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Cellular and Humoral Immune Responses to Heat Shock Protein 65 Are Both Involved in Promoting Fatty-Streak Formation in LDL-Receptor Deficient Mice

Jacob George, MD,* Arnon Afek, MD,† Boris Gilburd, PhD,‡ Yehuda Shoenfeld, MD,‡
Dror Harats, MD§

Tel Aviv, Israel

OBJECTIVES This study was designed to determine the role of cellular and humoral immune responses to heat shock protein 65 (HSP65) in murine atherosclerosis.

BACKGROUND Inflammatory processes appear to influence the progression of atherosclerosis. Immunization with HSP65 was previously shown to induce arteriosclerosis in rabbits and to enhance fatty-streak formation in mice. However, it has not been demonstrated directly whether HSP65-reactive antibodies and lymphocytes are separately capable of influencing lesion formation.

METHODS Low density lipoprotein-receptor deficient (LDL-RD) mice were immunized with HSP65 or control bovine serum albumin (BSA). Lymph-node cells, splenocytes and immunoglobulin G (IgG) were obtained from the immunized mice and transferred separately to six groups of syngenic LDL-RD mice.

RESULTS Adoptive transfer of HSP65-reactive lymph node cells increased fatty-streak formation in comparison with mice treated with BSA-primed cells. Similarly, transfer of splenocytes reactive with HSP65 led to enhanced fatty-streak generation compared with mice injected with BSA-sensitized splenocytes. Repeated intraperitoneal administration of IgG from serum of HSP65-immunized mice (every 10 days) enhanced fatty-streak formation in mice in comparison with their anti-BSA-IgG injected littermates.

CONCLUSIONS Antibodies and lymphocytes reactive to HSP65 promote fatty-streak formation in mice, providing direct evidence for the proatherogenic properties of cellular and humoral immunity to HSP65. (J Am Coll Cardiol 2001;38:900–5) © 2001 by the American College of Cardiology

Atherosclerosis is a process in which inflammatory reactions appear to play a dominant role (1,2). This notion has been further extended to incriminate autoimmune factors as involved in the progression of atherosclerotic plaque (3). As such, modified lipoproteins (oxidized low density lipoprotein [LDL]) and cross-reactive autoantigens (heat shock protein [HSP] 60/65) have been proposed as triggering antigens (3).

The HSPs are a family of molecules expressed by cells in response to stressful insults, and display a high degree of sequence preservation from bacteria to humans. Heat shock proteins have been named "chaperonines" because of their role in maintenance of protein stability by protecting against unfolding (4,5). However, their overexpression, that of HSP60 in particular, has been shown to be associated with a variety of autoimmune disorders in experimental animals (6). In recent years, it has been shown by a set of clinical and experimental studies that the immune response against bacterial HSP65 cross-reacts with mammalian HSP60 and could play a proatherogenic role in animals and humans (3). According to this hypothesis, infectious or toxic agents trigger an anti-HSP65 immune response that cross-reacts with the host HSP60 and results in acceleration of atherosclerosis. The principal experimental evidence for the role of HSP60/65 autoimmunity in atherosclerosis stems from two animal studies showing enhancement in arteriosclerosis in rabbits (7) and in fatty-streak formation in C57BL/6 mice fed an atherogenic diet (8).

Partial characterization of the mechanisms involved in promotion of atherogenesis has been demonstrated by showing the ability of bacterial and human HSP60 to activate vascular endothelium and smooth muscle cells and to modulate macrophage TNF-alpha and matrix metalloproteinase expression (9,10). Additionally immunoglobulin G (IgG) antibodies against HSP60 have been shown to mediate endothelial cytotoxicity (11,12). However, no study to date has demonstrated the impact of antigen (HSP65)-specific humoral and cellular mechanisms on atherogenesis in vivo.

In this study we analyze separately the roles of the cellular and humoral immune responses to HSP65 and determine their relative contribution to early atherosclerosis in LDL-receptor deficient (LDL-RD) mice.

From the *Department of Cardiology and the Cardiovascular Research Laboratory, Tel Aviv Medical Center, Israel; †Institute of Pathology, ‡The Research Unit of Autoimmune Diseases, and the §Institute of Lipid and Atherosclerosis Research, Sheba Medical Center, Tel Hashomer, Sackler Faculty of Medicine, Tel Aviv University, , Israel.
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Abbreviations and Acronyms

BSA	= bovine serum albumin
cpm	= counts per minute
ELISA	= enzyme-linked immunosorbent assay
HSP	= heat shock protein
ICAM	= intercellular adhesion molecule
IFA	= incomplete Freund's adjuvant
IgG	= immunoglobulin G
LDL-RD	= low density lipoprotein receptor-deficient
OD	= optical density
PBS	= phosphate-buffered saline
SI	= stimulation index
Th	= T-helper
VCAM-1	= vascular cell adhesion molecule-1

MATERIALS AND METHODS

Mice. Twelve-week-old female LDL-RD mice (hybrids of the C57BL/6J and 129Sv strains) were created by homologous recombination as described by Ishibashi et al. (13). The mice were obtained from the Jackson Laboratory (Bar Harbor, Maine) and bred at the local animal house by repeated (at least seven generations) brother-sister matings. Low density lipoprotein receptor deficiency was confirmed by repeated polymerase chain reactions (13). The mice were maintained on 12-h dark/12-h light cycles and were allowed access to food and water *ad libitum*. Animals were kept in standard conditions.

Study design. EXPERIMENT 1. Low density lipoprotein receptor-deficient mice were immunized subcutaneously in the hind footpad with 25 μg of HSP65 or control bovine serum albumin (BSA) (25 μg) emulsified in incomplete Freund's adjuvant (IFA). The mice were boosted twice under the same protocol following three and six weeks and were fed a chow diet. Twelve weeks after the first immunization, hearts were removed for determination of atherosclerosis and spleen cells from each group were pooled and collected. Splenocytes obtained from a single cell suspension were incubated for three days in the presence of Concavalin A (Sigma Chemical). Cell viability was evaluated by Trypan blue in each of the experiments. The cells (1×10^7) from HSP65 or BSA-immunized mice were transferred by a single intraperitoneal injection to nontreated female LDL-RD mice (10 per group).

EXPERIMENT 2. Draining lymph node cells were removed from mice that were immunized subcutaneously once in the hind footpad with either HSP65 or BSA (50 μg/mouse). The lymph node cells from each group (HSP65 or BSA) were collected and pooled, after which they were stimulated in vitro with HSP65 (10 μg/ml) for three days. Lymphocytes were then transferred by a single intraperitoneal injection to 12-week-old nontreated female mice (1×10^7/mouse). The recipient mice injected with lymph-node cells from either HSP65- or BSA-immunized mice were fed a chow diet and sacrificed 12 weeks afterwards.

To exclude the possibility of graft-versus-host disease that would likely influence atherosclerosis, mice were clinically assessed during the study at weekly intervals for the following parameters: weight loss, fur texture, posture, skin integrity and eye inflammation.

EXPERIMENT 3. Total IgG was purified from HSP65- or BSA-immunized mice (the mice that served as splenocyte donors and contained hyperimmune serum). Twelve-week-old LDL-RD mice were injected intraperitoneally with IgG from serum of HSP65 or BSA-immunized mice (100 μg/mouse per dose) every 10 days until sacrifice, 12 weeks following the initial injection. Such a protocol was shown by a preliminary experiment to yield consistently high levels of anti-HSP65 antibodies in the sera of the mice throughout the 12 weeks of the experiment.

The study was aimed at evaluating fatty streaks rather than advanced atherosclerosis induced by an atherogenic diet, as early lesions are more likely to be influenced by immunologic manipulations (8,14). Furthermore, avoidance of high-fat diet feeding prevents excessive LDL cholesterol elevations and keeps the LDL-RD mice at ranges of cholesterol approximating human levels (~180 mg/dl).

Cholesterol level determinations. Total plasma cholesterol levels were determined by an automated enzymatic technique (Boehringer Mannheim, Germany) at the end of the experiment.

Proliferation assays. Splenocytes (experiment 1) or draining lymph-node cells (experiment 2) were collected from immunized mice. The assays were performed as previously described (8), with minor modifications. Briefly, 1×10^6 cells/ml were incubated in triplicates for 72 h in 0.2 ml of culture medium in microtiter wells in the presence of different concentrations of HSP65. Proliferation was measured by the incorporation of [^{3}H] thymidine into deoxyribonucleic acid during the final 12 h of incubation. The results were computed as stimulation index (SI): the ratio of the mean counts per minute (cpm) of the antigen to the mean background cpm obtained in the absence of the antigen. Standard deviation was always $<$10% of the mean cpm.

Detection of anti-HSP65 antibodies by enzyme-linked immunosorbent assay (ELISA). Recombinant mycobacterial HSP65 or recombinant HSP60 (purchased from Stressgene) in phosphate-buffered saline (PBS) (pH 7.2) was coated onto flat-bottom 96-well ELISA plates (Nunc Maxisorp, Denmark) by overnight incubation (1 μg/ml). After washings with 0.02% PBS Tween and blocking with 1% BSA in PBS, sera or IgG were added in different concentrations and incubated for 1 h at room temperature. Alkaline-phosphatase-conjugated goat antimouse IgG (DAKO Ltd., High Wycombe, United Kingdom) was added and incubated for 1 h at room temperature followed by four washings with PBS/Tween. After extensive washing, 1 mg/ml p-nitrophenyl-phosphate (Sigma) in 50 mM carbonate buffer containing 1 mM $MgCl_2$ pH 9.8 was added as a substrate. The reaction was stopped after 30 min

by adding 1 M of NaOH. The optical density (OD) was read at a 405 nm wavelength in a Titertek ELISA reader.

Purification of mouse IgG. Immunoglobulin G from pooled sera of either HSP65 or BSA hyperimmunized mice was purified by protein G-Sepharose column (Pharmacia, Uppsala, Sweden).

Assessment of atherosclerosis. Quantification of atherosclerotic fatty-streak lesions was done by calculating the lesion size in the aortic sinus as previously described (15). 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 Inc., Elkhart, Indiana) and frozen. Every other section (5 to 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 area per section was counted with a grid by an observer unfamiliar with the tested specimen.

Immunohistochemistry. Immunohistochemical staining of 5-μm thick cryostat-cut sections of the aortic sinus was done as follows. Sections were fixed in cold acetone for 10 min, air-dried and washed twice in TBS. Endogenous peroxidase activity was blocked by incubating slides in a solution of 3% hydrogen peroxidase for 5 min. All incubations were performed at room temperature in humidified chambers. Slides were incubated with the appropriate primary antibody overnight, followed by 4 to 6 h of incubation with secondary horseradish peroxidase-labeled goat antirat IgG antibody (1:200 dilution in TBS). Sections were visualized by chromogenic detection (AEC substrate system No. K0697; Dako Corp, Carpinteria, California). AEC was used as a chromogen. After treatment, sections were washed with TBS and counterstained with hematoxylin. Vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule (ICAM-1) and E-Selectin were detected using the respective rat monoclonal antimurine antibodies (antibody dilution was 1:100, No. 1510-01; Southern Biotechnology Associates, Inc., Birmingham, Alabama). Primary antibodies bound to tissue were visualized after the binding of antirat immunoglobulin coupled to horseradish peroxidase. Control slides were incubated with nonspecific primary antisera, or in some cases without the primary antibody. In no case did control slides show a positive signal. Vascular cell adhesion molecule-1 surface coverage was expressed as the plaque positive area divided by the total plaque area as previously described (16). Six sections from six different mice in the HSP65 or BSA lymph node cell transfer recipients were chosen for analysis.

For assessment of the expression of HSP60/65 on endothelial cells, frozen aortic sinus sections were incubated with IgG from HSP65- (shown to be cross-reactive with HSP60) or BSA-immunized mice and probed with the Histomouse kit in accord with the manufacturer's instructions.

Statistical analysis. Differences between individual parameters were compared using the two-sided Student *t* test; $p < 0.05$ was considered statistically significant. Results are expressed as mean ± SEM.

RESULTS

Preliminary studies have shown that immunization with BSA (emulsified in IFA) did not promote fatty-streak formation in LDL-RD mice in comparison with nonimmunized mice. Thus, we have chosen BSA as a control irrelevant antigen throughout the experiments.

All mice appeared healthy throughout the experiments and no signs of graft-versus-host disease (weight loss, hunching, baldness, loss of skin integrity or eye inflammation) were evident.

Transfer of spleen cells, lymph node cells or antibodies to HSP65 does not alter cholesterol levels. Administration of splenocytes did not influence the cholesterol levels in LDL-RD mice. Mean cholesterol levels for the group of mice injected with HSP65-lymph node cells were 194 ± 33 mg/dl in comparison with values of 186 ± 29 in the T-BSA group. Levels were also similar in the Spl-HSP65 (injected with total splenocytes from HSP65-immunized animals) mice (190 ± 26 mg/dl) as compared with the Spl-BSA mice (193 ± 31 mg/dl). Immunoglobulin G administration did not affect cholesterol levels (196 ± 34 mg/dl in the IgG-HSP65 mice and 189 ± 29 mg/dl in the IgG-BSA mice).

Characterization of HSP65 reactivity in the transferred cells and IgG. The reactivity of lymphocytes from the HSP65-immunized mice and that of the purified IgG to HSP65 was characterized before their transfer to the noninjected mice.

Reactivity to HSP65 (25 μg/ml) of lymphocytes obtained from mice that were immunized with HSP65 was pronounced, with a mean SI of 5.6 ± 1.2 (Fig. 1A). The proliferative response to HSP65 (25 μg/ml) of splenocytes from mice repeatedly immunized with HSP65 was significant, with a mean SI of 4.9 ± 0.9 (Fig. 1B). In vitro stimulation with (mammalian) HSP60 (25 μg/ml) was also capable of inducing proliferation of HSP65-injected lymph node cells (mean SI of 3.2 ± 0.8) and splenocytes (SI of 2.9 ± 0.7), confirming the cross-reactivity between mycobacterial HSP65 and mammalian HSP60.

Immunoglobulin G binding to solid-phase-bound HSP65 was significant, starting from OD values of 1.7 ± 0.3 at a concentration of 100 μg/ml (Fig. 1C). To confirm cross-reactivity, plates were coated, in parallel, with recombinant HSP 60. Mean IgG binding to HSP60 at a concentration of 100 μg/ml was 0.8 ± 0.2 (OD 405 nm).

The effect of cell and IgG transfer on fatty-streak formation and VCAM-1 expression. Early fatty streaks in mice immunized and boosted with HSP65 were significantly larger (25,000 ± 6,380 μm^2) than their control immunized littermates (2,000 ± 1,400 μm^2; $p < 0.01$) (Fig. 2). Mice transferred with HSP65-reactive lymph node cells developed fatty streaks that were significantly larger than

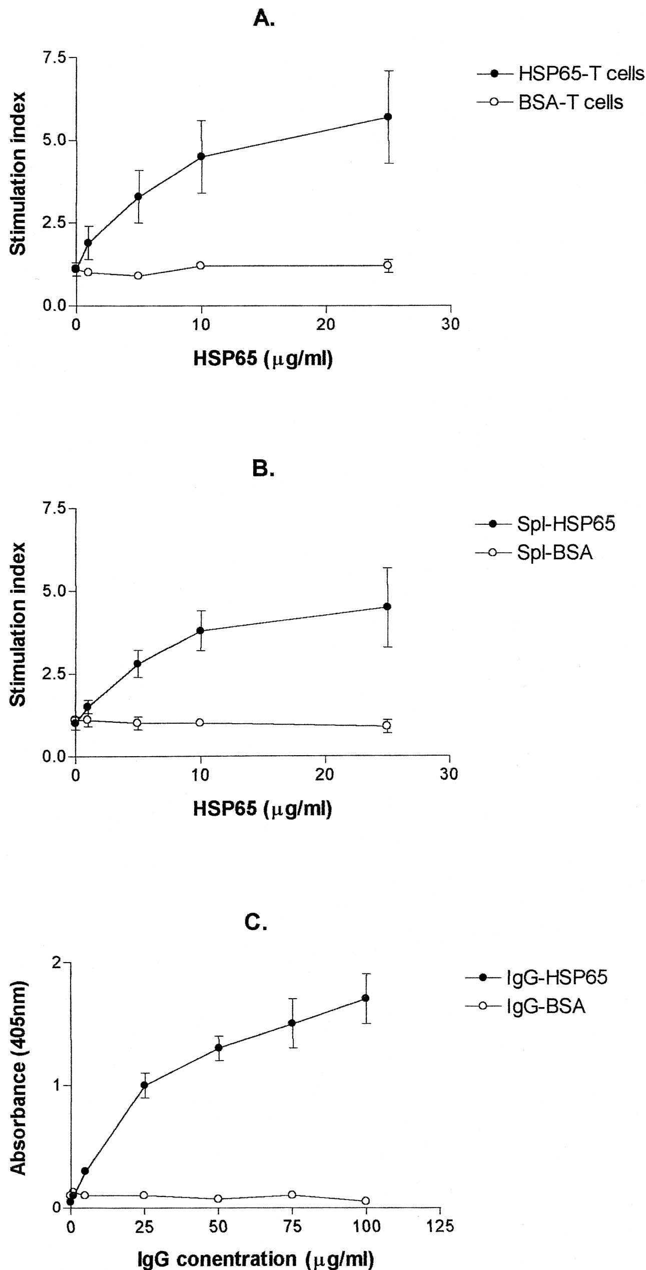

Figure 1. Characterization of heat shock protein 65 (HSP65) reactivity of lymphocytes and immunoglobulin G (IgG) employed for the transfer experiments. Reactivity of lymph-node cells **(A)** and splenocytes (Spl) **(B)** was determined by thymidine incorporation, whereas IgG binding was defined by enzyme-linked immunosorbent assay. BSA = bovine serum albumin.

their control BSA-injected littermates (Table 1). Sera from mice administered with lymph-node-reactive HSP65 did not display reactivity to HSP65 by ELISA (data not shown). Mice administered with anti-HSP65 splenocytes had larger fatty streaks in comparison with the animals given spleen cells reactive with BSA. Anti-HSP65 antibodies were undetectable in the sera of mice injected with HSP65-reactive splenocytes (data not shown).

Immunoglobulin G from serum of HSP65-immunized mice also enhanced fatty-streak size as compared with control IgG administration. Immunoglobulin G anti-HSP65 levels obtained upon sacrifice were 1.2 ± 0.2 (70% of the binding evident in the donor mice immunized with HSP65).

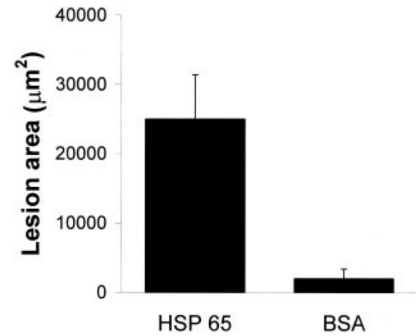

Figure 2. Fatty-streak formation in mice immunized with heat shock protein 65 (HSP65) or bovine serum albumin (BSA). Sections from mice immunized and boosted twice with HSP65 or BSA were stained with oil-red O. Lesion area was assessed by an unbiased observer using a grid.

Expression of VCAM-1 was increased compared with controls in slides obtained from mice transferred with anti-HSP65 lymph node cells. Mean surface coverage of plaque with VCAM-1 in the lymph node-HSP65 recipients was 45 ± 7% versus 31 ± 1% ± 6% in the lymph-node BSA recipients ($p < 0.05$). No differences in the extent of staining to ICAM-1 and E-Selectin were evident between the groups.

Immunoglobulin G from HSP65-immunized mice (cross-reactive with mammalian HSP60) but not control IgG immunostained aortic sinus endothelial cells, thus confirming the expression of HSP60 (Fig. 3).

DISCUSSION

In the current study we provide evidence for the proatherogenic role of the HSP65-specific cellular and humoral responses in mice. Immunization with HSP65 in a protocol similar to the one we employed in C57BL/6 mice (8) led to acceleration of fatty-streak formation in the LDL-RD mice. Adoptive transfer of lymphocytes obtained from lymph nodes (representing the primary immune response to HSP65) and from splenocytes (marking a tertiary response to HSP65) increased the size of the fatty-streak lesions in the recipient mice.

Evidence linking HSP65 with atherogenesis. Recently, several laboratories have provided evidence for the involve-

Table 1. Effect of Cell and IgG Transfer on Atherogenesis in LDL-RD Mice

	Anti-HSP65	Anti-BSA
Lymph node cell transfer (μm^2)	17,500 ± 4,750*	3,575 ± 770
Spleen cell transfer (μm^2)	20,000 ± 3,850†	5,000 ± 2,825
IgG transfer (μm^2)	17,000 ± 3,250‡	6,010 ± 2,800

*$p < 0.01$ compared with control; †$p = 0.01$ compared with control; ‡$p < 0.05$ compared with control.
BSA = bovine serum albumin; HSP65 = heat shock protein 65; IgG = immunoglobulin G.

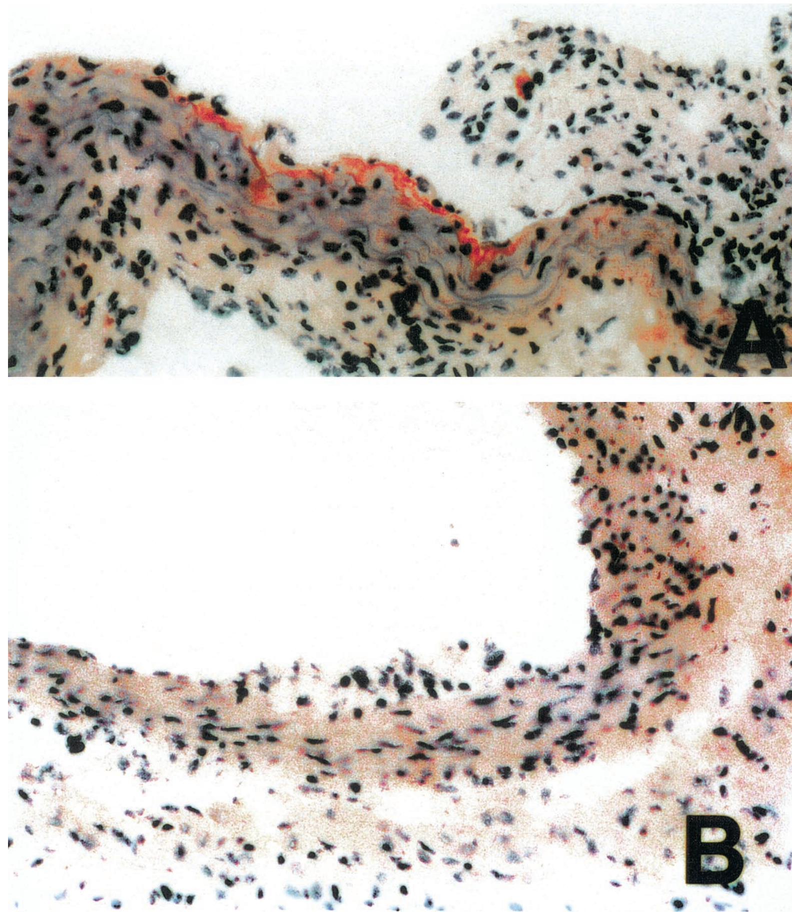

Figure 3. Immunohistochemical detection of heat shock protein (HSP)60/65. Immunoglobulin G (IgG) was purified from the sera of mice immunized with HSP65. Purified IgG was cross-reactive with HSP60 employing enzyme-linked immunosorbent assay. The IgG from HSP65 immunized mice was used to immunostain aortic sinus sections from low density lipoprotein receptor-deficient mice with the Histomouse kit. (A) Positive staining of the endothelial cells; (B) a similar section stained with control mouse IgG.

ment of HSP60/65 in atherogenesis. Clinical observations implied that the anti-HSP65 antibodies were associated with enhanced carotid atherosclerosis as detected by sonographic studies (3). Experimental data demonstrated that immunization of rabbits and mice with mycobacterial HSP65 led to arteriosclerotic lesions (7,8). The induced lesions were rich in CD3-positive cells, thus attesting for their inflammatory phenotype. It has been postulated that stress-induced expression of host HSP60 targeted by an anti-HSP65 immune response elements acts to enhance atherosclerosis. However, both these studies in which mycobacterial HSP65 was used for immunization could not address questions that pertain to the mechanisms mediating the proatherogenic effect. In the present study we have shown that HSP65-reactive lymph node and spleen cells and IgG were potent in promoting fatty-streak formation in the LDL-RD mouse model.

HSP expression in plaques of LDL RD mice as a trigger of T cell activation. A recently perceived paradigm holds that T-helper (Th) cells could be differentiated functionally according to their cytokine secreting profile (17). Thus, Th 1 cells are discerned by production of cytokines such as IFN-gamma and IL-2, whereas Th 2 cells favor production of IL-4 and IL-10. The dichotomy has also recently been observed in hypercholsterolemic apoE-deficient mice (18). In atherosclerosis, it appears that a predominant Th 1 pattern is accompanied by increased atherosclerosis (19,20). A possible proatherogenic effect of anti-HSP65 lymph node and spleen cells could thus derive from ligation of these cells by (cross-reactive) HSP60 expressed by the host (the mouse) at areas of shear stress. The existence of this interaction is evident by the ability of IgG from HSP65- but not from BSA-immunized mice to immunostain the endothelial surface of aortic sinus sections (Fig. 3). Once ligation of the T-cell receptor occurs, a shift towards proatherogenic (Th 1) cytokine secretion could follow, which may result in accelerated atherosclerosis. Evidence for the role of T cells in atherogenesis has been presented by several authors (14,21–23). Our study, however, provides the first evidence for antigen (HSP65)-specific T-cells influencing fatty-streak formation in vivo.

Anti-HSP65 antibodies enhance fatty-streak formation. We have observed in the present study that IgG anti-HSP65 antibodies could accelerate fatty streaks in recipient mice. Immunoglobulin G HSP65 antibodies can be formed in response to infections and react with self-HSP-60. In

humans, levels of anti-HSP65 antibodies correlate with carotid atherosclerosis (24) and their sustained presence appears to predict mortality among atherosclerotic patients (25). These observations are supported by in vitro studies: anti-HSP60/65 antibodies have functional properties that render them potentially atherogenic as they mediate endothelial cytotoxicity (11,12). These observations may provide a mechanism of action of these antibodies that may induce endothelial expression of adhesion molecules, followed by monocyte attraction and foam-cell formation. Furthermore, it would be interesting to study the combined effect of cellular and humoral immune responses to HSP65, as they may act synergistically on atherosclerosis progression.

Conclusions and prospects. In conclusion, we provide a direct proof that the lymphocytes and IgG reactive with HSP65 can promote fatty-streak formation in the LDL-RD mouse. These data also justify a separate analysis of the association between cellular immunity to HSP65 and atherosclerosis in humans, similar to the studies evaluating the relation of anti-HSP65 antibodies and atherosclerosis. Furthermore, this study provides additional evidence regarding the operability of autoimmune mechanisms in the early stages of atherosclerosis.

Reprint requests and correspondence: Dr. Dror Harats, Institute of Lipid and Atherosclerosis Research, Sheba Medical Center, Tel-Hashomer 52621, Israel. E-mail: dharats@post.tau.ac.il.

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