

Effect of Hyperglycemia and Hyperlipidemia on Atherosclerosis in LDL Receptor-Deficient Mice

Establishment of a Combined Model and Association With Heat Shock Protein 65 Immunity

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Diabetes and atherosclerosis have been proposed to be influenced by immune and autoimmune mechanisms. A common incriminated antigen in both disorders is the heat shock protein (HSP)-60/65. In the current study, we established a model combining hyperglycemia with hyperlipidemia in LDL receptor-deficient (LDL-RD) mice and assessed its possible influences on lipid profile, HSP60/65, and atherogenesis. LDL-RD mice were injected either with streptozotocin to induce hyperglycemia or with citrate buffer (control). When hyperglycemia was induced, both study groups were challenged with a high-fat (Western) diet for 6 weeks. Plasma fasting glucose, lipid profile, and antibody levels to HSP65 and oxidized LDL were assessed. At death, the spleens from both groups were evaluated for their proliferative response to HSP65 and the consequent cytokine production. The extent of atherosclerosis was assessed at the aortic sinus. Plasma glucose, cholesterol, and triglyceride levels were elevated in mice injected with streptozotocin compared with control mice. Atherosclerotic lesions were significantly larger in the streptozotocin-injected hyperglycemic LDL-RD mice ($132 \pm 23 \times 10^5 \mu\text{m}^2$) in comparison to their normoglycemic littermates ($20 \pm 6.6 \times 10^5 \mu\text{m}^2$; $P < 0.0001$). Both humoral and cellular immune response to HSP65 was more pronounced in streptozotocin-injected mice. When challenged with HSP65 *in vitro*, splenocytes from streptozotocin-injected mice favored the production of the T-helper (TH)-1 cytokine γ -interferon. In conclusion, we have established a mouse model that combines hyperglycemia with diet-induced hyperlipidemia in LDL-RD mice and studied its effect on atherosclerosis progression. The accelerated atherosclerotic process is associated with heightened immune response to HSP65 and a shift to a TH1 cytokine profile. *Diabetes* 49:1064–1069, 2000

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apo, apolipoprotein; ELISA, enzyme-linked immunosorbent assay; HSP, heat shock protein; IFN- γ , γ -interferon; IL, interleukin; LDL-RD, LDL receptor-deficient; oxLDL, oxidized LDL; PBS, phosphate-buffered saline; SI, stimulation index; STZ, streptozotocin; TH, T-helper.

It is well established that patients with diabetes experience accelerated atherosclerosis and its recognized complications (1–3). Several mechanisms have been suggested to play a role in mediating the additive effects of hyperglycemia on atherogenesis. These include increased oxidative stress and formation of advanced glycated end products (1–3). However, despite intensive research in the field, the association between hyperglycemia and atherogenesis, which may well be multifactorial, remains unresolved.

The involvement of the immune system in atherosclerosis has been the subject of considerable interest in recent years (4–6). The idea stems from the participation of effector cells of the immune system in the initial stages of atherosclerosis (i.e., T-cells, macrophages, and endothelial cells) (7). The immunologic view of atherosclerosis paved the way for the notion that modification of self-antigens, or a cross-reactive response towards inherent proteins, can drive an autoimmune response that could act to accelerate the progression of atherosclerosis (5). Accordingly, heat shock protein (HSP)-65 (derived from a bacterial origin and cross-reactive with mammalian HSP60) has been shown to evoke an immune-mediated response in rabbits (8) and mice (9), thus leading to enhanced atherosclerotic lesion formation. These observations were reinforced by the detection of HSP65 antibodies in the sera of patients with verified carotid atherosclerosis (10) and the expression of HSP60 in rabbits and humans atherosclerotic lesions (11).

HSP60/65 also has been proposed as a target antigen in type 1 diabetes (12). This assumption has gained predominant support in animal models, whereas in humans, further evidence is still lacking. Accordingly, nonobese diabetic (NOD) mice and streptozotocin-injected animals develop a heightened autoimmune response to HSP65 (12–16), which is proposed to contribute to pancreatic β -cell destruction and consequent hyperglycemia.

Recently, a model that combines hyperlipidemia and hyperglycemia induced by streptozotocin was established in apolipoprotein (apo)-E-deficient mice, a mouse model of spontaneous hyperlipidemia (17). The model supported the idea that the combination of both risk factors synergizes to promote atherosclerosis. Interestingly, when hyperglycemia was induced by using intraperitoneal streptozotocin (STZ) in

LDL receptor-deficient (LDL-RD) mice fed a high-fat high-cholesterol diet for the long period of 6 months, no effect was evident on atherosclerosis progression (18). The long period of atherogenic diet possibly masked the effect of hyperglycemia on atherogenesis.

In the current study, we aimed to accomplish 2 goals: 1) to establish a mouse model that combines short-term diet-induced hyperlipidemia with hyperglycemia, thus investigating the possible interactions between the 2 risk factors; and 2) to test the possible association of HSP65 autoimmunity in the development of the combined model.

RESEARCH DESIGN AND METHODS

Animals and diets. Male and female LDL-RD mice (C57BL/6 background) (15 mice in each study group) were obtained from the Jackson Laboratory (Bar Harbor, ME) and bred in a local animal house (Sheba Medical Center, Tel-Hashomer, Israel). The LDL-RD mice were created by homologous recombination as previously described (19).

Study protocol. At 6 weeks of age, 1 group ($n = 15$) received 50 mg/kg intraperitoneal STZ (Sigma, St. Louis, MO) in citrate buffer (0.05 mol/l; pH 4.5). Mice were injected with STZ for 5 consecutive days. Control mice ($n = 15$) received citrate buffer. Hyperglycemia was induced 2 weeks after the initial STZ injection. At 4 weeks, all mice were provided with an atherogenic Western-type diet for 6 weeks (TD 96125, Harlan Teklad [Madison, WI]; 42% of calories from fat, 43% from carbohydrates, and 15% from protein). An additional control group ($n = 15$) received intraperitoneal STZ and were fed a standard diet throughout the study. Every 4 weeks, blood was obtained from the retro-orbital sinus and put into tubes containing anticoagulant (1 mmol/l EDTA) to determine fasting plasma glucose, cholesterol, triglyceride levels, and antibodies to HSP65 and oxidized LDL (oxLDL).

Determination of glucose levels. Before each blood withdrawal, all mice were fasted for 4 h. Glucose levels were measured by a MediSense blood glucose sensor (MediSense, Bedford, MA).

Determination of lipid profile. Total plasma cholesterol and triglyceride levels were determined using an automated enzymatic technique (Boehringer Mannheim, Mannheim, Germany). HDL cholesterol levels were determined with HDL cholesterol reagent (Sigma).

Antigens and monoclonal antibodies. Recombinant HSP65 was purchased from Dr. M. Singh (Braunschweig, Germany). LDL and copper oxLDL were prepared as previously described (20). Rat monoclonal antibodies [H129.19 (L3T4)Rat anti-mouse CD3⁺] were purchased from PharMingen (San Diego, CA).

Proliferation assays of splenocytes to HSP65. At the end of the experiment, lymphocyte proliferation was assayed using spleens obtained at death as previously described (12), with minor modifications. Briefly, 1×10^6 cells per milliliter were incubated in triplicate for 72 h in 0.2 ml culture medium in microtiter wells in the presence of 1.2–10 μ g/ml HSP65. 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 counts per minute of the antigen to the mean background counts per minute obtained in the absence of the antigen.

Cytokine production on in vitro stimulation with HSP65. Medium was collected 24, 48, or 72 h after incubation of splenocytes from both study groups with HSP65 (10 μ g/ml). The medium was assayed for interleukin (IL)-4, IL-10, or γ -interferon (IFN- γ) production using a commercial kit (R&D Systems, Minneapolis, MN).

Detection of anti-HSP65 and anti-oxLDL antibodies by enzyme-linked immunosorbent assay. Recombinant HSP65 (1 μ g/ml) was coated onto flat-bottom 96-well enzyme-linked immunosorbent assay (ELISA) plates (Nunc Maxisorp, Roskilde, Denmark) overnight, and ELISA was performed as previously described (9). Ninety-six well polystyrene plates were coated with either copper oxLDL, native LDL (at a concentration of 5 μ g/ml in phosphate-buffered saline [PBS]), or PBS overnight, and ELISA was performed as previously described (9). The optical density was read at a 405-nm wavelength in a Titertek ELISA reader (S.L.T. Laboratory Instruments, Vienna, Austria). Levels of anti-oxLDL antibodies were calculated as binding to native LDL subtracted from the binding to oxLDL.

Assessment of atherosclerosis. Quantification of atherosclerotic fatty streak lesions was done by calculation of lesion size in the aortic sinus as previously described (21). Lesion areas per section were counted using a grid by an observer unfamiliar with the tested specimen.

Immunohistochemistry. Immunohistochemical staining was performed with rat anti-mouse CD3 (PharMingen). Cryostat sections (5 μ m thick) of the aortic

sinus were prepared as described previously (22). CD3⁺ cells were counted and averaged from a total of 4–5 sections (per mouse per group) taken from different mice in each group. Only lymphocytes within aortic plaques were counted.

Statistical analysis. Comparison between STZ- and citrate-treated mice was performed by the Student's *t* test, and the Mann-Whitney *U* test was used to compare independent values according to whether the distribution was parametric. $P < 0.05$ was accepted as statistically significant. Values are presented as means \pm SD unless otherwise specified.

RESULTS

Glucose concentrations and body weight. To induce diabetes, LDL-RD mice were injected intraperitoneally with 50 mg/kg body wt of STZ for 5 consecutive days, and 4 weeks after the treatment, the mice were fed a high-fat high-cholesterol Western diet. This treatment induced the combined hyperglycemia and hyperlipidemia in this mouse model throughout the experiment. Mean glucose levels were similar both in control and STZ-treated mice before STZ injection: 82 ± 26 mg/dl in STZ-treated mice vs. 96 ± 23 mg/dl in citrate-treated control mice. Higher levels of plasma glucose were detected in STZ-treated mice 4 weeks after injection (Fig. 1). At the end of the experiment (10 weeks after injection), serum glucose in STZ-treated mice was 386 ± 21 mg/dl compared with 80 ± 4 mg/dl in citrate-treated mice ($P < 0.0001$). Similar findings were observed in female mice (data not shown). No differences were evident with respect to glucose levels between standard diet-fed hyperglycemic and Western diet-fed hyperglycemic mice (Fig. 1). No significant difference in body weight gained during the study was observed (4.8 ± 3.2 vs. 4.2 ± 2.2 g for STZ-treated and control mice, respectively).

Lipid profile in hyperglycemic hyperlipidemic mice. The mean cholesterol and triglyceride levels were similar in both groups before STZ injections (237 ± 35 mg/dl in the STZ-treated mice and 226 ± 31 mg/dl in the control group). At death, after 6 weeks on a high-fat diet (Western type), cho-

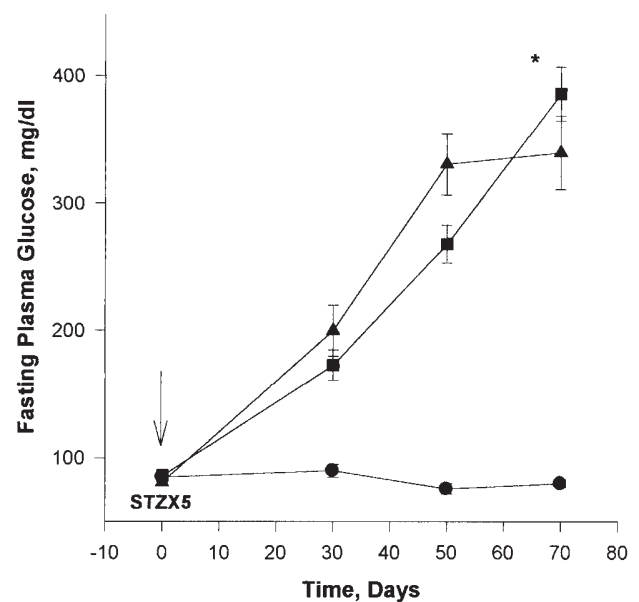


FIG. 1. Plasma glucose levels in STZ- and citrate-treated mice. Plasma from LDL-RD mice ($n = 15$) treated with STZ standard diet (▲), STZ Western diet (■) ($n = 15$), or citrate buffer (●) ($n = 15$) were assayed by a blood glucose sensor kit for the concentrations of glucose, as described in RESEARCH DESIGN AND METHODS. Data are presented as means \pm SE. * $P < 0.0001$.

TABLE 1
Lipid profile in STZ- and citrate-treated mice

Group	n	Total cholesterol (mg/dl)			Triglyceride (mg/dl)		
		0 Weeks	4 Weeks	10 Weeks	0 Weeks	4 Weeks	10 Weeks
STZ + standard diet	15	250 ± 28	276 ± 45	345 ± 42	259 ± 70	248 ± 42	272 ± 25
Western diet	15	226 ± 31	185 ± 45	979 ± 243	226 ± 42	197 ± 50	140 ± 44
STZ + Western diet	15	237 ± 35	248 ± 63	1,707 ± 364*	200 ± 50	226 ± 42	558 ± 256*

Data are means ± SD. Measurement of total cholesterol and triglyceride levels was performed on the plasma taken from the retro-orbital plexus of hyperglycemic and normoglycemic mice. The determination was done as described in RESEARCH DESIGN AND METHODS. * $P < 0.0001$.

lesterol levels in the STZ-injected mice were significantly higher ($1,707 \pm 91$ mg/dl) than in the citrate-treated mice (980 ± 67 mg/dl; $P < 0.0001$). Plasma triglyceride levels were also higher in the STZ-treated mice (538 ± 250 mg/dl vs. 140 ± 44 mg/dl in citrate-treated control mice) ($P < 0.0001$). On the other hand, STZ injection in standard diet-fed mice had no effect on plasma lipid levels (Table 1). HDL levels at time 0 were 120 ± 24 vs. 121 ± 36 mg/dl for STZ-treated and control mice, respectively. HDL levels were lower at the end of the study in both groups (71 ± 29 vs. 78 ± 21 mg/dl). Similar findings were observed in female mice (data not shown).

Antibodies to HSP65 and oxLDL in hyperglycemic hyperlipidemic mice. STZ-induced hyperglycemia in the hyperlipidemic LDL-RD mice was associated with elevated levels of anti-HSP65. Whereas in the beginning of the study, no differences in antibody levels were detected, after 30 days (before starting the Western diet), the levels in STZ-injected mice were higher (mean absorbance ± SD, 0.03 ± 0.01) in comparison to citrate buffer-injected mice (0.04 ± 0.02 ; $P = 0.31$). At death, anti-HSP65 levels in STZ-injected mice were significantly higher (mean absorbance ± SD, 0.46 ± 0.02) in comparison to citrate buffer-injected mice (0.22 ± 0.01 ; $P < 0.009$)

(Fig. 2A). No significant differences were evident in anti-HSP65 levels between the STZ-injected standard diet-fed group and citrate-treated group ($P = 0.748$).

Anti-oxLDL levels increased in hyperlipidemic LDL-RD mice (citrate-treated). However, when hyperglycemia was induced, anti-oxLDLs failed to increase and their levels were similar to those obtained at baseline (Fig. 2B). Similar findings were observed in female mice (data not shown).

Cellular immune response to HSP65. Proliferative response of splenocytes from STZ-injected mice to HSP65 was significantly elevated (SI of 4.73 ± 0.80) in comparison to the value obtained for the citrate-injected mice (SI of 1.88 ± 0.48 ; $P < 0.001$) (Fig. 3).

Cytokine production from splenocytes in response to in vitro HSP65 stimulation. When stimulated with HSP65 ($10 \mu\text{g/ml}$) in vitro, splenocytes from STZ-treated mice significantly increased the secretion of the T-helper (TH)-1 cytokine IFN- γ (from 26.7 ± 5.8 to 485 ± 55 pg/ml). No detectable change in the pattern of the IL-4 cytokines was evident. IL-10 secretion diminished in the STZ-injected mice from 14 ± 8.6 to 2.8 ± 2.8 pg/ml in the presence of HSP65). This trend favoring TH1 cytokine shift was not as pronounced in

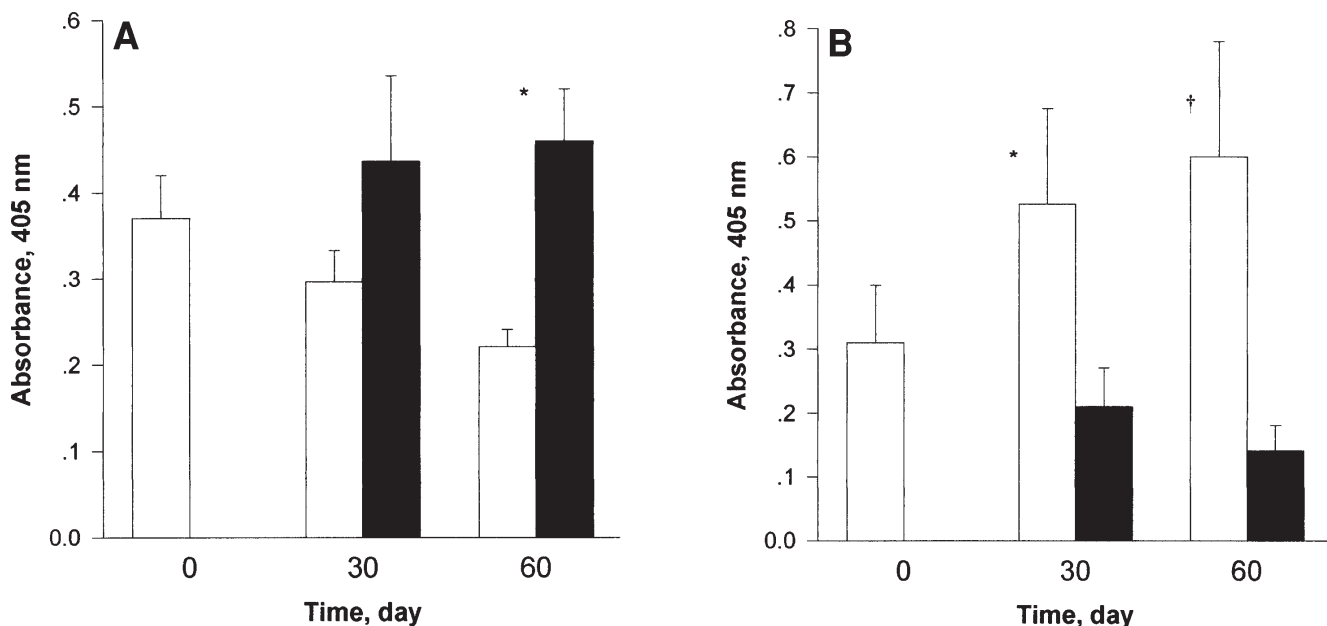


FIG. 2. Kinetics of anti-HSP65 (A) and anti-oxLDL (B) antibody production. HSP65 or oxLDL was detected in STZ-treated (■, $n = 10$) and citrate-treated (□, $n = 10$) mice, as described in RESEARCH DESIGN AND METHODS. Data are presented as means ± SE. * $P < 0.009$; † $P < 0.0001$.

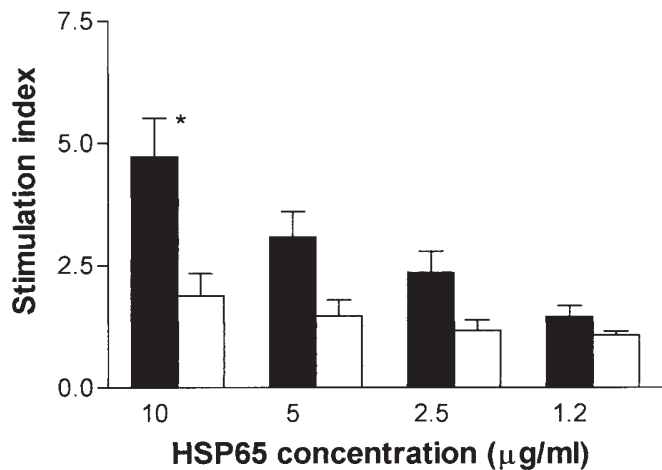


FIG. 3. Proliferative response from diabetic hyperlipidemic and hyperglycemic mice. At the end of the study, spleens from STZ-treated (■) or citrate-treated (□) mice (3 from each group) were removed, and the lymphocytes were isolated and stimulated in HSP65 (1.2–10 µg/ml), as described in RESEARCH DESIGN AND METHODS. Results are presented as mean SI ± SE. * $P < 0.001$.

the citrate-treated mice before and after HSP65 stimulation for IFN- γ (from 141 ± 38.4 pg/ml before to 305.5 ± 58.3 pg/ml after) and for IL-10 (from 14 ± 8.6 pg/ml before to 2.8 ± 2.8 pg/ml after). These results are summarized in Table 2.

Aortic lesion formation in hyperlipidemic hyperglycemic mice. Atherosclerotic lesion formation was considerably increased in the aortic sinus from STZ-injected males (mean aortic lesion, $132 \pm 23 \times 10^5 \mu\text{m}^2$) in comparison to the citrate-treated mice ($20 \pm 6.6 \times 10^5 \mu\text{m}^2$; $P < 0.0001$) (Figs. 4 and 7). A similar trend was evident in female mice (data not shown). A significant positive correlation was evident between fasting glucose levels and cholesterol levels (Fig. 5A). A significant positive correlation ($R = 0.64$; $P < 0.001$) was noted between the atherosclerotic lesions at the aortic sinus and the fasting plasma glucose levels at the end of the study (Fig. 5B). A significant positive correlation ($R = 0.61$; $P = 0.005$) was detected between the atherosclerotic lesion at the aortic sinus and the plasma cholesterol levels at the end of the study (Fig. 6). Lesions in mice with hyperlipidemia and hypergly-

cemia contained more CD3⁺ lymphocytes (5–8/lesion) compared with nondiabetic hyperlipidemic mice (1–3/lesion).

DISCUSSION

In the present study, we found that LDL-RD mice faced with the double challenge of STZ-induced hyperglycemia and diet-induced hyperlipidemia develop atherosclerotic lesions that exceeded those induced by a high-fat diet alone. This model, which combines both traditional and established risk factors, is a tool by which to study the accelerating effect of hyperglycemia on atherosclerosis in a mouse model. Several authors reported apparently nonoverlapping results when using induction of hyperglycemia and hyperlipidemia in mice. In the first report by Kunjathoor et al. (24), naive BALB/c and C57BL/6 mice were challenged with STZ-induced hyperglycemia and a high-fat diet. The BALB/c but not the C57BL/6 mice were found to develop larger fatty streaks, suggesting a genetic component in the progression of the disorder. However, the drawbacks of the model are the small size of the lesions induced in the naive animals and the use of a proinflammatory cholate-containing high-fat diet that may affect lesion formation in an unrelated mechanism. A combined model has also been tested by Reaven et al. (18) in LDL-RD mice treated with STZ. In this study, no differences were evident between STZ-treated and control mice with respect to lesion development. However, the mice were supplemented with a high-fat diet for a relatively long period of 6 months, suggesting that the significant hyperlipidemia overwhelmed the influence of the induced hyperglycemia, making it logical to use a shorter dietary program like the one used in the current study.

Recently, Park et al. (17) demonstrated acceleration of atherosclerosis in apoE-deficient mice injected with STZ, omitting the need for high-fat diet feeding in this mouse with spontaneous hyperlipidemia. Our study provides complementary evidence for the possible synergistic effects of both hyperlipidemia and hyperglycemia and may corroborate the relationship of diabetes and atherosclerosis.

An additional advantage of the current model over previously described combined models (17,18,24) is that both risk factors are induced exogenously (hyperglycemia by STZ and hyperlipidemia by high-fat diet feeding). The model allows investigation of the effects of different timings of both risk factors on their synergism with respect to lesion formation. The second

TABLE 2

Cytokine concentration in the medium after in vitro stimulation in STZ- and citrate-injected mice

Antigen	Bovine serum albumin	HSP65	Concanavalin A
IFN- γ (pg/ml)			
Citrate	141.5 ± 38.4	$305.5 \pm 58.3^*$	$3,895 \pm 58.0$
STZ	$26.7 \pm 5.8\ddagger$	$485 \pm 55.0\ddagger$	$4,91 \pm 54.0$
IL-4 (pg/ml)			
Citrate	ND	ND	88 ± 23.3
STZ	ND	ND	84 ± 19.5
IL-10 (pg/ml)			
Citrate	25.7 ± 11.8	8.2 ± 3.5	883.5 ± 55.6
STZ	14 ± 8.6	2.8 ± 2.8	365 ± 17.7

Data are means \pm SD of 3 independent experiments. Conditioned media after splenocyte stimulation with 10 µg/ml HSP65 were collected 48 h after incubation. Cytokine concentrations were determined by ELISA kits, as described in RESEARCH DESIGN AND METHODS. * $P < 0.05$ compared with bovine serum albumin; $\ddagger P < 0.001$ compared with bovine serum albumin. ND, not detectable.

goal of the present study was to investigate the autoimmune aspect of the combined model to elucidate whether HSP65-driven immune response can add to the accelerated atherosclerosis evident in diabetics. The basis for this hypothesis lies in previous observations that pertain to the proatherogenic role of HSP65 immunization in rabbits (8) and mice (9; reviewed in 25). Furthermore, anti-HSP65 has been found to be associated with increased carotid atherosclerotic plaques, and in vitro studies support a cytotoxic role for anti-HSP65 antibodies on cellular constituents of the atherosclerotic lesion (26,27). Type 1 diabetes and its equivalent in animal models are considered autoimmune in origin, leading to a selective destruction of pancreatic β -cells. HSP65 has been proposed as a target candidate antigen in the immune-mediated destruction (12–16). Herein, we wished to study the progression of atherosclerosis in diabetic hyperlipidemic mice and to study the association with immunity to HSP65. Indeed, we have found a significantly enhanced humoral and cellular immune response to HSP65 in the mouse with the combined model. Moreover, when in contact with HSP65 in vitro, splenocytes from STZ-injected mice favored the secretion of the TH1 cytokine IFN- γ . It is important to notice that whereas there was no significant difference in SI with different concentrations of HSP65 in the citrate-treated group, a dose-dependent response curve was detected in the STZ-treated mice. This observation is in accord with the observation of Albers et al. (28), which noticed that coinjection of STZ with ovalbumin drove a predominant TH1 response in an unrelated model. These findings are consistent with the proatherogenic role of IFN- γ in atherosclerosis, which was reported in the study by Gupta et al. (29). These authors showed that crossing apoE-deficient mice with animals lacking the IFN- γ receptor results in double transgenic mice that develop significantly fewer lesions in comparison with their apoE-deficient littermates.

Our hypothesis is that STZ-induced hyperglycemia may be associated with the production of HSP65-reactive antibodies and T-cells with concomitant expression of self-

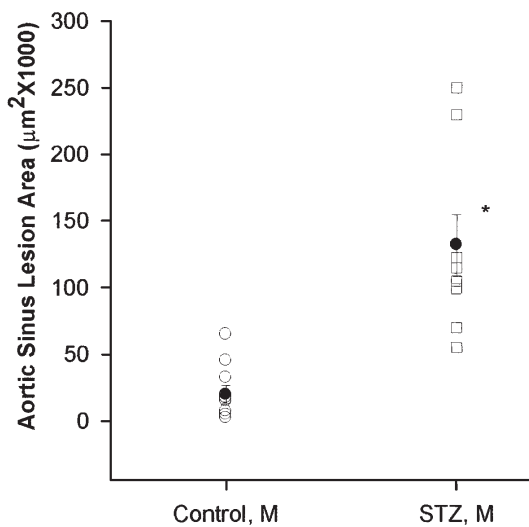


FIG. 4. Atherosclerotic lesion size in STZ- and citrate-treated mice. At the end of the experiment, hearts were removed from control ($n = 10$) and STZ-treated ($n = 10$) mice. Serial 10- μm sections performed at the aortic sinus region were stained with Oil-red O (Sigma, St. Louis, MO). The values represent mean aortic lesion size \pm SD. * $P < 0.0001$.

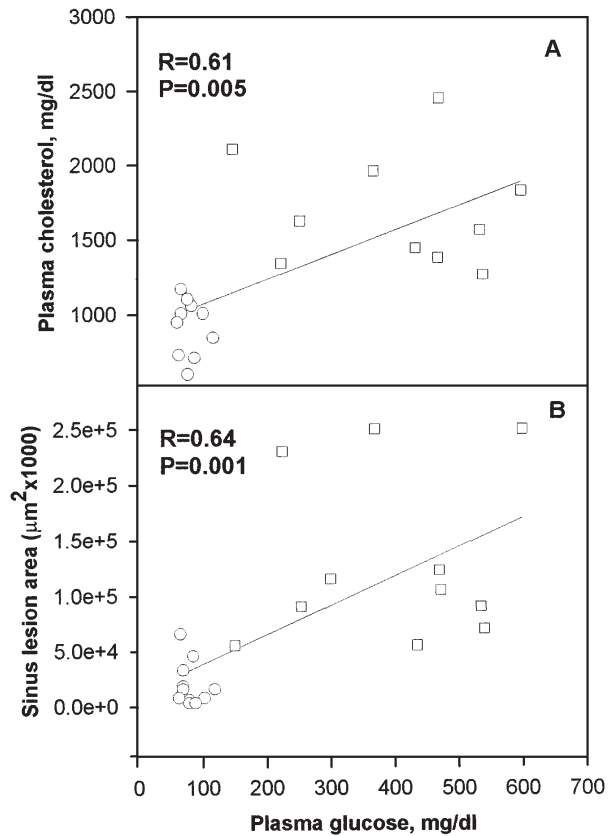


FIG. 5. Correlation between glucose and cholesterol levels and glucose and sinus atherosclerotic lesions. Plasma glucose levels were correlated with plasma cholesterol levels (A) and sinus atherosclerotic lesion area (B) at the end of the experiment in STZ-treated (\square) and citrate-treated (\circ) mice.

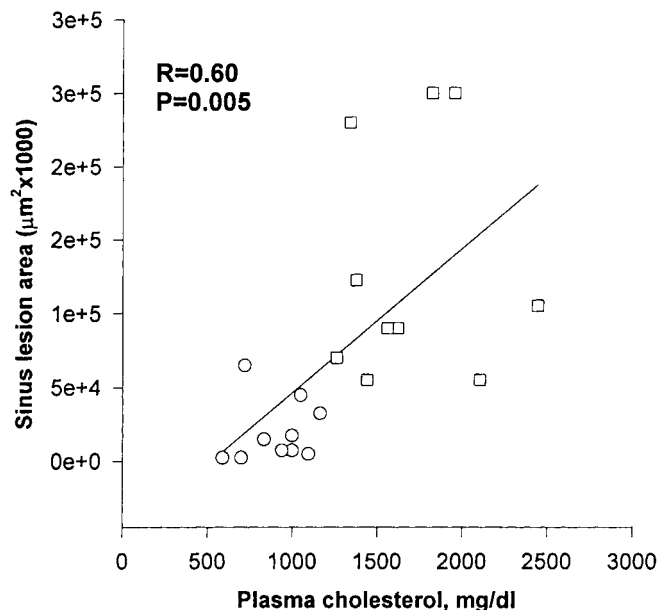


FIG. 6. Correlation between the atherosclerotic lesions and plasma cholesterol levels. Plasma cholesterol levels were correlated with sinus atherosclerotic lesion area at the end of the experiment in STZ-treated (\square) and citrate-treated (\circ) mice.

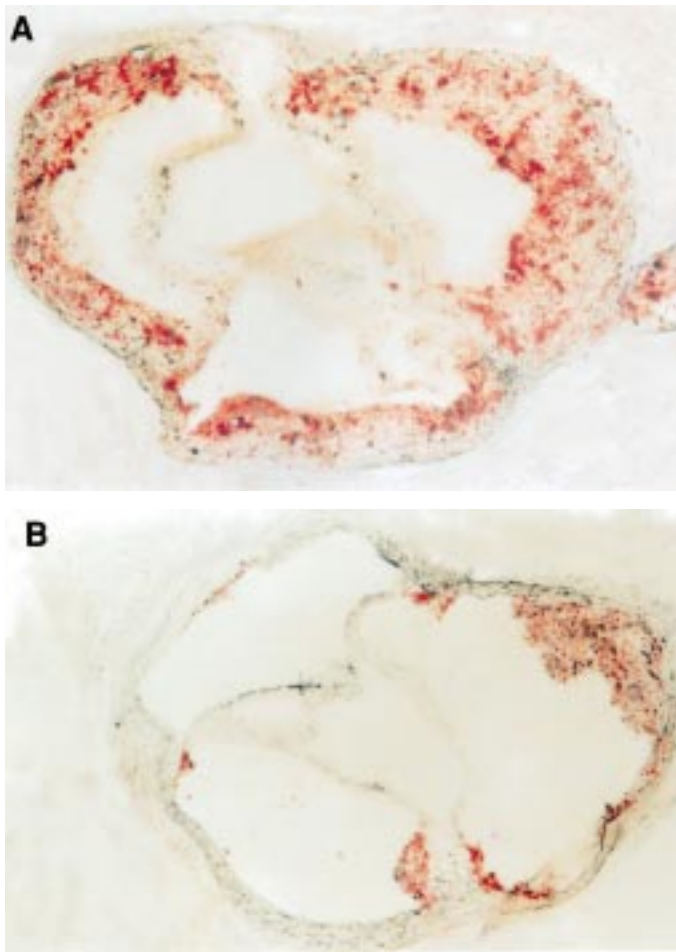


FIG. 7. Atherosclerotic lesions in STZ- and citrate-treated mice. **A** representative Oil-red O-stained section from a male LDL-RD mouse injected with STZ (**A**) in comparison with a section from a citrate-treated littermate (**B**) is shown.

HSP60 in the pancreas and arterial wall of mice. When the HSP65-reactive T-cells face the overexpressed HSP60 within aortic tissue, they tend to shift their cytokine towards the proatherogenic type 1 response and consequently experience enhanced lesion formation. This result can be additive to other direct and indirect effects of hyperglycemia and hypercholesterolemia in these mice. To exclude these effects, we intend to treat these mice with insulin and a hydroxymethylglutaryl-CoA reductase inhibitor.

In conclusion, in the present study, we have shown accelerated atherosclerosis in a model that combines high-fat diet-induced hyperlipidemia with STZ-induced hyperglycemia in the LDL-RD mice. The combined model suggests a possible additive role for hyperglycemia on atherosclerosis progression by dyslipidemia effects or other mechanisms. Autoimmunity to HSP65 may play a role of being involved in mediating the additive effect of hyperglycemia on atherogenesis.

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