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Non-obese diabetic (NOD) mice exhibit an increased cellular immune response to glycated-LDL but are resistant to high fat diet induced atherosclerosis

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Abstract

Diabetes mellitus is one of the major risk factors for atherosclerosis. In recent years several murine models have been developed in an attempt to reproduce the accelerated atherosclerosis by combining induced hyperglycemia with hyperlipidemia. In the present study we wished to examine the effect of spontaneous hyperglycemia and hyperlipidemia induced by high fat diet on atherosclerosis development and on markers of the immune system in diabetes prone NOD mice. We tested two high fat dietary regimens (with or without cholate supplementation) in female NOD mice that either developed or did not develop diabetes. Plasma fasting glucose, lipid profile, antibodies to oxidized-LDL and glycated-LDL were assessed. The spleens from both groups were evaluated for their proliferative response. The extent of atherosclerosis was assessed at the aortic sinus. It was found that the two high fat dietary regimens were insufficient to elicit atherosclerosis in the diabetic and non-diabetic NOD mice. The diabetic hyperlipidemic NOD mice displayed an increased cellular immune response to glycated-LDL in comparison with their non-diabetic littermates. The immune response towards copper oxidized LDL was similar in both groups despite an increased susceptibility of LDL extracted from diabetic hyperlipidemic mice to undergo copper induced oxidation. We conclude that the NOD mouse is highly resistant to atherosclerosis even in the presence of hyperglycemia–hyperlipidemia and increased susceptibility to copper induced LDL oxidation. © 2001 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Atherosclerosis; Hyperglycemia; NOD; Oxidation; Mouse; Glycated-LDL

1. Introduction

Patients with diabetes mellitus exhibit accelerated atherosclerotic lesion formation that significantly affects their long term morbidity and mortality [1,2]. Clinical studies are limited in providing sufficient data on the mechanisms mediating the enhanced atherosclerosis, as these patients are treated with medications that influence their lipid profile and glucose levels. Among the etiologic determinants suggested to lead to enhanced atherosclerosis in diabetics, a state of heightened oxidative stress has been pinpointed as a major contributing factor [3]. Various forms of lipoprotein modification have been proposed to play a dominant role in atherosclerosis [4,5]. Oxidation as well as glycation of LDL have both been shown to result in a modified form of the lipoprotein that serves as a target for antibodies in atherosclerotic patients [6–11]. However, despite the association between the various immune markers and atherosclerosis, no data has yet supported a cause and effect relationship.

Animal models have recently been developed in which diabetes and hyperlipidemia were combined to study their roles in the accelerated atheroscleotic process. Convenient models by which to assess the relative importance of hyperlipidemia and diabetes are the transgenic or nontransgenic mice. In an earlier study

Abbreviations: Con A, Concavaline A; ELISA, enzyme linked immunosorbent assay; Exp, experiment; NOD, non-obese diabetic; LDL-RD, LDL receptor deficient; SI, stimulation index.

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[12], wild type BALB/c and C57BL/6 mice susceptible for atherosclerosis, fed with high fat diet (paigen type), showed extensive oil-red-O staining fatty streak aortic sinus lesion. In the same study, BALB/C mice injected with Streptozotocin to develop hyperglycemia and fed a high fat diet, exhibited significant aortic fatty streak lesions compared with BALB/C mice fed with chow diet only. Subsequent authors have made use of transgenic atherosclerosis-prone mouse models such as the apoE and LDL-receptor deficient (LDL-RD) mice [13,14]. We have recently established a mouse model of accelerated atherosclerosis in LDL-RD mice injected with streptozotocin and fed a high fat diet. The enhanced lesion progression in this mouse model was associated with an increased cellular and humoral immune response to heat shock protein 65 (HSP65) [15]. The principal disadvantage of the above mentioned mouse models is the employment of the β-cell cytotoxic exogenous agent streptozotocin, unlike diabetes in humans that develops spontaneously.

The non-obese diabetic (NOD) mouse is a model of type I diabetes displaying an autoimmune process culminating in spontaneous hyperglycemia [16–18]. Within 16–20 weeks about 60% of female NOD mice develop diabetes which, similar to humans, involves autoimmunity to islet cells as well as insulitis [16]. Thus, hyperglycemic and non-hyperglycemic NOD mice can be compared as well as their reaction to various interventions. In the current study, we wished to investigate whether induction of hyperlipidemia in diabetic NOD mice is associated with an increased susceptibility to atherosclerosis and with altered cellular and humoral responses to modified (glycated and oxidized) forms of LDL.

2. Materials and methods

2.1. Experimental design

2.1.1. Animals

Female NOD mice were kindly provided by Professor I.R. Cohen (The Weizman Institute, Rehovot, Israel). Mice were housed in the local animal house (Sheba Medical Center). Bedding, food, and water were autoclaved before use. The mice were maintained on 12 h-dark/12 h-light cycles.

The mice were maintained for 6 weeks on a normal chow diet: 4.5% fat by weight (0.02% cholesterol). The mice were divided into two groups, In the first group, at the age of 6 weeks, the diet was switched to a 'paigen' type diet (PD) containing: 1.25% cholesterol, 7.5% casein and 0.5% (w/w) sodium-cholate (Harlan, Teklad Premier Laboratory Diets, Madison, WI). In the second group, at the age of 6 weeks, the diet was switched to a western type diet (WD) containing 42% of calories from fat, 43% from carbohydrates, 15% from protein (TD 96125, Harlan Teklad).

At 16–20 weeks 60% of the NOD female mice were found to be hyperglycemic and the rest were normoglycemic as shown by fasting glucose levels and hemoglobin-A1c (HbA1c). Ten hyperglycemic and ten normoglycemic NOD mice from each group (PD and WD, total 40 mice) were chosen for further experimentation until sacrifice, at the age of 24 weeks.

In parallel, a separate control group of female NOD mice was fed a normal chow diet throughout the entire 24 weeks. Preliminary studies conducted in our laboratory clearly showed that these mice fed normal chow diet do not develop atherosclerotic lesions, even when they are hyperglycemic for a prolonged period of time.

2.2. Determination of glucose and HbA1c levels

Before each blood withdrawal, all mice were fasted for 4 hours. Glucose levels were measured by MediSense blood glucose sensor (MediSense, USA).

HbA1C levels were determined in the sera of all mice upon sacrifice employing a kit (Unimate 5 HbA1c, Hoffmann-La Roche, CH-4070 Basel).

We tested the urine every 4 weeks for glucose, keton bodies and PH, using Medi-test combi-9 kit (Mechery-Nagel, Duren, Germany).

2.3. Lipid profile

Total plasma cholesterol and triglyceride (TG) levels were determined using an automated enzymatic technique (Boehringer Mannheim, Germany). HDL cholesterol levels were determined by HDL cholesterol reagent (Sigma). Non-HDL cholesterol, LDL-C and VLDL-C, where calculated by the Friedewald equation.

2.4. Lipoprotein oxidation

Lipoproteins ($d = 1.063$ g/ml, top fraction) were isolated from pooled plasma of three mice from each group and three pools of lipoproteins from each group were analyzed. Lipoproteins were incubated at a concentration of 50 μg/ml in PBS, pH 7.4, with 15 μmol/l $CuSO_4$. Lipid oxidation was measured as diene conjugation formation at a wavelength of 234 nm at 37°C in the dark [19].

2.5. Preparation of native LDL, copper induced oxidized LDL and glycated LDL

Blood for lipoprotein isolation was collected in EDTA (1mg/ml) from mice after 12 h of fasting. LDL ($d = 1.019–1.063$ g/l) was isolated from the plasma after density adjustment with KBr, by preparative ultracentrifugation at 50 000 rpm for 22 h, using a type 50

rotor as previously described [19,20]. LDL preparations were washed by ultracentrifugation, dialyzed against 0.15 mol/l EDTA (pH 7.4), passed through an Acrodisc filter (0.22 μm pore size) to remove aggregates, and stored under nitrogen in the dark. Copper oxidation of LDL was performed by incubation of post-dialyzed LDL (1 mg of protein/ml in EDTA-free PBS) with copper sulfate (10 μM) for 24 h at 37°C. Lipoprotein oxidation was confirmed by analysis of thiobarbituric acid-reactive substances (TBARS) [19].

Glycated-LDL was prepared by incubating native LDL in PBS containing 80 mmol/l glucose in a nitrogen-saturated atmosphere for 7 days at room temperature. The glycated LDL fractions were separated from the glucose-incubated LDL by a desalting column [21].

2.6. Detection of anti-oxLDL and anti-glycated LDL antibodies by ELISA

Polystyrene plates with 96 wells (Nunc Maxisorp, Denmark) were coated with either copper-oxLDL, glycated-LDL (at a concentration of 10 μg/ml in PBS) or native LDL overnight at 4°C. After washing four times with PBS containing 0.05% Tween and 0.001% aprotinin (Sigma, USA), the plates were blocked with 2% bovine serum albumin (BSA) for 2 h at room temperature. Diluted (1:50) serum fractions were added in PBS containing 0.05% Tween and 0.2% BSA. After additional overnight incubation at 4°C, the sera were washed and alkaline phosphatase-conjugated goat anti-mouse IgG (Jackson Immuno-Research Laboratory, USA) was added (diluted 1:10 000 in PBS 0.05% Tween–0.2% BSA) for 1 h at room temperature. After extensive washing, 1 mg/ml *p*-nitrophenyl-phosphate (Sigma, USA) in 50 mmol/l carbonate buffer containing 1 mmol/l $MgCl_2$ (pH 9.8) was added as a substrate. The reaction was stopped after 30 min by adding 1 mol/l NaOH. Absorbance was detected at a 405 nm wavelength in a Titertek ELISA reader (S.L.T Laboratory Instruments, Vienna, Austria) and results expressed as absorbance at 405 nm. Anti-oxidized and anti-glycated LDL antibody levels were calculated as binding to native LDL subtracted from oxidized and glycated-LDL binding, respectively.

2.7. Spleen cell proliferation assays

Splenocytes were obtained from three mice in each group at the end of the study for proliferation studies as previously described [20]. Briefly, 1×10^6 cells/ml were incubated in triplicates for 72 h in 0.2 ml culture medium in microtiter wells in the presence or absence of Con-A (2.5 μg/ml), native, oxidized and glycated LDL (all at a concentration of 10 μg/ml). Proliferation was measured by the incorporation of $[^3H]$thymidine into DNA during the final 12 h of incubation. The results were computed as stimulation index (SI): the ratio of the mean cpm of the antigen to the mean background cpm obtained in the absence of the antigen.

2.8. Assessment of aortic sinus atherosclerosis

Quantification of atherosclerotic fatty streak lesions was done by calculating the lesion size in the aortic sinus as previously described [22,23]. The heart and upper section of the aorta were removed from the animals and the peripheral fat cleaned carefully. The upper section was embedded in O.C.T compound (Miles, Elkhart, IN, USA) and frozen. Every other section (5–10 μm thick) throughout the aortic sinus (400 μm) was taken for analysis. Sections were evaluated for fatty-streak lesions after staining with oil-red O. Lesion areas per section were measured using a grid by an observer unfamiliar with the tested specimen

2.9. Statistical analysis

Comparison between diabetic and non-diabetic NOD mice on a high fat diet was performed by Student's *t*-test and the Mann–Whitney *U* test was used to compare independent values. $P < 0.05$ was accepted as statistically significant. Values are presented as mean ± S.D. unless otherwise specified.

3. Results

3.1. Glucose and HbA1c levels in female NOD mice on a high fat diet that developed or did not develop diabetes

In the paigen fed diet group, mean glucose level in hyperglycemic NOD mice ($n = 10$) was 488 ± 26 mg/dl compared to 136 ± 8 mg/dl ($P < 0.0001$) in the normoglycemic NOD mice ($n = 10$). A similar finding was noted in the western fed diet group compared to control (394 ± 25 ($n = 10$) vs. 152 ± 3 mg/dl ($n = 10$); $P <$ 0.0001, respectively) (Fig. 1).

To confirm the presence of sustained hyperglycemia in the diabetic group and to assure the normoglycemia in the non-diabetic group we measured HbA1c levels prior to sacrifice of the mice. We found that hyperglycemic NOD mice displayed significant elevation of HbA1c concentrations in comparison to normoglycemic NOD mice.

In the paigen fed diet group HbA1c was $10.8 \pm$ 0.24% in the hyperglycemic hyperlipidemic NOD mice as compared to 3.5 ± 0.08% in the normoglycemic NOD mice ($P < 0.0001$), In the group fed western diet HbA1c was 9 ± 0.2% in the hyperglycemic hyperlipidemic mice as compared to 3 ± 0.02%, in the normo-

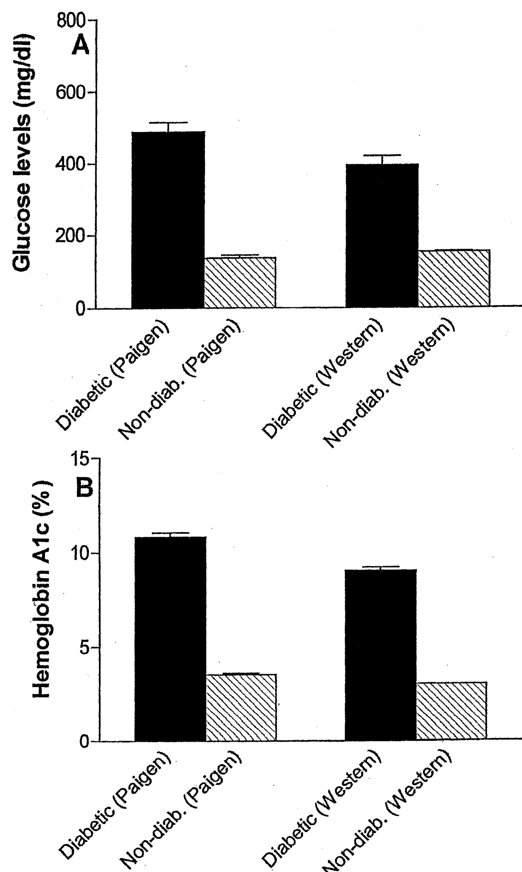

Fig. 1. Glucose levels in diabetic and non-diabetic NOD mice. Plasma from female NOD mice were fed a paigen or western-type diet (A). Upon sacrifice, sera from both groups were tested for HbA1c levels as described in Section 2. Results are presented as mean ± S.D.

glycemic mice($P < 0.0001$),(Fig. 1). No ketoacidosis was detected during the experiment.

3.2. Lipid profile

Significant hypercholesterolemia developed in the hyperglycemic NOD mice fed paigen diet, mean levels of cholesterol were 338 ± 2.0 mg/dl as compared to 131 ± 7.0 mg/dl ($P < 0.0001$) in the normoglycemic mice. A similar finding was observed in the group fed western diet, (262 ± 51 vs. 132 ± 2.5 mg/dl; $P < 0.0001$, respectively). Plasma triglyceride levels in the NOD mice fed paigen diet, that developed hyperglycemia and hyperlipidemia compared to normoglycemic mice, was 113 ± 2.0 vs. 94 ± 3.0 mg/dl ($P = 0.0002$). Similar findings were observed in the NOD mice fed western diet that developed hyperglycemia and hyperlipidemia compared to nonrmoglycemic mice (246 ± 49 vs. 93 ± 4.3 mg/dl; $P < 0.0001$, respectively). HDL levels were similar for all the groups studied on the paigen fed diet and in the western fed diet, 94 ± 3 mg/dl for hyperglycemic mice and 90 ± 2.0 mg/dl for normoglycemic mice ($P =$ n.s). Non-HDL cholesterol, LDL-C and VLDL-C, where calculated from total cholesterol, triglyceride and HDL cholesterol, according to Friedewald equation. (Table 1).

3.3. Determination of conjugated dienes.

LDL-fraction from hyperglycemic hyperlipidemic NOD mice in the paigen fed diet group was significantly more susceptible to in-vitro oxidation as compared to LDL from normoglycemic mice. This was expressed by a shorter lag phase for conjugated diene formation (57 ± 2.5 min for the hyperglycemic group and 80 ± 0.6 min for the normoglycemic group, $P = 0.001$) (Fig. 2). Similar findings were observed in the group fed western diet (data not shown).

Table 1
Lipids levels in diabetic and non-diabetic NOD mice[a]

Group	Cholesterol (mg/dl)	Triglyceride (mg/dl)	HDL (mg/dl)	VLDL-C (mg/dl)	Non-HDL-C (mg/dl)	LDL-C (mg/dl)
(A) Paigen diet						
Diabetic	338 ± 2.0	113 ± 2.0	94 ± 3.0	23 ± 1.0	242 ± 19	206 ± 24
Non-diabetic	130 ± 7.0	94 ± 3.0	90 ± 2.0	18 ± 1.0	39 ± 7.0	24 ± 7.0
P (diabetic/non-diabetic)	<0.0001	0.0002	n.s.	0.001	<0.0001	<0.0001
(B) Western diet						
Diabetic	263 ± 51	246 ± 49	93 ± 4.0	41 ± 8.0	173 ± 47	132 ± 53
Non-diabetic	132 ± 2.5	93 ± 4.0	96 ± 2.0	19 ± 1.0	22 ± 4.0	4.0 ± 1.0
P (diabetes/non-diabetes)	<0.0001	0.0001	n.s	0.003	<0.0001	<0.0001

[a] Total cholesterol levels were performed on the plasma taken from the retroorbital plexus of diabetic ($n = 10$) and non-diabetic ($n = 10$) mice given a paigen diet (A) and diabetic ($n = 10$) and non-diabetic ($n = 10$) mice given a western diet (B). Data are presented as mean ± S.E.

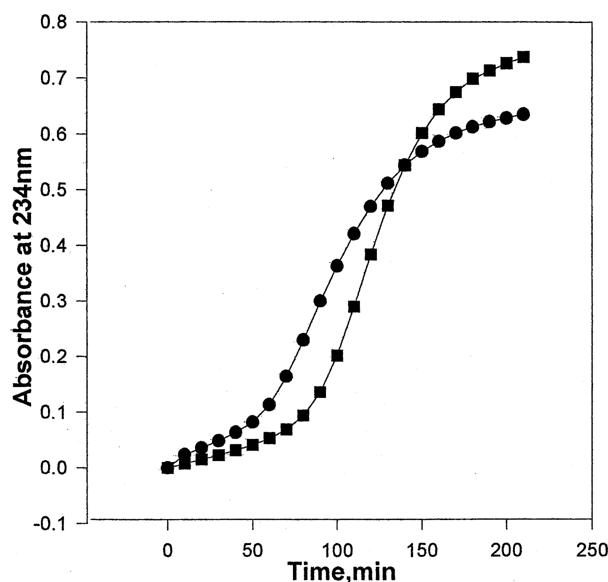

Fig. 2. Determination of conjugated diene formation in diabetic and non-diabetic NOD mice. Lipoproteins (1.063 top) were isolated at the end of the trial from diabetic (circles) and non-diabetic (squares) NOD mice fed high fat diet. Conjugated diene formation during incubation of 50 μg/ml protein with 15 μmol/l $CuSO_4$ was measured at 234 nm. Results are presented as mean ± S.D.

3.4. Anti-glycated and anti-oxLDL antibody level

Anti-oxLDL antibody levels did not differ between hyperglycemic hyperlipidemic and normoglycemic NOD mice in the group fed paigen diet (mean OD of 0.28 ± 0.04 in the hyperglycemics as compared to mean OD of 0.27 ± 0.05 in the normoglycemics; $P =$ n.s) (Fig. 3). Similar findings were observed in the group fed western diet, (data not shown). Anti-glycated LDL antibody levels tended to be higher in the hyperglycemic hyperlipidemic NOD mice fed with paigen diet (mean OD of 0.30 ± 0.05) as compared to normoglycemic mice (OD of 0.23 ± 0.04; $P =$ n.s) (Fig. 3). Similar findings were observed in the group fed western diet, (data not shown).

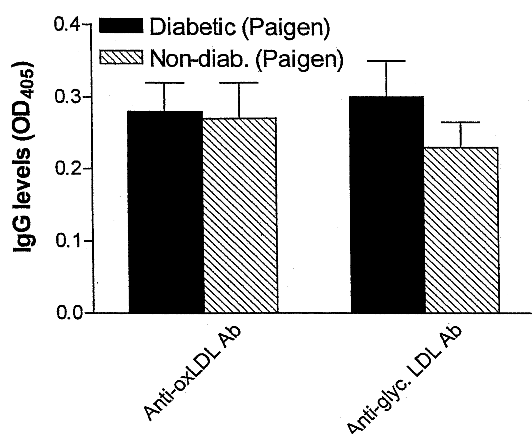

Fig. 3. Antibodies to oxidized and glycated LDL in diabetic and non-diabetic NOD mice. Sera obtained at the end of the study was evaluated by ELISA for levels of IgG antibodies to oxidized and glycated-LDL. Results are presented as mean ± S.E.

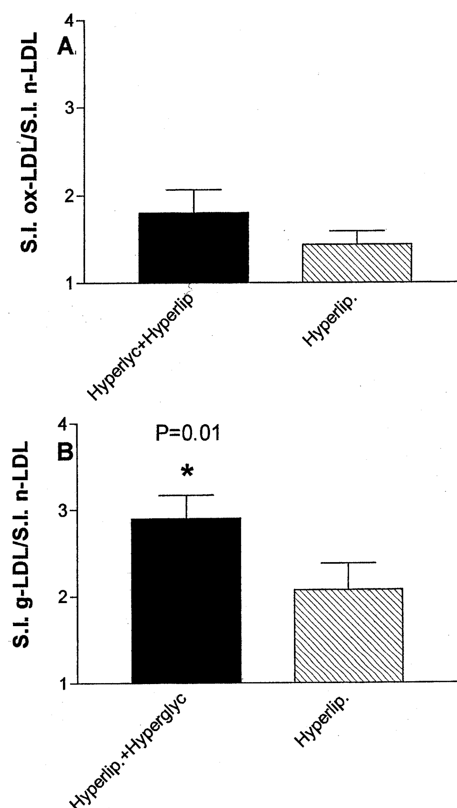

Fig. 4. Cellular immunity to oxidized and glycated LDL in diabetic and non-diabetic NOD mice. Splenocytes from mice in both groups were obtained upon sacrifice and incubated with 10 μg/ml of either oxidized (A) or glycated LDL (B) as described in Section 2. Results are presented as mean ± S.D. of triplicates ($n = 3$ in each group).

3.5. The cellular immune response to glycated and oxLDL

Native LDL did not elicit a cellular immune response in any of the NOD mice studied, as thymidine uptake was not altered in the absence or presence of native LDL (data not shown). No differences were evident in the extent of cellular immune response to oxLDL between diabetic and non-diabetic NOD mice in the group fed paigen diet (mean SI of 1.8 ± 0.15 in the former as compared to 1.43 ± 0.08 in the latter; $P =$ n.s).

An elevated cellular immune response to glycated LDL (mean SI of 2.9 ± 0.15) was found in hyperglycemic hyperlipidemic NOD mice fed with paigen diet compared to normoglycemic NOD mice (SI of 2.06 ± 0.17; $P < 0.01$) (Fig. 4). Similar findings were observed in the group fed western diet (data not shown).

Normolipemic diabetic and non-diabetic NOD mice did not display an increased cellular immune response to glycated or oxidized LDL (data not shown).

3.6. Aortic sinus atherosclerosis in NOD mice

Despite the presence of severe hyperglycemia and hyperlipidemia none of the mice, neither in the paigen fed diet nor in the western fed diet developed atherosclerotic lesions (Fig. 5).

4. Discussion

The aim of the current study was to investigate whether the NOD mouse, which features genetically dictated tendency to insulinitis and hyperglycemia, when combined with hyperlipidemia could serve as an appropriate model to induce atherosclerosis. For this purpose we have fed NOD mice with two forms of diet one of which contains cholic-acid and the second-cholate free. We reasoned that the presence of sustained spontaneous hyperglycemia, when developed in a NOD mice fed high cholesterol, high fat diet would result in accelerated fatty streak formation.

Among the wild type mouse strains, BALB/c mice, which are relatively resistant to atherosclerosis, appear to develop increased fatty streaks when hyperglycemia is induced by the β-cell toxin streptozotocin [12]. In contrast, the lesions in the more atherosclerosis-susceptible C57BL/6 mice are not influenced by a similar regimen of diabetes induction [12], suggesting that genetic factors are also involved in dictating the tendency to develop accelerated atherosclerosis in hyperlipidemic diabetic mice. The creation of transgenic apoE and LDL-receptor deficient mice has provided new tools by which to improve the models combining hyperglycemia and hyperlipidemia, as atherosclerosis in these animals more closely resembles human lesions [13–15]. However, conflicting effects on atherosclerosis were also obtained in these combined models [13,14]. Moreover, the use of a cytotoxic agent to induce hyperglycemia has drawbacks with respect to the assessment of atherosclerosis as a disorder with inflammatory characteristics [24].

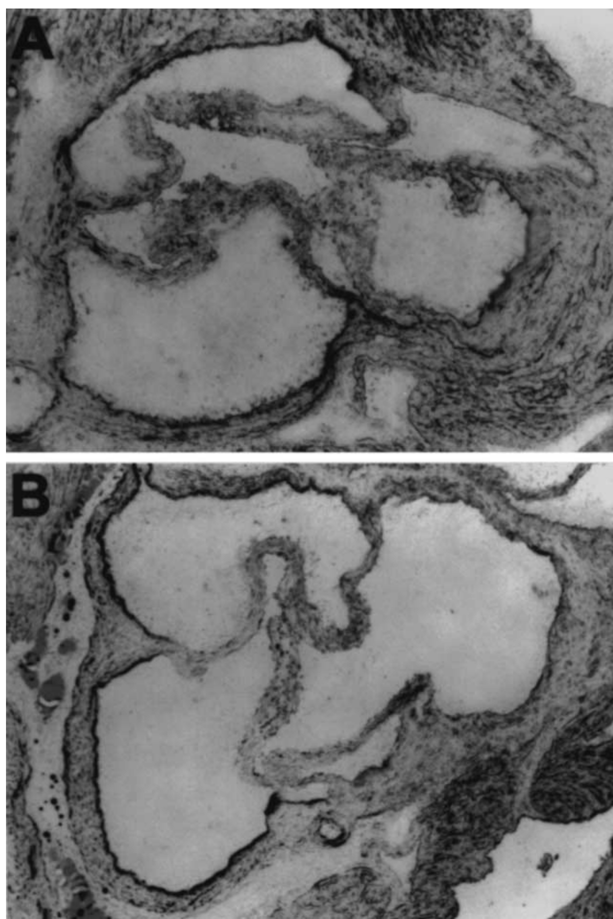

Fig. 5. Aortic sinus atherosclerosis in NOD mice. A representative oil-red O stained section from a diabetic (A) in comparison with a section from a non-diabetic (B) NOD mouse fed a high fat diet.

We have found that even high fat diet fed NOD mice that developed sustained hyperglycemia evident by very high concentrations of HbA1c, did not exhibit fatty-streak lesion formation. This observation was obtained despite the use of two forms of high fat diet that were given for 18 weeks, a time period that is sufficient to elicit fatty streaks in wild type C57BL/6 mice.

In recent years, considerable interest and research have been directed to the involvement of the immune system in atherosclerosis [24,25]. The creation of the apoE and LDL-RD mice has facilitated research in this field as they allowed expanded modes of immune modulation. Oxidized LDL has attracted major attention as a trigger of immune mediated responses and as a candidate participant in atherosclerosis progression [5]. Supporting this concept is the finding of anti-oxidized LDL antibodies in association with carotid atherosclerosis in humans [6] as well as in hyperlipidemic mice [26]. In our study, the presence of hyperlipidemia and hyperglycemia was not associated with elevated levels of oxidized LDL antibodies, despite a considerably increased susceptibility of LDL to in vitro oxidation in the diabetic NOD mice. Similarly, the cellular immune response to oxidized LDL was not affected by the development of hyperglycemia in the hyperlipidemic NOD mice.

Glycation of LDL by blood glucose represents a non-enzymatic reaction, which can also proceed under euglycemic conditions (reviewed in Refs. [27,28]). Glycated LDL is different from the native LDL form both by the glycation of the apoB lysine residues and also by the lipid composition [27]. Interestingly, advanced glycosylation end products (AGE) were found in atherosclerotic plaques from normoglycemic rabbits [29] and severe hypercholesterolemia per se can trigger glycated LDL formation to an extent similar to that found in diabetics [30]. Moreover, it appears that glycated LDL could be capable of influencing atheroscle-

rosis in a similar manner to oxidized LDL [27]. In parallel, antibodies to glycated LDL have been found in the sera of patients with type I and type II diabetes but were not associated with vascular complications [9–11]. In the current study we have measured the humoral immune response to glycated LDL and found higher but not significant levels in diabetic NOD mice in comparison to their euglycemic littermates. This observation is supported by our data, showing for the first time that a cellular immune response to glycated LDL was evident in the NOD mice with diabetes but not in hyperlipidemic NOD mice with no hyperglycemia. These findings imply that glycated LDL in diabetic hyperlipidemic mice may be an influential determinant that is capable of eliciting a respective immune response. In recent years, immune responses toward various antigens have been shown to influence atherosclerosis [31] and it is possible that these reactions could be involved in the progression of the plaque in a more susceptible strain.

In conclusion, we have found that in the NOD mouse, feeding a high fat diet as well as development of diabetes are insufficient to break the resistance to early atherosclerosis that is probably genetically predetermined. Autoimmune responses to glycated but not oxidized LDL were more pronounced in the diabetic hyperlipidemic as compared with euglycemic NOD mice.

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