EXPERIMENTAL STUDIES

Oral Tolerance With Heat Shock Protein 65 Attenuates \textit{Mycobacterium Tuberculosis}-Induced and High-Fat-Diet-Driven Atherosclerotic Lesions

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OBJECTIVES
The goal of this study was to explore the efficacy of oral tolerance with heat shock protein (HSP) 65 in two apparently non-overlapping models of murine atherosclerosis.

BACKGROUND
Atherosclerosis is considered to be a chronic inflammatory process. Autoimmune mechanisms have been shown to influence atherogenesis in experimental animal models. Heat shock protein 65 is a candidate antigen thought to drive a proatherogenic immune-mediated response. Mucosal tolerance is a therapeutic means of accomplishing immune unresponsiveness toward a given antigen by feeding it before active induction of the disorder.

METHODS
Low-density lipoprotein receptor deficient mice were fed with different doses of HSP65 every other day for 10 days. Feeding with either bovine serum albumin (BSA) or phosphate buffered saline (PBS) served as control. One day after the last feeding, mice were challenged either by immunization with heat killed \textit{Mycobacterium tuberculosis} or by a high fat diet.

RESULTS
Lymphocyte reactivity from mice fed with HSP65 and immunized either against HSP65 or \textit{M. tuberculosis} was significantly reduced in comparison with BSA-fed mice. Moreover, co-incubation of splenocytes—from mice with tolerance induced with HSP65 but not BSA—with HSP65-reactive lymphocytes resulted in the suppression of HSP65 reactivity by the latter cells. Interleukin-4 production by HSP65-fed and immunized mice was increased upon priming with respective protein. Early atherosclerosis was attenuated in HSP65-fed mice, compared with either BSA- or PBS-fed mice, regardless of the method employed to induce fatty streaks (\textit{M. tuberculosis} immunization or high-fat diet).

CONCLUSIONS
Oral tolerance induced with HSP65 could prove to be a novel means of suppressing atherogenesis. (J Am Coll Cardiol 2002;40:1333–8) © 2002 by the American College of Cardiology Foundation

The relatively modern view of atherosclerosis is of a chronic inflammatory process, wherein cellular and humoral components act to bring about gradual vessel obstruction (1,2). Recently, this concept has been further extended by the detection of antigen-driven immune responses toward various plaque antigens. Accordingly, modified forms of low-density lipoprotein (LDL), heat shock proteins (HSP), and phospholipid binding proteins have been shown to drive humoral and cellular reactions and, thus, to influence the progression of atherosclerosis (3).

Heat shock proteins are a family of approximately 25 molecules with highly conserved structures that serve protective roles (4). However, HSP (i.e., HSP65) has been implicated as a target autoantigen in several experimental autoimmune diseases (i.e., arthritis, type I diabetes) (5,6). There are abundant data to support the involvement of HSP60/65 in atherogenesis: anti-HSP65 as well as anti-HSP60 antibodies have been demonstrably associated with atheromatous lesions in humans (7,8). Studies conducted in rabbits (9) and mice (10–12) show that the generation of an HSP65-induced immune response by immunization with the recombinant protein or with an HSP65-rich preparation of \textit{Mycobacterium tuberculosis} enhances atherogenesis.

Mucosal “tolerization” (i.e., the intentional induction of tolerance by means of chemical or biological agents) is a method by which a state of unresponsiveness is induced in the host immune system against a given antigen (13). Because organ-specific autoimmune diseases have been shown to be triggered by distinct antigens, tolerization has been employed in order to block these responses to bring about attenuation of disease outcome in experimental models.

As autoimmune processes have been shown to participate in atherosclerosis, with abundant evidence pointing to HSP65 as a possible antigenic candidate; we therefore reasoned that tolerization of the immune response to this antigen would result in reducing atherosclerosis.

METHODS

Mice. Low-density lipoprotein receptor deficient (LDL-RD) female mice (14), age six weeks, were fed either normal chow-diet containing 4.5% fat by weight (0.02% cholesterol) or an atherogenic diet (Western type diet, TD 96125,
Harlan Teklad; 42% of calories from fat, 43% from carbohydrates, 15% from protein).

Cholesterol level determinations. Total plasma cholesterol levels were determined by using an automated enzymatic technique (Boehringer Mannheim, Germany).

Study design. Two experiments were designed in which atherosclerosis was induced either by a high-fat diet or by immunization with heat-killed *M. tuberculosis* (10,12).

In the first experiment, LDL-RD mice were fed by a nasogastric tube, five doses (every other day) of recombinant Mycobacterial HSP65 in phosphate buffered saline (PBS) in three different concentrations (50 μg/dose, 5 μg/dose, and 0.5 μg/dose). Control mice were either fed a control antigen (bovine serum albumin [BSA] 50 μg) or not fed. One day after the last feeding, all mice were challenged by an immunization with heat killed suspension of *M. tuberculosis* (10 mg/ml; 100 μg/mouse) emulsified in incomplete Freund’s adjuvant to induce fatty streaks (10). Mice were euthanized 12 weeks after the last feeding.

In the second experiment, the mice were fed five doses of HSP65 (100 μg, 10 μg, 1 μg, and 0.1 μg/mouse) or BSA (100 μg/mouse) every other day, and the Western diet was substituted for chow one day after the final feeding. An additional group that was fed no antigen served as control. Mice were euthanized five weeks after the initiation of the high-fat diet.

Proliferation assays. Draining inguinal lymph nodes (taken 8 days after immunization) or splenocytes (taken upon euthanization) were collected from four HSP65-, *M. tuberculosis*-, and PBS-immunized mice for the proliferation studies. The assays were performed as previously described (12). Briefly, 1 × 10⁶ lymph node cells/ml were incubated in triplicates for 72 h in 0.2 ml of culture medium in microtiter wells in the presence 10 μg/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: the ratio of the mean cpm of the antigen to the mean background cpm obtained in the absence of the antigen. Standard deviations were always <10% of the mean counts per min.

To test efficacy of tolerance in vitro, splenocytes from HSP65-tolerized mice were co-cultured with lymph node cells obtained from mice immunized against HSP65 in the presence of 10 μg/ml of HSP65 for 72 h. The next steps, including thymidine uptake, were performed similarly to the similar proliferation assays, as described previously. Interferon-gamma (IFN-γ), interleukin-4 (IL-4), and tumor growth factor-beta (TGF-β) secretion by tolerized lymph node cells. Conditioned medium was obtained from the lymph node proliferation experiments after 48 h of culture in the presence of HSP65. IFN-γ, IL-4, and TGF-β concentrations were determined by enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer’s suggestions (R&D Systems).
Detection of anti-HSP65 antibodies and of IgG isotypes. Recombinant HSP65 (1 μg/ml) in PBS (pH 7.2) was coated onto flat bottom 96-well ELISA plates (Nunc, Denmark) by overnight incubation, and the assay was performed as previously described (12).

IgG isotypes in the sera of HSP65-tolerized and nontolerized mice were determined by an ELISA kit (phosphatase; Southern Biotechnology Associates, Birmingham, Alabama) according to the manufacturer’s suggestions.

Assessment of atherosclerosis and immunohistochemistry. The quantification of atherosclerotic fatty streak lesions was done by calculating the lesion size in the aortic sinus as previously described (15), with a few modifications. Briefly, 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 OCT medium and frozen. Every other section (10 μm thick) throughout the aortic sinus (400 μm) was taken for analysis. The extent of atherosclerosis was evaluated at the level of the aortic sinus. Processing and staining of the tissue with oil-red O was carried out according to Paigen et al. (15).

Immunohistochemical staining for CD3 cell number was done on 5 μm–thick frozen sections of aortic sinus. Rat anti-mouse CD3 primary antibodies were used for probing. Slides were developed with the three amino-9-ethylcarbonasole substrate. Sections were counterstained with hematoxylin. Spleen sections were used as a positive control. Staining in the absence of first or second antibody was used as a negative control.

Statistical analysis. All parameters were compared employing a one-way analysis of variance, followed by a Fisher-protected least significant difference test. P < 0.05 was accepted as statistically significant. Results are presented as mean ± SEM.

RESULTS

Oral feeding with different doses of HSP65 or with control antigen (BSA) did not alter the lipid profile. These results were consistent in both experiments: levels of total cholesterol and triglycerides did not differ between groups (data not shown), and animal weight also did not differ between groups (data not shown).

The effect of HSP65 tolerization was tested in vivo and in vitro. Mice fed five doses of HSP65 (50 μg)—the concentration found most potent—or control antigen (BSA) were immunized against HSP65, and proliferation was assessed using thymidine uptake. We have found that lymphocytes from HSP65-fed mice exhibited significantly reduced proliferation to HSP65 (52% at 10 μg HSP65) compared with BSA-fed mice (Fig. 1A). Similarly, lymphocytes from HSP65-fed M. tuberculosis immunized mice were significantly less reactive to HSP65 than cells from animals fed BSA (Fig. 1B). Spleenocytes from HSP-tolerized mice were capable of significantly reducing the reactivity of lymph node cells from HSP65 to the antigen upon their co-incubation (Fig. 2).

To evaluate whether regulatory cells were induced by oral tolerization with HSP65, we assessed cytokine production by lymph node cells of treated versus non-treated animals. We have found that lymph node cells from HSP65-tolerized LDL-RD mice produced significantly (3.8 times) more IL-4 than non-tolerized cells (p < 0.01) (Fig. 3). IFN-γ levels in the cultured medium did not differ between mice that were tolerized (mean of 3,121 ± 438 pg/ml) and mice that were not tolerized (2,904 ± 515 pg/ml) with HSP65 (p = 0.77). TGF-β levels produced by

Figure 2. In vitro induction of tolerance to heat shock protein (HSP) 65 by co-culture of tolerized splenocytes with HSP65 responder lymph node cells. To assess in vitro tolerization, 10⁷ splenocytes from HSP65 or bovine serum albumin (BSA)-fed (i.e., mice that were fed 5 times with HSP65, 50 μg/dose or BSA) were incubated in the presence of 10⁴ lymph node cells from Mycobacterium tuberculosis immunized mice in the presence of different concentrations of HSP65. The figure represents average values obtained from three studies, each repeated twice. *p < 0.05. Hatched bar = BSA-fed splenocytes; solid bar = HSP-fed splenocytes.

Figure 3. Induction of T helper 2 cytokines by orally tolerized mice. Lymph node cells were obtained from heat shock protein (HSP) 65 tolerized low-density lipoprotein receptor deficient mice immunized with HSP65 upon priming in vivo with 10 μg/ml HSP65 for 48 h. Interleukin-4 (IL-4) levels in the conditioned medium were assessed by a capture enzyme-linked immunosorbent assay kit as described in the Methods section. *p < 0.01.
lymph node cells from tolerized and non-tolerized mice were below the detection threshold.

To explore the possible effect of the change in cytokine (Th2 dominance) on the IgG isotype patterns, we have made solid phase assessment of total IgG isotypes and HSP65 isotypes. No detectable differences were evident in the levels of total IgG isotypes between HSP65-tolerized and non-tolerized cells (Fig. 4A). Additionally, no skewing of the anti-HSP65 IgG isotype pattern was observed in HSP65-immunized mice between the tolerized and non-tolerized groups (Fig. 4B).

In the first experiment, fatty streaks were significantly reduced in mice fed either 50 μg (n = 9; mean lesion size of 8,600 ± 1,600 μm²), 5 μg (n = 8; 22,500 ± 2,950 μm²), or 0.5 μg (n = 16; 26,700 ± 2,950 μm²) HSP65, compared with BSA (n = 6; 49,300 ± 5,400 μm²) (p < 0.0001, p < 0.001, respectively) (Fig. 5A).

Because no differences were evident when BSA was compared with PBS (Fig. 5A and 6), the next experiment was carried out employing BSA as control. In the second experiment, when Western diet was employed, lesion size in BSA-fed mice was 76,900 ± 11,700 μm² (n = 10). In comparison with BSA administration, prior feeding with HSP65 reduced atherosclerosis when doses of 100 μg (n = 13; 30,000 ± 8,000 μm²), 10 μg (n = 13; 34,200 ± 6,900 μm²), and 1 μg (n = 13; 36,300 ± 6,300 μm²) (p < 0.01 for all) were employed (Fig. 5B and 6). Oral feeding with 0.1 μg/dose of HSP65 did not significantly reduce fatty streaks compared with control.

Oral tolerance did not alter the number of plaque-infiltrating CD3 lymphocyte positive cells between the different groups (0 to 4/positive cells/fatty streak; data not shown). Similarly, no differences were evident with regard to plaque Mac-1 positive cells between tolerized and non-tolerized mice.
DISCUSSION

In the current study, we have shown, for the first time, application of oral tolerization with HSP65 as a novel means of suppressing atherosclerosis. In previous studies, we successfully employed oral tolerance in a model of fatty streak induction by immunization with heat killed suspension of \textit{M. tuberculosis} \textsuperscript{10–12}. In the present model, cellular immunity to the mycobacterial HSP65 is involved in promoting fatty streaks, similar to the induction of adjuvant arthritis in rats. Thus, we sought to induce a state of unresponsiveness of the cellular immune response to HSP65 in order to reduce the development of atherosclerosis. In the parallel model of adjuvant arthritis, amelioration of the inflammatory joint lesions was indeed accomplished by prior feeding with HSP65 \textsuperscript{16}. More intriguing, however, are the results obtained in the second experiment, in which feeding HSP65 led to suppression of high-fat-diet-induced atherosclerosis. In this model of atherosclerosis, spontaneous reactivity to HSP65 is not evident, as in the \textit{M. tuberculosis}-and HSP65-driven fatty-streak model.

**Oral feeding of HSP65 and suppression of cellular immunity to HSP65.** Several mechanisms are operable in oral tolerance and appear to be dependent on antigen dosing and preparation. It is thought that whereas high-dose feeding leads to clonal deletion/anergy \textsuperscript{17,18}, low doses induce regulatory cells \textsuperscript{19,20} capable of altering cytokine production. We monitored the tolerizing effect of HSP65 feeding by several methods. In vivo, we explored the cellular reactivity to HSP65 and found it was significantly diminished in HSP65-fed compared with BSA-fed mice. These findings were reinforced by in vitro studies showing that co-incubation of HSP65-tolerized splenocytes with HSP65-reactive lymphocytes is capable of reducing their proliferation to the antigen.

We have recently found that antigen-driven cellular immune responses are capable of transferring atherosclerosis, regardless of the lipid profile \textsuperscript{21}. These results were supported by transfer experiments employing immunodeficient apoE deficient mice \textsuperscript{22}. In the model of HSP65-driven fatty streaks, lymphocyte reactivity appears to play a proatherogenic role \textsuperscript{23}. Thus, tolerization to HSP65 can intuitively explain the reduction in fatty streaks, and our observations could support the operability of mechanisms that promote clonal anergy/deletion as responsible for the reduced fatty streaks.

**Oral tolerance with HSP65 and modulation of T-helper cytokine production.** However, as could be observed, there is no strict correlation between the extent of atherosclerosis reduction and the suppression of immune responses to HSP65. Thus, additional mechanisms may be present. We have chosen to explore the possibility that regulatory cells are induced, capable of producing Th2 and Th3 cytokines or, alternatively, downregulating Th1 cytokines upon encountering HSP65. We have found that oral feeding of HSP65 induced the production of lymphocytes capable of significantly increasing the production of the Th2 cytokine IL-4. These were not paralleled by a suppressive effect on Th1 cytokine secretion, as evidenced by the unaltered IFN-\textgamma production. These results are consistent with previous reports implying that both of these Th2 cytokines are essential for the protection afforded by oral tolerance \textsuperscript{24}. The relevance to atherosclerosis can be understood when considering the observations by Huber et al. \textsuperscript{25} showing that IL-4 plays a protective role in atherosclerosis. The lack of significant Th1 suppression despite elevated Th2 cytokines in response to HSP65 is also evident by the lack of change in the total IgG isotype pattern in the serum as well as in the HSP65-specific IgG isotype distribution in the immunized mice.

\textbf{Figure 6.} Atherosclerosis in heat shock protein (HSP) 65 and bovine serum albumin (BSA) tolerized low-density lipoprotein receptor deficient (LDL-RD) mice. Representative Oil-red O stained section from BSA (A) and HSP65 (B) and fed LDL-RD mice.
TGF-β represents the most commonly described cytokine associated with favorable oral tolerance (13). Interestingly, TGF-β production was, similar to IFN-γ, not influenced by oral tolerance with HSP65. This observation can be explained by data showing that TGF-β levels are significantly reduced in atherosclerosis (26). Because the mice employed for the current study are hyperlipidemic and atherosclerosis prone, we speculate that this propensity may have been responsible for the lowering of TGF-β to levels that could not be counterregulated by oral tolerization.

From the above observations, it is apparent that no conclusive evidence can be obtained as to the exact mechanism mediating the tolerizing effect on atherosclerosis. Moreover, the effect of HSP65 feeding may not be specific, considering that the protective effect on atherosclerosis is maintained even when proliferative responses to HSP65 are not significant. A modulating effect on Th2 cytokine up-regulation could also be operable and perhaps even dominant. The use of additional antigens considered as candidate autoantigens in atherosclerosis may provide partial answers to these questions.

**Conclusions.** In conclusion, we have found that atherosclerosis-prone mice can be tolerized to HSP65, resulting in suppression of plaque formation. Several mechanisms, including clonal anergy and induction of regulatory cells capable of inducing Th2 cytokine dominance, are probably involved in the beneficial effect. This means of selectively modulating the immune system by mucosal tolerance may prove to be a novel therapeutic strategy in atherosclerosis.

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**References**


