**Enhanced Fatty Streak Formation in C57BL/6J Mice by Immunization With Heat Shock Protein-65**

Jacob George, Yehuda Shoenfeld, Arnon Afek, Boris Gilburd, Pnina Keren, Aviv Shaish, Juri Kopolovic, Georg Wick, Dror Harats

**Abstract**—Recent data suggest that the immune system is involved in atherogenesis. Thus, interest has been raised as to the possible antigens that could serve as the initiators of the immune reaction. In the current work, we studied the effects of immunization with recombinant heat shock protein-65 (HSP-65) and HSP-65–rich *Mycobacterium tuberculosis* (MT) on early atherogenesis in C57BL/6J mice fed either a normal chow diet or a high-cholesterol diet (HCD). A rapid, cellular immune response to HSP-65 was evident in mice immunized with HSP-65 or with MT but not in the animals immunized with phosphate-buffered saline (PBS) alone. Early atherosclerosis was significantly enhanced in HCD-fed mice immunized with HSP-65 (n=10; mean aortic lesion size, 45 417±9258 μm²) or MT (n=15; 66 350±6850 μm²) compared with PBS-injected (n=10; 10 028±3599 μm²) or nonimmunized (n=10; 9500±2120 μm²) mice. No fatty streak lesions were observed in mice fed a chow diet regardless of the immunization protocol applied. Immunohistochemical analysis of atherosclerotic lesions from the HSP-65– and MT-immunized mice revealed infiltration of CD4 lymphocytes compared with the relatively lymphocyte-poor lesions in the PBS-treated or nonimmunized mice. Direct immunofluorescence analysis of lesions from HSP-65– and MT-immunized mice fed an HCD exhibited extensive deposits of immunoglobulins compared with the fatty streaks in the other study groups, consistent with the larger and more advanced lesions found in the former 2 groups. This model, which supports the involvement of HSP-65 in atherogenesis, furnishes a valuable tool to study the role of the immune system in atherogenesis. (Arterioscler Thromb Vase Biol. 1999;19:505-510.)

**Key Words:** atherosclerosis ■ autoantibodies ■ heat shock protein-65 ■ oxidized LDL ■ *Mycobacterium tuberculosis*

Atherosclerosis is a multifactorial process, the hallmark of which is an accumulation of lipids within the vessel wall, accompanied by mononuclear cell infiltration and proliferation of smooth muscle cells.¹ In recent years, it has become apparent that the immune system plays an important role in the earliest and subsequent stages of atherosclerosis, as manifested by the finding of activated T cells and immunoglobulins within the lesions at different stages.²⁻⁵ These observations have prompted the search for possible (auto)antigenic candidates against which a local immune response is presumed to be triggered. The 2 major antigens proposed by different groups are heat shock protein (HSP)-60/65 and oxidized LDL (oxLDL).⁶⁻⁸

The association of oxLDL with accelerated atherosclerosis has been suggested by direct evidence pointing to its existence within atherosclerotic plaques in humans and animals.⁹¹⁰ Furthermore, circumstantial data suggest that T-lymphocyte clones extracted from human atherosclerotic lesions recognize ox-LDL.¹¹ However, experimental studies in animals indicate that immunization with homologous oxLDL elicits a protective, rather than a deleterious, effect on the development of atherosclerosis and neointimal formation.¹²⁻¹⁵

Probably the most illustrative evidence for the existence of an autoimmune reaction in atherosclerosis was provided in recent work by Wick and coworkers (Xu et al¹⁶), showing that normocholesterolemic rabbits immunized with recombinant HSP-65 or with *Mycobacterium tuberculosis* (MT; an HSP-65–rich bacterium) developed atherosclerosis when fed a normal chow diet. These same authors have subsequently reinforced these findings by showing increased expression of HSP-60 within atherosclerotic lesions¹⁷ and documenting increased humoral response to the antigen in humans with carotid atherosclerosis and coronary heart disease.¹⁸¹⁹

The aim of the present study was to extend these findings to a mouse model known to be genetically more resistant to atherosclerosis than are rabbits and to study the nature of the immune response toward HSP-65 and oxLDL. The advantage of having a mouse model with accelerated atherosclerosis induced by immunization would be the ability to conduct controlled studies with a large number of animals to further
elucidate the role of the immune system in atherosclerosis. We show herein that C57BL/6J mice fed a high-cholesterol diet (HCD) develop significantly enhanced fatty streak accumulation when immunized with recombinant HSP-65 or with MT containing the HSP-65.

Methods

Mice
Female C57BL/6J mice, 8 weeks old, were purchased from Tel Aviv University, Tel Aviv, Israel.

Diets
Mice were fed either an HCD (containing 1.25% cholesterol and 0.5% sodium cholate; TD 88051, Harlan Teklad) or a conventional mouse diet (Chow). The diet and water were provided ad libitum. During the study, mice remained healthy, as evidenced by coat condition and body weight gain.

Antigens and Reagents: Recombinant HSP-65
Antigens and reagents for recombinant HSP-65 were purchased from Dr M. Singh, Braunschweig, Germany.

Adjuvant
Incomplete Freund’s adjuvant (IFA; HSP-65–free) was obtained from DIFCO Laboratories and was fortified by adding 5 mg/mL MT, and the mixture was sonicated on ice before emulsification.

Preparation of LDL and OxLDL
Blood for lipoprotein isolation was collected in EDTA from mice after 12 hours of fasting. LDL (density =1.019 to 1.063 g/mL) was isolated from plasma as previously described, and the amount of KBr, by preparative ultracentrifugation at 50,000 rpm/min for 22 hours with a type 50 rotor. LDL preparations were treated with added KBr, by preparative ultracentrifugation at 50,000 rpm/min for 22 hours with a type 50 rotor. LDL preparations were washed after ultracentrifugation, dialyzed against 0.15 Molar EDTA (pH 7.4), passed through an Acrodisc filter (0.22-micron pore size) to remove aggregates, and stored under N2, in the dark.

LDL oxidation was performed by incubation of predialyzed LDL (1 mg protein/mL in EDTA-free PBS) with CuSO4 (10 micromolar/L) for 24 hours at 37°C. Lipoprotein oxidation was confirmed by analysis of the conjugated-diene content of the lipoproteins.

Experimental Design
A pilot study performed before the main experiment indicated that immunization of C57BL/6J mice with either HSP-65 or MT did not lead to fatty streak formation when mice were fed a chow diet. Thus, in the main experiment, most of the animals were fed the HCD. Mice were immunized subcutaneously 3 times (every 3 weeks) with either HSP-65 (10 mg/mL), MT (5 mg/mL), or PBS, all emulsified in the background cpm obtained in the absence of the antigen. The SDs were always<10% of the mean cpm.

Proliferation Assays of Draining Lymph Node Lymphocytes From Immunized Mice
Draining inguinal lymph nodes were collected from 4 HSP-65–, 4MT–, and 4PBS–immunized mice killed 11 days after the primary immunization. The assays were performed as previously described11 with minor modifications. In brief, 106 cells/mL were incubated in triplicate for 72 hours in 0.2 mL of culture medium in microtiter wells in the presence of 2 or 10 ng/mL HSP-65. Cell proliferation was measured by the incorporation of [3H]thymidine into DNA during the final 12 hours of incubation. The results were computed as a stimulation index (SI), as the ratio of the mean counts per minute (cpm) of the antigen to the mean background cpm obtained in the absence of the antigen. The SDs were always<10% of the mean cpm.

Detection of Anti–HSP-65 Antibodies
Recombinant HSP-65 (1 µg/mL) in PBS (pH 7.2) was coated onto flat-bottom, 96-well ELISA plates (Nunc Maxisorp) by overnight incubation at 4°C as previously described. After washing with 0.02% PBS–TWEEN and blocking with 1% BSA in PBS, sera were added in different dilutions (1:50, 1:100, and 1:200 in PBS) and incubated for 1 hour at room temperature. Peroxidase-conjugated rabbit anti-mouse IgG (Dako Ltd, High Wycombe, UK) was added, and incubation was continued for 1 hour at room temperature, followed by 4 washes with PBS/TWEEN. Finally, 100 µL of citrate phosphate buffer (0.1 mol/L, pH 4.2) containing 0.53 mg/mL 2,2’-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) (Sigma Chemical Co) was added, and absorbance was measured after 30 minutes at 490 nm in a Titertek ELISA reader.

Exhibition assays were performed to confirm the specificity of anti–HSP-65 antibodies and to check for a possible cross-reactivity with oxLDL, an important immunogen in atherosclerosis. The concentration of HSP-65–immunized mouse serum resulting in the half-maximal binding to HSP-65 was determined, and different inhibitors (ie, HSP-65, oxLDL, or BSA) were applied in increasing concentrations.

Detection of Anti-OxLDL Antibodies
Ninety-six-well polystyrene plates (Nunc) were coated with either copper-oxidized LDL, native LDL (at a concentration of 5 µg/mL in PBS), or PBS alone overnight at 4°C. After 4 washes with PBS containing 0.05% Tween and 0.001% aprotinin (Sigma), the plates were blocked with 2% BSA for 2 hours at room temperature. Serum fractions were diluted to 1:50 in PBS–0.05% Tween–0.2% BSA and added to the wells. After additional overnight incubation the plates were washed, and alkaline phosphatase–conjugated goat anti-mouse IgG (Jackson ImmunoResearch laboratories Inc), diluted 1:10 000 in PBS–0.05% Tween–0.2% BSA, was added for 1 hour at room temperature. After extensive washing, 1 mg/mL p-nitrophenyl phosphate (Sigma) in 50 mmol/L carbonate buffer containing 1 mmol/L MgCl2, pH 9.8, was added as a substrate. The reaction was stopped after 30 minutes by adding 1 mol/L NaOH. The optical density was read at a 405-nm wavelength in a Titertek ELISA reader (SLT

<table>
<thead>
<tr>
<th>Table 1. Experimental Immunization Groups</th>
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<tr>
<td>Immunizing Antigen/Adjuvant</td>
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<tr>
<td>-----------------------------</td>
</tr>
<tr>
<td>HSP-65 IFA</td>
</tr>
<tr>
<td>HSP-65 IFA</td>
</tr>
<tr>
<td>MT IFA</td>
</tr>
<tr>
<td>MT IFA</td>
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<tr>
<td>PBS IFA</td>
</tr>
<tr>
<td>None</td>
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Cholesterol Levels
At the end of the experiment, 1 to 1.5 mL of blood was obtained by cardiac puncture; 1000U/mL heparin was added to each sample. Total plasma cholesterol levels were determined by using an automated enzymatic technique (Boehringer Mannheim).
Laboratory Instruments). Levels of anti-oxLDL antibodies were calculated as the level of binding to native LDL subtracted from that for the binding to oxLDL.

Assessment of Atherosclerosis
Quantification of atherosclerotic fatty streak lesions was done by calculation of lesions size in the aortic sinus as previously described\(^{22}\) with a few modifications. In brief, the heart and upper portion of the aorta were removed from the animals and the peripheral fat was carefully removed. The upper section was embedded in OCT medium and frozen. Every other section (10-µm thick) throughout the aortic sinus (400 µm) was taken for analysis. The distal portion of the aortic sinus is recognized by the 3 valve cusps that form the junction of the aorta to the heart. Sections were evaluated for fatty streak lesions after being stained with oil red O. Lesion areas per section were counted on a grid by an observer who was unfamiliar with the tested specimen.

Immunohistochemistry
Immunohistochemical staining (antibodies to mouse CD4, CD8a, and macrophages; all from Serotek) of cryostat sections (5-µm thick) of the aortic sinus was done as described previously.\(^{22}\) For demonstration of oxLDL epitopes in the lesions of the mice, the sections were fixed (with methanol and subsequently with acetone) and blocked before incubation with mouse monoclonal antibodies specific for MDA-modified lysines of LDL (MDA2; obtained by immunization of BALB/c mice with MDA-LDL; a kind gift of Dr S. Yla-Herttuala, Helsinki, Finland\(^{23}\)). The assay was performed using the Histomouse-SP bulk kit (Zymed Laboratories Inc) for detection of mouse primary antibodies on mouse tissues. The results were evaluated by light microscopy.

Direct Immunofluorescence to Detect Murine Bound Antibodies
Mouse hearts were perfused with NaCl and quick-frozen in LN\(_2\). Cryostat sections (5 µm) were stained with FITC-conjugated, affinity-purified goat anti-mouse IgG and IgM F(ab\(^\prime\))\(_2\) (Sigma) and then examined under a fluorescence microscope.

Statistical Analysis
Data are presented as mean±SEM. Statistical differences were determined by 1-way ANOVA to determine interactions between immunization groups. \(P<0.05\) was accepted as statistically significant.

Results

Body Weights
Initial body weights for C57BL/6J mice were 22±2 g (mean±SEM). Final average body weights were 24 to 25 g. No statistically significant differences were found between weights in the various immunization groups at the end of the experiment.

Cholesterol Levels
Average cholesterol levels in the plasma of the C57BL/6J mice fed an HCD were 2.549±0.226 mg/mL compared with their littermates fed a chow diet (0.704±0.062 mg/mL). No differences in the cholesterol levels were evident between the experimental groups given the same diet.

Proliferative Responses of Draining Lymph Node Cells to HSP-65
Significant SIs to 10 µg/mL HSP-65 were evident in both the HSP-65– (7.9±0.55) and MT– (2.95±0.13) immunized mice but not in the PBS-immunized mice (1.07±0.13; Figure 1). The results of the SI measurements were also significant for lower concentrations of HSP-65 in the culture medium (2 µg/mL).

Antibodies to HSP-65
Anti–HSP-65 levels in the sera of chow-fed, MT-immunized and HSP-65–immunized mice were higher than those in the chow-fed, nonimmunized mice (Table 2). The anti–HSP-65 levels in the HSP-65–immunized mice were considerably higher than those in their MT-immunized littermates. Levels of HSP-65 antibodies were significantly lower in HCD-fed, MT-immunized mice compared with their chow-fed, MT-immunized littermates.

Table 2. Anti–HSP-65 and OxLDL Antibody Levels

<table>
<thead>
<tr>
<th>Immunization Group</th>
<th>Anti–HSP-65</th>
<th>Anti–OxLDL</th>
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<tbody>
<tr>
<td>HCD+HSP-65</td>
<td>1.26±0.151*</td>
<td>0.323±0.051</td>
</tr>
<tr>
<td>Chow+HSP-65</td>
<td>1.23±0.153*</td>
<td>0.331±0.072</td>
</tr>
<tr>
<td>HCD+MT</td>
<td>0.263±0.045</td>
<td>0.326±0.055</td>
</tr>
<tr>
<td>Chow+MT</td>
<td>0.468±0.085*</td>
<td>0.74±0.121*</td>
</tr>
<tr>
<td>HCD+PBS</td>
<td>0.359±0.207</td>
<td>0.616±0.031*</td>
</tr>
<tr>
<td>HCD+none</td>
<td>0.431±0.167</td>
<td>0.678±0.172†</td>
</tr>
<tr>
<td>Chow+none</td>
<td>0.211±0.035</td>
<td>0.441±0.040</td>
</tr>
</tbody>
</table>

Antibody levels were determined in sera of mice taken at the end of the experiment. Results are expressed as mean±SEM. *\(P<0.001\) compared with nonimmunized, chow-fed mice.
†\(P<0.05\) compared with chow-fed, nonimmunized mice.

Figure 1. Proliferative responses of draining lymph node cells to HSP-65. Lymph node cells were collected from 4 injected mice 11 days after the primary immunization (with either HSP-65, MT, or PBS) and incubated with HSP-65 (at concentrations of 2 or 10 µg/mL) as described in Methods. Results are presented as SIs and are mean±SEM.
Early Development of Atherosclerosis in the Aortic Sinuses

The mice that were fed a chow diet did not develop early fatty streaks in their aortas regardless of the immunization protocol applied. Mean aortic lesion size in the nonimmunized, HCD-fed mice was $9500 \pm 2120 \, \mu m^2$. Significantly enhanced fatty streak formation, when compared with nonimmunized mice, was evident in the HSP-65– (45 417 $\pm 9258 \, \mu m^2; P < 0.05$) and MT– (66 350 $\pm 6850 \, \mu m^2; P < 0.05$) immunized mice. Lesion development in the PBS-immunized mice (10 028 $\pm 3599 \, \mu m^2$) was similar to that in their nonimmunized littermates (Figures 3 and 4).

Immunohistochemistry

Lesions from mice in all experimental groups were found to contain macrophages. The lesions of HSP-65– and MT-immunized mice appeared more mature and contained significantly more macrophages. Fatty streaks in the aortic sinus sections from the HCD-fed, MT- and HSP-65-immunized mice were found to possess larger numbers of CD4+ lymphocytes (with minor amounts of CD8+ lymphocytes). Only low numbers of CD4+ and CD8+ lymphocytes were evident in the lesions of the PBS-immunized or nonimmunized mice fed an HCD.

More lesions from the HCD-fed, MT-immunized mice were found to exhibit MDA-LDL–specific epitopes, evident by staining with a monoclonal anti–MDA-LDL antibody. Direct immunofluorescence studies displayed significantly greater IgG deposition in the lesions from MT and HSP-65 immunization groups and in comparison with lesions taken from PBS-immunized or nonimmunized, HCD-fed mice. The larger quantities of lymphocytes and immunoglobulins found in the MT- and HSP-65–immunized mice were consistent with the more advanced lesions found in these groups.

Discussion

The principal aim of the current study was to establish an inbred small-animal model of atherosclerosis by immunization with either recombinant HSP-65 or the HSP-65–rich MT, a goal that has successfully been accomplished in normcholesterolemic rabbits.16 An additional purpose was to investigate the role of the immune response toward HSP-65 and oxLDL after immunization with heat-killed preparations of MT, which are known to contain HSP-65, or with the recombinant protein only. This finding is of particular interest, in view of the current controversy as to the principal autoantigen that triggers the immune response within atherosclerotic plaques.5,7 It has been suggested5,8,16,17 that the expression of HSP, probably due to various stressful insults,
results in a respective autoimmune response that accelerates atherosclerosis. Further support for this hypothesis is the induction of arteriosclerosis by immunization of normocholesterolemic rabbits with HSP-65 and MT,16 or equivalently, by infections (Marek’s disease in chickens; cytomegalovirus, herpes simplex virus, and Chlamydia pneumonia in humans; reviewed in Reference 24). A leading notion holds that oxidation of LDL is an important factor in atherosclerosis, assuming that the subsequent cellular and immune response toward it plays a protective role.12–15

In the present study, we have shown that HCD-fed C57BL/6J mice immunized with MT or with HSP-65 developed accelerated fatty streak formation in their aortic sinuses compared with their PBS-immunized or nonimmunized littermates fed a similar diet. Both MT- and HSP-65–immunized mice exhibited a clear and specific cellular immune response toward HSP-65, evident by the proliferation of lymph node cells to the protein. Furthermore, it was apparent that the Chow-fed mice immunized with MT, the HCD-fed mice, and particularly the HSP-65–immunized animals, developed higher levels of antibodies to HSP-65 compared with MT-immunized mice fed an HCD. Antibodies to oxLDL were also found to be lower in the HCD-fed, MT-immunized mice when compared with their Chow-fed littermates. A possible explanation for the reduced oxLDL and HSP-65 levels in the MT-immunized mice became apparent during the immunohistochemistry studies. The lesions in the C57BL/6J mice immunized with MT or with HSP-65 and fed an HCD contained large deposits of immunoglobulins, which could not be detected in the lesions of their littermates (nonimmunized mice fed an HCD). We suggest that immunization with MT or HSP-65 induces humoral and cellular immune reactions against HSP-65, which then exerts a cross-reactive effect on arterial endothelial cells expressing HSP-60 due to feeding of the HCD. Indeed, HSP-65 and oxLDL antibodies can be detected in mice immunized with MT, as well as in those fed the HCD. The unexpectedly low titers of anti-oxLDL and anti–HSP-65 antibodies in the HCD-fed, MT-immunized mice can be explained either by the occurrence of immune complexes or by the deposition of the antibodies in the fatty streaks. The levels of HSP-65 antibodies in the HSP-65–immunized mice were so high that deposition in the plaque did not influence the overall levels in the mouse sera. A very recent report supports this concept, showing that patients with acute myocardial events have significantly lower titers of anti–HSP-65 antibodies compared with subjects with coronary heart disease. This observation suggests that the expression of HSP-60 by the infarcted heart “consumes” the anti–HSP-65 previously present in the sera of these patients. These findings, combined with the in vitro assays demonstrating the potential of anti–HSP-60 antibodies to mediate endothelial cytotoxicity, lend support to the role of the humoral response in promoting the accelerated atheroerotic process. Recent studies have raised the issue of immunomodulatory roles of anti-oxLDL antibodies in the pathogenesis of atherosclerosis, suggesting that these antibodies, when induced by immunization with homologous oxLDL, are associated with the suppression of atherosclerosis in animals. These findings and the data from the current study suggest that antibodies against “injurious” oxLDL may lead to protection from atherosclerosis, whereas a humoral response to protective HSP-60 accelerates atherogenesis. An interesting observation was that despite the lower anti–HSP-65 antibody levels in the MT-immunized mice in comparison with HSP-65–immunized animals, atherosclerosis was significantly enhanced in both groups. It is possible that antibodies of different specificity are induced by the 2 immunization protocols. Additionally, immunization with MT may have a more pronounced effect on the production of proinflammatory cytokines that may then exert an additive effect on atherogenesis in this mouse model. The role of the cellular immune system in atherogenesis is also controversial. Several authors have shown that suppression of cellular immunity resulted in accelerated atherosclerosis in animals. Yet treatment with monoclonal antibodies to CD4 was found to suppress atherogenesis in mice, suggesting that the cellular immune response is involved in the progression of atherosclerosis. Two recent studies imply that apoE-deficient mice (an atherosclerosis-prone strain) develop similarly mature atherosclerotic lesions when deprived of their T- and B-cell functions by cross-breeding with recombinase-activating gene−1− and −2−deficient mice.

How can these observations be reconciled with our current results pointing to the relative abundance of CD4 cells within lesions of MT- and HSP-65–immunized mice compared with the scarcity of these immunopotent cells in the nonimmunized mice? It appears that atherosclerosis is a multifactorial process involving several cellular components. Exogenous factors such as infections and encounters with additional antigens by various routes are likely to influence the progression of atherogenesis, in addition to the known major causes, such as hypercholesterolemia and genetic susceptibility. In our study, lymph node reactivity to HSP-65 was pronounced in MT- and HSP-65–immunized mice, and the lesions from these same animals were rich in CD4 cells. It may thus be proposed that the anti–HSP-65–specific T cells generated after immunization became localized in areas known to preferentially express HSP (ie, endothelial cells faced with the “burden” of hypercholesterolemia-induced formation of LDL), where they released mediators that propagated early atherosclerosis. It is noteworthy that in the current model, unlike in the rabbit model, normocholesterolemic mice immunized with HSP-65 did not develop fatty streaks. Thus, the primary initiating factor in the mouse is hypercholesterolemia, which exerts a “stressful insult” on the endothelial cells, leading to HSP expression. The immune response to HSP-65 thus acts to enhance the severity of the lesions induced by the HCD.

The results of the current study are in line with the several studies documenting infectious agents associated with accelerated atherosclerosis. Thus, Chlamydia pneumonia and herpes simplex virus are examples of infectious agents that have been associated with accelerated atherosclerosis. In general, it has been suggested that infections elicit a nonspecific immune response that also acts at the local level (the atherosclerotic lesion), leading to an accumulation of inflammatory cells that attract macrophages, thus leading to increased uptake of lipids and accelerated atherosclerosis.

In conclusion, we have shown that immunization of mice with recombinant HSP-65 or with heat-killed, HSP-rich MT can lead to accelerated fatty streak formation. This small-
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animal model furnishes an improved tool for studying the contribution of the immune system to the development of atherosclerosis over the current atherosclerosis-prone murine models, which have been genetically manipulated. Moreover, this model could be applied as a means of studying immunomodulatory interventions in atherogenesis.

Acknowledgments

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