1640 containing 11.1 mM glucose and supplemented with 5% (vol/vol) carbon dioxide at 37°C. The medium was changed on days 2 and 4. Islets were cultured with or without 1 mmol/L NMMA, which is able to inhibit NO production. Following incubation, media were collected for assay of nitrite levels and islets collected for immunohistochemistry.Media samples (80 µl) were deproteinized by adding 20 µl 35% sulphosalicylic acid (Sigma, Milan, Italy). Samples were incubated for 30 min at 0°C and subsequently centrifuged for 20 min at 12,000*g*. Ten microliters of 0.5% naphtylethylendiamine dihydrochloride (Sigma, Milan, Italy), 5% sulphanilamide (Sigma, Milan, Italy), and 25% concentrated H_3PO_4 were added to the supernatant [Green et al., 1982; Sternesjö et al., 1995]. The reaction was carried out at 60°C for 15 min, and the absorbance at 546 nm was measured in a spectrophotometer against a standard curve.

General Morphology

At sacrifice, animals were anaesthetized with ether and the pancreas removed and processed. Samples were fixed in Bouin's solution and embedded in paraffin for light microscopy. Serial sections (5 µm thick) were stained with

haematoxylin-eosin for general morphology, and a semiquantitative evaluation of infiltration was performed. Only sections containing six or more islets were selected, and at least 50 islets per pancreas were evaluated. The degree of inflammatory infiltration was scored from 0–5, as shown in Table 1. The evaluation was carried out by two different investigators. A third investigator scored the slides without knowing to which group the slides belonged.

Immunocytochemistry

Tissue from the tail of the pancreas from each animal was frozen and cryosectioned. Sections were stained by the avidin-biotin peroxidase indirect staining method. The monoclonal antibodies used were antimacrophage clone EBM11 (Dakopatts, Milan, Italy) for activated macrophages, clone BM8, which detects murine pan macrophages, and CD11b (clone 5C6), which is useful for myeloid cell–endothelial cell interaction studies because it detects the integrin C3bi (CD11b) molecule and is useful for studying type 1 diabetes (BMA Biomedicals AG, Augst, Switzerland). The secondary antibody was biotinylated goat antirat antibody. The primary Ab was substituted with rat nonimmune serum for the negative control.

The number of immunostained cells was quantified. At least ten ducts per pancreas were viewed. Sections of 5 μm thickness were observed, and the immunoreactive elements on alternate sections were determined at a magnification of ×400 using an eyepiece with a square-ruled grid with a total area of 0.062 mm2 and counted with an M4 image analysis system (Imaging-Brock University, St. Catherine, Ontario, Canada) in 60 different areas. This allowed the number calculation of immunoreactive cells per square millimeter ± SEM to be calculated.

TABLE I. Grading Scores for Pancreatic Islet Infiltration

Grading score	Morphological appearance
0	Normal islets without infiltration
1	Infiltrates in small foci at the islet periphery
2	Infiltrates surrounding the islets
3	Inflammatory cells infiltrating <50% of the islet, without cell parenchymal derangement
4	Extended infiltration invading the islets with diffuse cell destruction and prominent cytoarchitectural derangement
5	Islet atrophy because of β cell loss

Ultrastructure

Small cubes of tissue taken from the tail of the pancreases were fixed in 2.5% glutaraldehyde in a 0.1 M phosphate buffer, postfixed in 0.1% OsO_4 in the same buffered solution for 1 h, and then dehydrated and embedded in epoxy resins. Counterstained (uranyl acetate and lead citrate) ultrathin sections were observed under an electron microscope (EM 109; Zeiss, Oberchoken, Germany).

Statistical Analysis

Results are presented as means ± SD. A Student's *t*-test (level of significance set at $P < 0.05$) or analysis of variance (ANOVA-2) was used.

RESULTS

Glycemia

Results are shown in Figure 1. Untreated control NOD mice showed an increase in glycemic levels. The highest values in this group were reached at week 25 (at sacrifice), when blood glucose levels considerably exceeded 12 mmol/l in all mice ($P < 0.001$ vs. C57BL6/J controls and vs. silica-treated animals).

Silica-treated animals did not show an increase in glycemic levels throughout the experiment; the values in this group were similar to the values in control C57BL6/J animals.

CIT-treated NOD animals showed increased levels of glycemia, with values exceeding 12 mmol/L, but these values were slightly lower than the values found in untreated NOD controls. NMMA-treated animals showed values comparable with those found in diabetic NOD controls ($P < 0.001$ vs. C57BL6/J and silica-treated NOD animals). C57Bl6/J control animals were normoglycemic throughout the experiment.

Enzymes

SOD. Results are shown in Figure 2. Total superoxide dismutase levels in control untreated C57BL6/J animals were considerably higher when compared with those found in all NOD groups ($P < 0.0001$). In particular, the lowest levels of this enzyme were found in 25-week-old untreated diabetic controls. NMMA

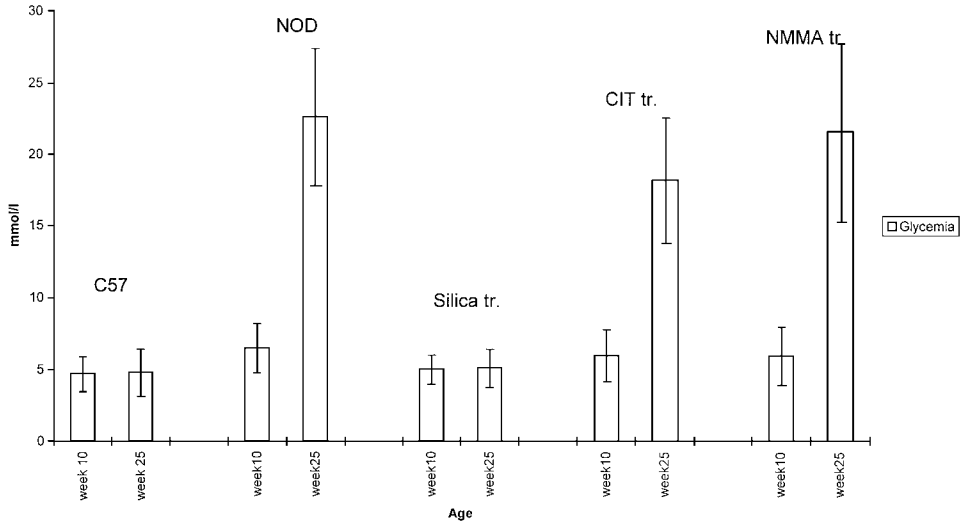

Fig. 1. Figure showing glycemic levels. Values are expressed as millimole/liter and are means ± SD. CIT tr., citiolone-treated animals; C57, C56BL6/J; NMMA tr., NMMA-treated animals; NOD, untreated NOD controls; Silica-tr., silica-treated animals.

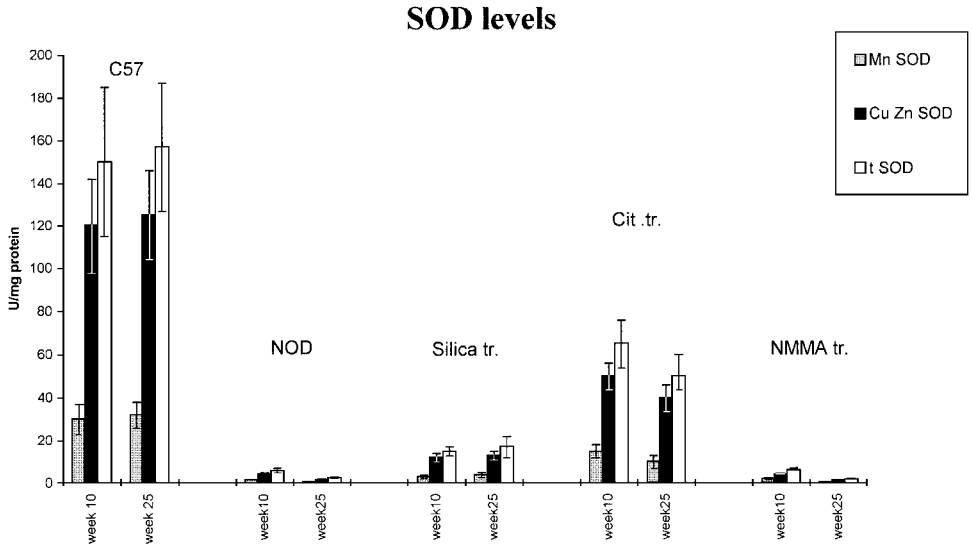

Fig. 2. Figure showing SOD levels. Values are expressed as units per milligram of protein and are means ± SD. CIT tr., citiolone-treated animals; C57, C56BL6/J; NOD, untreated NOD controls; NMMA tr., NMMA-treated animals; Silica-tr., silica-treated animals; t SOD, total SOD.

administration to NOD animals did not alter SOD levels, which remained at extremely low levels, comparable with the levels found in untreated NOD controls. Silica pretreatment slightly enhanced SOD values ($P < 0.01$ vs. untreated NOD controls) and in particular was able to increase CuZn-SOD values. CIT treatment increased SOD values significantly ($P < 0.0001$ vs. untreated NOD and NMMA-treated; $P < 0.001$ vs. silica-treated animals). CuZn-SOD levels, in this experiment, were affected more than Mn-SOD values by type 1 diabetes progression ($P < 0.001$).

CAT. Results are shown in Figure 3. Catalase values were decreased by type 1 diabetes progression in untreated NOD animals and were increased by CIT treatment up to values higher than the values found in C57BL6/J animals ($P < 0.01$ vs. untreated NOD). Silica-treated animals had normal CAT levels. NMMA treatment was not able to counteract the decrease of CAT levels in these animals.

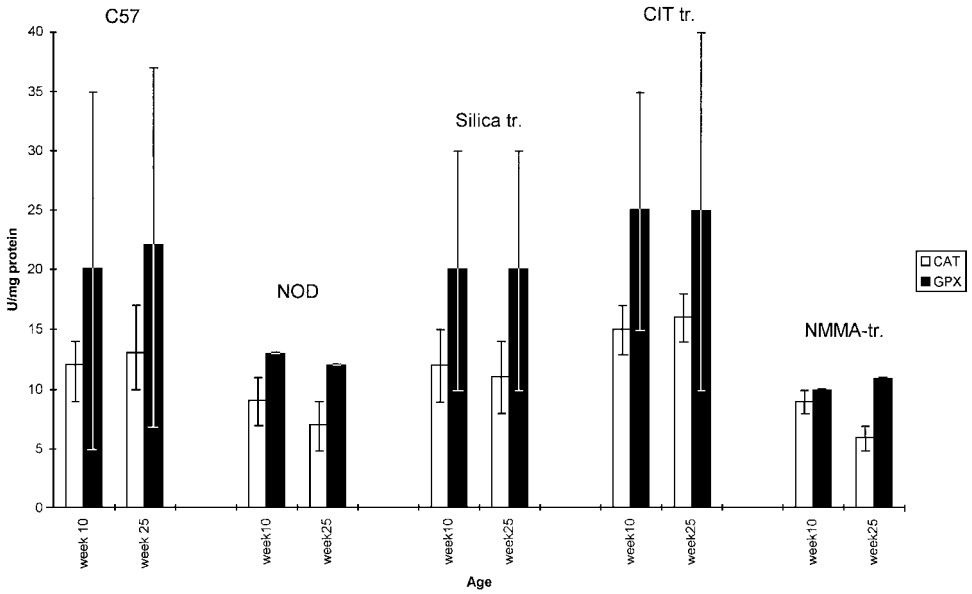

Fig. 3. Figure showing catalase (CAT) and glutathione peroxidase (GPX) levels. Values are expressed as units per milligram of protein and are means ± SD. CIT tr., citiolone-treated animals; C57, C56BL6/J; NMMA tr., NMMA-treated animals; NOD, untreated NOD controls; Silica-tr., silica-treated animals.

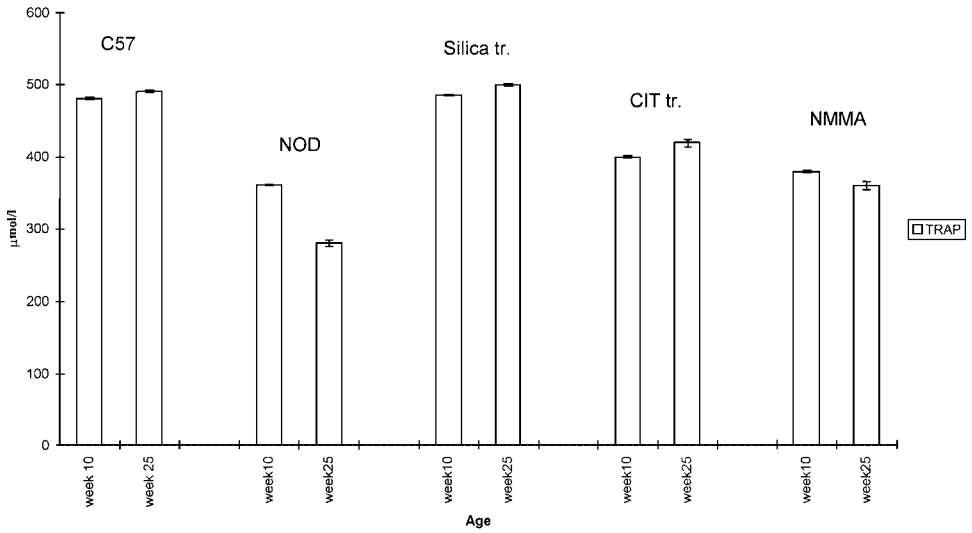

Fig. 4. Figure showing plasma antioxidant capacity levels (TRAP). Values are expressed as micromoles per liter and are means ± SD. CIT tr., citiolone-treated animals; C57, C56BL6/J; NMMA tr., NMMA-treated animals; NOD, untreated NOD controls; Silica-tr., silica-treated animals.

GPX. Results are shown in Figure 3.

Glutathione peroxidase values in NOD animals were found to be lower than those of C57BL6/J mice ($P < 0.001$). Treatment with CIT or silica restored these values to normal (i.e., to levels found in C57BL6/J animals), but NMMA treatment did not counteract the decrease observed in GPX levels with diabetes progression ($P < 0.001$ vs. C57BL6/J).

TRAP. Results are shown in Figure 4. Plasma antioxidant capacity levels were decreased significantly in untreated NOD animals compared with C57BL6/J controls ($P < 0.001$). Silica, CIT, and NMMA treatments counteracted the decrease observed in untreated NOD animals significantly. In particular, CIT ($P < 0.01$ vs. untreated NOD) and silica treatment ($P < 0.001$ vs. untreated NOD) were the

TABLE II. Nitrite Accumulation in Culture Medium (Without or Plus NMMA 1 mmol/L) of Islets Originating From Untreated NOD Mice[a]

	A (week 10)	B (week 25)	*t*-test
Without NMMA			
C Females	25.4 ± 4.2	44.8 ± 9.2	B vs. A $P < 0.001$
			C vs. E $P < 0.0001$
D Males	16.8 ± 4.6	34.2 ± 6.0	B vs. A $P < 0.001$
			D vs. F $P < 0.0001$
Plus NMMA			
E Females	6.5 ± 2.0	10.5 ± 3.0	B vs. A $P < 0.01$
F Males	4.6 ± 1.4	9.6 ± 2.0	B vs. A $P < 0.01$

[a]Values, given as picomoles/islet × 6 days, are mean ± SD. Groups of 50–100 islets were cultured and used for the assay.

TABLE III. Mean of Insulitis Grading Scores

	Week 10	Week 25
C57BL6/J	0	0
NOD untreated	2.8	4.5
NOD silica-treated	1.0	1.2
NOD citiolone-treated	2.5	3.8
NOD NMMA-treated	2.6	4.5

most effective ways to restore the TRAP decrease. In fact, values of silica-treated animals were comparable with those found in control C57BL6/J.

Nitrite Accumulation

Results are shown in Table 2. Nitrite levels in culture media were increased in islets of control NOD mice and were decreased significantly in islets from NOD animals cultured with NMMA ($P < 0.0001$ vs. all groups).

Standard Light Microscopy

Ten-week-old untreated NOD animals (controls) showed varying intensity of periislet and periductal infiltration. Pancreatic vessels were filled with mononuclear elements, mainly clustered around ducts. The majority of the islets showed a periislet infiltration and, in a small number of animals, an intraislet infiltration also. In several animals, accumulation of infiltrating cells was evident also within the exocrine parenchyma. The degree of infiltration is shown in Table 3.

Silica-treated NOD animals showed only small foci of infiltration at the islet periphery. CIT-treated animals showed an islet and duct infiltration, although less intense when compared with untreated control NOD animals ($P < 0.01$).

NMMA-treated mice showed the same degree of infiltration as was seen in NOD untreated controls of the same age (Table 3).

Control C57Bl6J animals showed no signs of infiltration.

Twenty-five–week-old NOD control animals showed infiltrating elements in or around the islets of Langerhans as well as around ducts only rarely. Actually, the endocrine parenchyma of these animals was of a small dimension because of loss of the islet β cells (with the exception of silica-treated animals). NMMA-treated animals showed the same degree of infiltration, and CIT-treated animals showed a slightly lower grading score (Table 3). Silica-treated animals also showed very weak infiltration at week 25. Islets from 25-week-old control C57Bl6J animals were of normal dimensions and showed no signs of infiltration.

Immunocytochemistry

Semiquantitative evaluations are shown in Table 4.

Ten-week-old NOD control animals and NMMA-treated NOD animals showed periislet and ductal cell immunoreactivity for all anti-macrophage antibodies used in this experiment. Other than BM8 (pan macrophages), EBM11 (activated macrophages) (Fig. 5) and CD11b-immunoreactive structures (Fig. 6) also were observed. The latter in particular were detected also on endothelia.

CIT-treated animals showed a similar pattern in that all antibodies were observed but in lesser intensity.

Silica-treated animals showed no immunoreactivity for monocyte/macrophages either at 10 or 25 weeks, indicating that silica treatment was able to deplete phagocytic cells.

TABLE IV. Semiquantitative Evaluations of Immunoreactive Elements[a]

Antibody	Strain	Week 10	Week 25
BM8	NODc	7.5 ± 1.0*,***	0.5 ± 0.1
	C57BL6/J	0.2 ± 0.1	0.2 ± 0.1
	NOD-silica	0.1 ± 0.1	0.1 ± 0.1
	NOD-CIT	6.0 ± 0.5*	1.8 ± 0.2
	NOD-NMMA	7.2 ± 0.5*,***	0.2 ± 0.2
EBM-11	NOD	5.5 ± 0.2*	0.6 ± 0.1
	C57BL6/J	0.2 ± 0.1	0.2 ± 0.1
	NOD-silica	0.1 ± 0.1	0.1 ± 0.1
	NOD-CIT	4.2 ± 0.2*	1.5 ± 0.1
	NOD-NMMA	4.4 ± 0.5*	0.2 ± 0.1
CD11b	NOD	4.5 ± 0.2**	1.0 ± 0.1
	C57BL6/J	0.2 ± 0.1	0.2 ± 0.1
	NOD-silica	0.1 ± 0.1	0.1 ± 0.1
	NOD-CIT	4.4 ± 0.6**	1.5 ± 0.5
	NOD-NMMA	3.6 ± 0.5**	0.2 ± 0.1

[a]Data are given as immunoreactive cells/square millimeter ± SEM. NODc, NOD untreated controls; NOD-CIT, NOD treated with citiolone; NOD-NMMA, NOD treated with NMMA (an NO inhibitor); NOD-silica, NOD treated with silica particles.
*Student's *t*-test, $P < 0.001$ vs. week 25.
**Student's *t*-test, $P < 0.01$ vs. week 25.
***Student's *t*-test, $P < 0.01$ vs. EBM11 and CD11b, week 10.

At week 25, control untreated NOD animals and NMMA-treated animals both showed no immunoreactivity. Only CIT-treated animals showed a weak immunoreactivity for BM8 and CD11b antibodies.

Transmission Electron Microscopy

Silica-treated animals showed no signs of infiltration. At the ultrastructural level, no macrophages were seen on TEM. Islets, ducts, and exocrine pancreas were completely devoid of infiltration, and the ultrastructure of islet cells was normal.

Citiolone-treated and NMMA-treated animals showed in particular both an islet and duct infiltration as well as islet β cell sufferance or loss at this level. The level of infiltration and destruction of islet β cells in NMMA-treated animals was similar to that observed in the pancreas of untreated NOD animals of the same age. Of particular interest was the finding of numerous trapped and marginating infiltrating elements in islet capillaries and of hypertrophic endothelia (Fig. 7).

In 25-week-old NOD control animals, islets were completely devoid of infiltrating elements and insulin-containing β cells. The same pattern was noted in NMMA-treated animals. CIT-treated animals showed a less severe destruction in some islets and the presence of infiltrating elements, including monocyte/macrophages (Fig. 8) and lymphocytes. Silica-treated animals showed a normal islet cytoarchitecture, the presence of insulin-containing β cells, and some periislet lymphocytes. Monocyte/macrophages were not encountered.

DISCUSSION

Ihm and Yoon [1990] demonstrated that macrophages are essential for development of β cell–specific cytotoxic effectors and insulitis in the NOD mouse. The present study demonstrates that early depletion of macrophages by treatment with silica not only is able to prevent insulitis and diabetes development in NOD mice but also is capable of increasing the antioxidant status. Other treatments, including free radical and nitrogen radical scavengers, are not capable of exerting these actions. In fact, citiolone, a free radical scavenger, is able to increase SOD significantly ($P < 0.001$ vs. untreated NOD, NMMA-treated, and silica-treated animals) and CAT and GPX values. Silica treatment also is capable of counteracting the TRAP decrease observed in untreated NOD mice, although it does not significantly block the blood glucose rise and insulitis progression in type 1 diabetes. Administration of an NO inhibithor (namely NMMA) in this study completely fails to counteract diabetes progression and enzyme decrease. The only effect exerted by NMMA is to decrease nitrite accumulation in cultured islet media and to partly counteract the TRAP decrease. NMMA administration to NOD animals did not alter SOD levels, which remained at extremely low amounts, comparable with those found in untreated NOD controls. As shown, silica administration was the only treatment in this study able to deplete macrophages (as demonstrated by immunocytochemistry) and consequently to significantly block the rise in blood glucose levels and the progression of insulitis. Moreover, silica-treated animals show enhanced SOD values ($P < 0.01$ vs. untreated NOD controls) and in particular increased CuZn-SOD values and the highest TRAP levels, demonstrating that depletion of macrophages also is able to improve the antioxidant status. This study shows

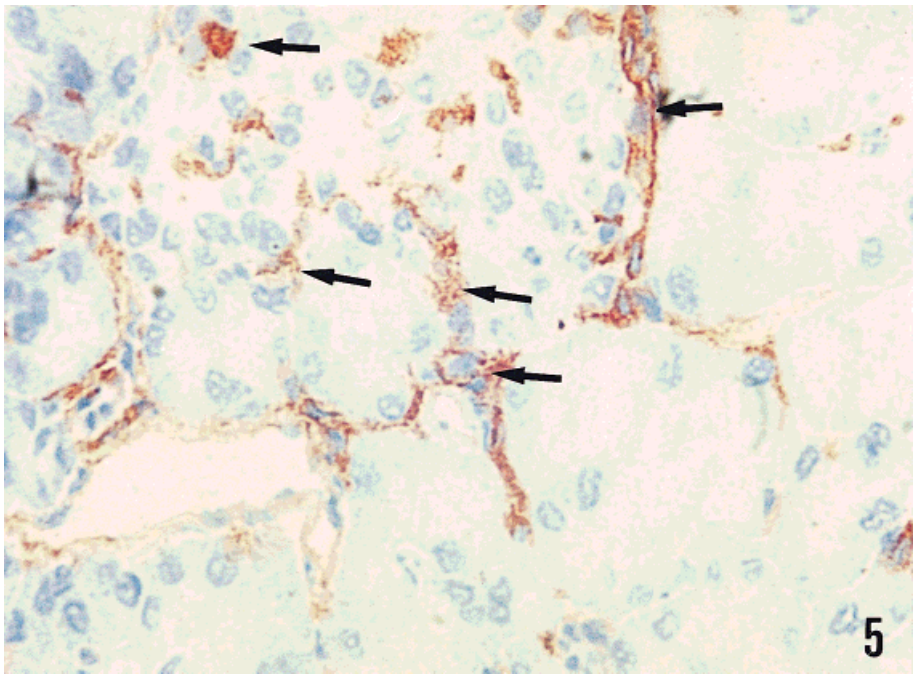

Fig. 5. Light micrograph showing EBM11 immunoreactive structures (arrows) in 10-week-old untreated NOD islet pancreas. ×450.

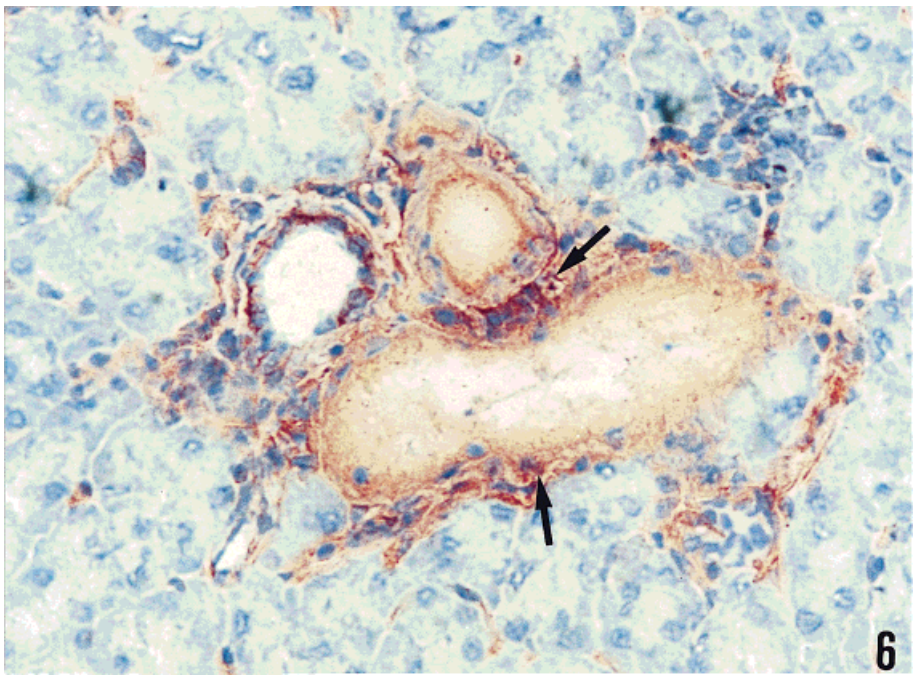

Fig. 6. Light micrograph showing CD11b immunoreactive cells in 10-week-old untreated NOD pancreas including duct cells and endothelia (arrows). ×400.

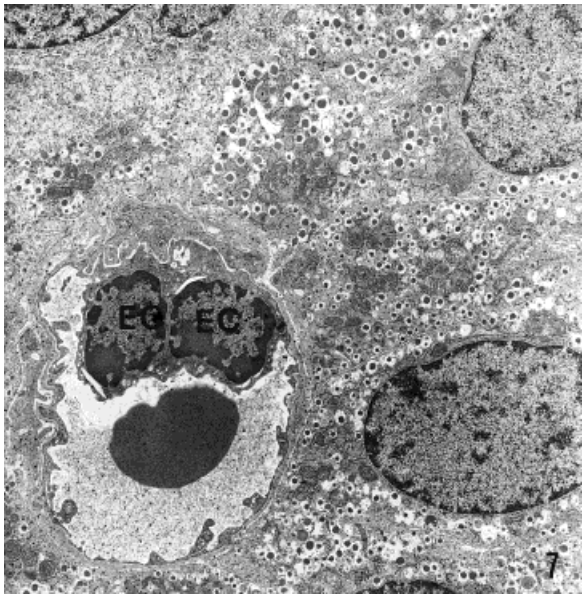

Fig. 7. Transmission electron micrograph showing hypertrophic endothelial cell (EC) of 10-week-old citiolone-treated NOD pancreas. ×3,000.

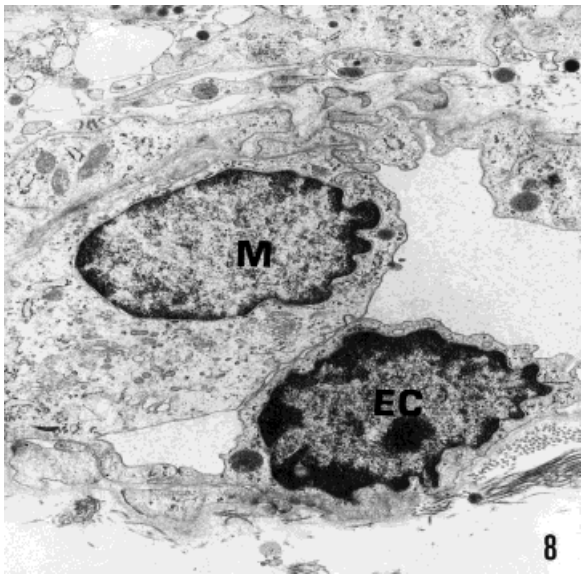

Fig. 8. Transmission electron micrograph showing a marginating macrophage (M) of 25-week-old citiolone-treated NOD pancreas. EC, endothelial cell. ×7,000.

that macrophages are obligatorily involved when the antioxidant status is affected. The reported findings are important in that both oxygen and nitrogen free radicals contribute to the islet β-cell destruction in NOD mice. In this regard, we already demonstrated that NOD mice islets possess extremely low levels of SOD levels, the first cellular defense against free radicals [Papaccio et al., 1995b].

Macrophages may present β cell antigen to helper T lymphocytes, which then induce β cell–specific immunological effectors. On the other hand, macrophages themselves may act as effectors secreting cytotoxic products. Ihm and Yoon [1990] did not exclude that early silica administration also could exert a direct depressive effect on cells other than macrophages. In this regard, we demonstrate that, at least for dendritic and endothelial cells, this seems to be unlikely [Papaccio et al., 1998]. On the other hand, late treatment with silica does not affect the destruction of β cells by preexisting effectors, in contrast to late anti-L3T4-antibody treatment [Ihm and Yoon, 1990].

If macrophages are essential for the development of β cell–specific effectors in the initial phase of insulitis in NOD mice, their depletion, as this study demonstrates, significantly increases the antioxidant status in plasma of NOD animals up to levels found in CIT-treated animals. This new finding demonstrates that macrophages are involved actively in decreasing TRAP levels and in altering the antioxidant status, which in untreated NOD animals is considerably lower than in silica-treated ($P < 0.001$) and in CIT-treated animals ($P < 0.01$). The increase in TRAP levels obtained by using a free radical scavenger (namely citiolone) is comparable with that obtained by early macrophage depletion. The latter stresses the direct involvement of macrophages in the free radical production processes, which has been reported several times as being responsible for the β cell damage [Papaccio et al., 1986; Sandler et al., 1987].

It seems apparent that the role of macrophages for type 1 diabetes development must also be regarded now in reference to its action in altering the antioxidant status.

ACKNOWLEDGMENTS

This study was supported by Regional (Campania Region) funds. The authors are grateful to Mr. Georgios Daphnys (Zoological Station) for his expert technical assistance. Prof. D. Zaccheo is gratefully acknowledged for criticisms and cooperation, and grant #96.02056.06 (CNR).

REFERENCES

Anderson JT, Cornelius JG, Jarpe AJ, Winter WE, Peck AB (1993): Insulin-dependent diabetes in the NOD mouse model II. β cell destruction in autoimmune diabetes is a TH2 and not a TH1 mediated event. Autoimmunity 15: 113–122.

Asamoto H, Akazawa Y, Tashiro S, Oishi M, Azuma T, Koide S, Sudo K, Yokota H, Tochino, Y (1984): Infiltration of lymphocytes in various organs of the NOD mouse. J Japan Diab Soc 27:775–781.

Charlton B, Bacelji JA, Mandel T (1988): Administration of silica particles or anti-LyT2 antibody prevents B cell destruction in NOD mice given cyclophosphamide. Diabetes 37:930–935.

Cornelius JG, Luttge BG, Peck AB (1993): Antioxidant enzyme activities in IDD-prone and IDD-resistant mice: A comparative study. Free Radic Biol Med 14:409–420.

Grankvist K, Marklund SL, Taljedal IB (1981): Cu-Zn superoxide dismutase, Mn superoxide dismutase, catalase and glutathione peroxidase in pancreatic islets and other tissues in the mouse. Biochem J 199:393–398.

Green LS, Wagner DA, Glogowski J, Skipper PL, Wishnok JS, Tannenbaum SR (1982): Analysis of nitrate, nitrite and [^{15}N] nitrate in biological fluids. Anal Biochem 126: 131–138.

Hänninen A, Jalkanen S, Salmi M, Toikkanen S, Nokolakaros G, Simell O (1992): Macrophages, use of T-cell receptors and endothelial cell activation in the pancreas at the onset of insulin-dependent diabetes. J Clin Invest 90: 1901–1910.

Hänninen A, Salmi M, Simell O, Jalkanen S (1993): Endothelial cell–binding properties of lymphocytes infiltrated into human diabetic pancreas. Implications for pathogenesis of IDDM. Diabetes 42:1656–1662.

Harboe M (1979): A method for determination of the hemoglobin content of tissue homogenates. Clin Chem Acta 92:229–234.

Hayakawa M, Yokono K, Nagata M, Hatamori N, Ogawa W, Miki A, Mizoguti H, Baba S (1991): Morphological analysis of selective destruction of pancreatic B cells by cytotoxic T lymphocytes in NOD mice. Diabetes 40:1210–1265.

Ihm S-H, Yoon J-W (1990): Studies on autoimmunity for initiation of β-cell destruction. VI. Macrophages essential for development of β-cell-specific cytotoxic effectors and insulitis in NOD mice. Diabetes 39:1273–1278.

Kolb-Bachofen V, Epstein S, Kiesel U, Kolb H (1988): Low-dose streptozocin-induced diabetes in mice. Electron microscopy reveals single cell insulitis before diabetes onset. Diabetes 37:21–27.

Lee K-U, Amano K, Yoon J-W (1988): Evidence for initial involvement of macrophage in development of insulitis in NOD mice. Diabetes 37:989–991.

Linn T, Strate C, Federlin K, Papaccio G (1994): Intercellular-adhesion-molecule-1 (ICAM-1) expression in the islets of non obese diabetic (NOD) and low-dose streptozocin-treated mouse pancreas. Histochemistry 97:371–374.

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951): Protein measurement with the Folin phenol reagent. J Biol Chem 193:265–275.

Marklund S, Marklund G (1974): Involvement of the superoxide anion radical in the autooxidation of pyrogallol and a convenient assay for superoxide dismutase. Eur J Biochem 47:469–474.

Miyagawa J-I, Hanafusa T, Miyazaki A, Yamada K, Fujino-Kurihara H, Nakajima H, Kono N, Nonaka K, Tochino Y, Tarui S (1986): Ultrastructural and immunocytochemical aspects of lymphocytic submandibulitis in the nonobese diabetic (NOD) mouse. Virchows Arch B Cell Pathol 51:215–225.

Oellerich M, Engelhardt P, Schaadt M, Diehl V (1980): Determination of methotrexate in serum by a rapid, fully mechanized enzyme immunoassay (EMIT). J Clin Chem Clin Biochem 18:169–174.

Oschilewski U, Kiesel U, Kolb H (1985): Administration of silica prevents diabetes in BB-rats. Diabetes 34:197–199.

Papaccio G, Pisanti FA, Frascatore S (1986): Acetyl- homocysteine thiolactone-induced increase of superoxide dismutase counteracts the effect of subdiabetogenic doses of streptozocin. Diabetes 35:470–474.

Papaccio G, Linn T, Federlin K, Volkman K, Esposito V, Mezzogiorno V (1991a): Further morphological and biochemical observations on early low dose streptozocin diabetes in mice. Pancreas 6:320–327.

Papaccio G, Frascatore S, Esposito A, Pisanti FA (1991b): Early macrophage infiltration in mice treated with low-dose streptozocin decreases islet superoxide dismutase levels: Prevention by silica pretreatment. Acta Anat 142: 141–146.

Papaccio G, Chieffi Baccari G, Mezzogiorno V, Esposito V (1993): Extraislet infiltration in NOD mouse pancreas: Observations after immunomodulation. Pancreas 8:459–464.

Papaccio G, Chieffi Baccari G, Strate C, Linn T (1995a): Pancreatic duct inflammatory infiltration in the non-obese diabetic (NOD) mouse. J Anat 185:465–470.

Papaccio G, Frascatore S, Pisanti F.A., Latronico M G V, Linn T (1995b): Superoxide dismutase in the non-obese diabetic mouse: A dynamic time-course study. Life Sci 56:2223–2228.

Papaccio G, Sellitti S, Salvatore G, Latronico MGV, Chieffi Baccari G (1996): The Harderian gland in autommune diabetes of the nonobese diabetic mouse. Microsc Res Tech 34:156–165.

Papaccio G, Latronico MGV, Pisanti FA, Federlin K, Linn T (1998): Adhesion molecules and microvascular changes in the nonobese diabetic (NOD) mouse pancreas. An NO-inhibitor ($_{L}$-NAME) is unable to block adhesion inflammation-induced activation. Autoimmunity 27:65–77.

Pereira B, Costa Rosa LFB, Safi DA, Bechara EJH, Curi R (1994): Superoxide dismutase, catalase and glutathione peroxidase activities in the lymphoid organs of diabetic rats. J Endocrinol 142:161–165.

Rice-Evans C, Miller NJ (1994): Total antioxidant status in plasma and body fluids. Methods Enzymol 234:279–293.

Sandler S, Andersson A, Hellerström C (1987): Inhibitory effects of interleukin-1 on insulin secretion, insulin biosynthesis and oxidative metabolism of isolated rat pancreatic islets. Endocrinology 121:1424–1431.

Signore A, Pozzilli P, Gale EAM, Andreani D and Beverly PCL (1989): The natural history of lymphocyte subsets infiltrating the pancreas of NOD mice. Diabetologia 32: 282–289.

Steiner L, Kronke K-D, Fehsel K, Kolb-Bachofen V (1997): Endothelial cells as cytotoxic effector cells: Cytokine-

activated rat islet endothelial cells lyse syngeneic islet cells via nitric oxide. Diabetologia 40:150–155.

Sternesjö J, Bendtzen K, Sandler S (1995): Effects of prolonged exposure in vitro to interferon-γ and tumour necrosis factor-α on nitric oxide and insulin production of rat pancreatic islets. Autoimmunity 20:185–190.

Suarez-Pinzon WL, Szabó Csaba, Rabinovitch A (1997): Development of autoimmune diabetes in NOD mice is associated with the formation of peroxynitrite in pancreatic islet β-cells. Diabetes 46:907–911.

Welsh N, Eizirik DL, Sandler S (1994): Nitric oxide and pancreatic β-cell destruction in insulin dependent diabetes mellitus: Don't take NO for an answer. Autoimmunity 18:285–290.

Werms SW, Lucchesi BR (1990): Free radicals and ischemic tissue injury. Trends Pharmacol Sci 11:161–166.

Wu G (1995): Nitric oxide synthesis and the effect of aminoguanidine and N^G-monomethyl-L-arginine on the onset of diabetes in the spontaneously diabetic BB rat. Diabetes 44:360–364.

Yagi N, Yokono K, Amano K, Nagata M, Tsukamoto K, Hasegawa Y, Yoneda R, Okamoto N, Moriyama H, Masatoshi M, Tominaga Y, Miyazaki J-I, Yagita H, Okumura K, Mizoguchi A, Miki A, Chizuka I, Maeda S, Kasuga M (1995): Expression of intercellular adhesion molecule 1 on pancreatic β cells accelerates β cell destruction by cytotoxic T-cells in murine autoimmune diabetes. Diabetes 44:744–752.