Vaccine-Induced Antibodies Inhibit CETP Activity In Vivo and Reduce Aortic Lesions in a Rabbit Model of Atherosclerosis


Abstract—Using a vaccine approach, we immunized New Zealand White rabbits with a peptide containing a region of cholesteryl ester transfer protein (CETP) known to be required for neutral lipid transfer function. These rabbits had significantly reduced plasma CETP activity and an altered lipoprotein profile. In a cholesterol-fed rabbit model of atherosclerosis, the fraction of plasma cholesterol in HDL was 42% higher and the fraction of plasma cholesterol in LDL was 24% lower in the CETP-vaccinated group than in the control-vaccinated group. Moreover, the percentage of the aorta surface exhibiting atherosclerotic lesion was 39.6% smaller in the CETP-vaccinated rabbits than in controls. The data reported here demonstrate that CETP activity can be reduced in vivo by vaccination with a peptide derived from CETP and support the concept that inhibition of CETP activity in vivo can be antiatherogenic. In addition, these studies suggest that vaccination against a self-antigen is a viable therapeutic strategy for disease management. (Arterioscler Thromb Vasc Biol. 2000;20:2106-2112.)

Key Words: CETP ■ lipoproteins ■ atherosclerosis ■ vaccine

There is a strong inverse relationship between the plasma concentration of cholesterol in HDLs (HDL-C) and the development of coronary heart disease (CHD). Several studies demonstrate that the plasma concentration of HDL-C is a more powerful predictor of CHD than either total plasma cholesterol or the plasma concentration of cholesterol in LDLs (LDL-C), indicating that the risk of CHD is increased by 2% to 3% for every 1% decrease in HDL-C. Although 33% of patients with CHD have low plasma levels of HDL-C as their primary lipid abnormality, there is currently no effective therapy for increasing the plasma concentration of HDL-C. Diet and moderate exercise are ineffective, statins afford only a modest 5% to 7% increase in HDL-C, and niacin has side effects and compliance profiles that limit its use.

One therapeutic approach that has been suggested for increasing plasma HDL-C concentrations is the inhibition of cholesteryl ester transfer protein (CETP) activity. CETP is a 74-kDa plasma glycoprotein that facilitates transfer of neutral lipids and phospholipids between lipoproteins and contributes to the regulation of plasma concentration of HDL-C. The expectation that inhibition of CETP activity would increase plasma HDL-C concentrations is based on several lines of evidence. CETP functions in the plasma to lower the concentration of HDL-C by moving cholesteryl esters from HDLs to VLDLs and LDLs. Transient inhibition of CETP activity in rabbits and hamsters by monoclonal antibodies, small molecules, or antisense oligonucleotides causes an increase in plasma HDL-C. Sustained inhibition of CETP expression with antisense oligonucleotides increased plasma HDL-C and reduced atherosclerotic lesions in a rabbit model of atherosclerosis. Transgenic mice and rats expressing CETP have decreased plasma concentrations of HDL-C. Human populations with reduced or absent CETP activity due to genetic mutations have markedly elevated plasma HDL-C. Collectively, these data suggest that reducing CETP levels or activity could provide a benefit for individuals at risk of developing CHD.

We devised an immunotherapeutic approach to reduce CETP activity in vivo using a vaccine to elicit antibodies that bind to and block the function of CETP. The vaccine, referred to as TT/CETP, is a 31-amino-acid synthetic chimeric peptide that contains an N-terminal cysteine, a T-cell epitope consisting of residues 830 to 843 of tetanus toxin, and a B-cell epitope consisting of residues 461 to 476 of human CETP (Figure 1A). The T-cell epitope from tetanus toxin was selected on the basis of its ability to bind promiscuously to many MHC haplotypes and elicit strong T-cell help for B cells. The sequence from human CETP used as a B-cell epitope to elicit specific anti-CETP antibodies was selected...
Coupling Kit (Biacore) and a flow rate of 5 μL/min. The association of the TT/CETP epitope to the native CETP molecule.7 This region of CETP is highly conserved between species, and mutagenesis studies indicate that in the native CETP molecule.7 This region of CETP is highly conserved between species, and mutagenesis studies indicate a requirement of this C-terminal sequence for lipid transfer activity.23 The N-terminal cysteine was added to the peptide sequence to allow chemical linkage of the peptide vaccine to carrier molecules, if desired.

We tested for the effects of TT/CETP administration on fatty-streak lesion development in a New Zealand White rabbit model of atherosclerosis as described below.

Methods

TT/CETP Vaccine Peptide Synthesis

The 31-amino-acid chimeric peptide (Figure 1A) was produced by solid-phase synthetic chemical methods (QCB and Multiple Peptide Systems) and was demonstrated to be >90% pure by high-performance liquid chromatography analysis.

Surface Plasmon Resonance

All binding experiments were performed at room temperature in HEPES-buffered saline (mmol/L: NaCl 150, MgCl₂ 1, CaCl₂ 1, HEPES 10, pH 7.2, with 0.005% polysorbate 20 surfactant) with a BIAcore instrument (Biacore AB). The anti-CETP monoclonal antibody TP224a was directly linked to a research-grade CM5 sensor chip. The association of the TT/CETP epitope to the native CETP molecule was demonstrated by the rapid increase in resonance units as buffer lacking the vaccine peptide flows over the sensor chip (arrow 1). The dissociation of the CETP peptide vaccine from TP2 was demonstrated by the slow decrease in resonance units as buffer lacking the vaccine peptide flows over the sensor chip (arrow 2).

Recombinant Human CETP

A Chinese hamster ovary (CHO) cell line engineered to express and secrete recombinant human CETP (rhuCETP) was obtained from Alan Tall (CHO line E8, Columbia University).

Recombinant Rabbit CETP

Recombinant rabbit CETP (rrbCETP) was produced in CHO cells engineered to express rabbit CETP as follows. A 1500-bp DNA fragment containing the rabbit CETP coding sequence was constructed from plasmid pUC19/rbCETP24 (a gift from L.B. Agellon and S. Yokoyama) containing the fifth codon of the mature protein to 73 bp 3′ to the termination codon and a hybridization product containing the first 4 codons of the mature protein. This DNA fragment was inserted into a eukaryotic expression vector and cotransfected with a plasmid encoding dihydrofolate reductase (DHFR) into a DHFR-CHO cell line. G418 was used to select rrbCETP-secreting clones. For method details, please see http://www.atvbaha.org

Production and Purification of Recombinant CETP

Both rhuCETP- and rrbCETP-producing CHO cell lines were grown on weighted collagen microspheres (Cellex) in roller bottles with DMEM/F12 1:1 supplemented with 0.5% FCS, 4 mmol/L glutamine, and penicillin/streptomycin at 37°C, 5% CO₂. Purification of rhuCETP and rrbCETP from conditioned medium was performed according to the method described by Weinberg et al.25

Anti-CETP Antibody ELISA

Plasma reactivity with rrbCETP was determined with an ELISA format with biotinylated rrbCETP bound to streptavidin-coated plates. Specifically bound antibodies were detected with horseradish peroxidase–conjugated anti-rabbit antibodies and visualized with a colorimetric substrate. For method details, please see http://www.atvbaha.org

Western Blot Analysis

rruCETP and rrbCETP, 0.2 μg per lane, were electrophoresed under reducing and denaturing conditions through a 4% to 20% SDS–polyacrylamide gel and transferred onto a polyvinylidene membrane (Millipore Corp). The membrane was washed twice in 1X PBS with 0.05% Triton X-100, then blocked with PBS, 0.5% gelatin, 1.0% BSA, 1% nonfat dry milk, 0.6% NP-40, and 0.9% Triton X-100 overnight, and probed with either TP2 or a mouse monoclonal antibody that binds to both human and rabbit CETP, or plasma from TT/CETP-vaccinated rabbits. The membranes were washed 3 times in PBS, 0.05% Triton X-100, and developed with horseradish peroxidase–conjugated goat anti-mouse Ig (Southern Biotechnology Associates) or goat anti-rabbit Ig (Jackson Immunoresearch Laboratories, Inc).

CETP Activity

Plasma CETP activity was determined with a commercial kit as directed (Roar Biomedical). The samples for every time point from individual rabbits were run in duplicate on 1 plate to eliminate interplate variability.

FPLC Lipoprotein Analysis

 Serum lipoproteins were separated with 2 Superose 6 HR 10/30 (Pharmacia) columns in tandem on an AKTA Explorer fast protein liquid chromatography (FPLC) workstation (Pharmacia). A buffer containing 0.5 mol/L sodium chloride, 50 mmol/L potassium phosphate, and 1 mmol/L EDTA, pH 7.4, was used for equilibration of the columns and as the mobile phase. For each separation, 200 μL of pooled sera was loaded and eluted at 0.5 mL/min. Absorbance at 280 nm and 260 nm was monitored, and 0.5-mL fractions were collected throughout the elution. Total cholesterol concentration was determined with a fluorometric cholesterol assay as directed (Molecular Probes) and plotted as a function of fraction number. The resulting peaks were analyzed with the program PeakFit (SPSS Science). The identity of the lipoprotein species corresponding to each cholesterol...
peak was confirmed by Western blot analysis using antibodies to apoA1 and apoB (data not shown).

**Lipoprotein Analysis**
The plasma total cholesterol and HDL-C concentrations from each rabbit were determined during the cholesterol-feeding portion of the experiment (weeks 19 to 32) with commercially available kits (Total-C kit, HDL-C kit; Sigma Chemical Co) as directed.

**Vaccination of Rabbits Fed a Normal Chow Diet**
Seven New Zealand White rabbits, cared for according to institutional guidelines, were vaccinated intramuscularly with 200 μg of TT/CETP in complete Freund’s adjuvant (CFA) (DIFCO)/PBS emulsion during week 1. The group was boosted intramuscularly during weeks 5, 8, 12, and 22 with 200 μg of the vaccine in an incomplete Freund’s adjuvant (IFA) (DIFCO)/PBS emulsion. Blood samples were collected from fasted rabbits at several time points for analysis of antibodies, CETP activity, and lipoproteins.

**Rabbit Cholesterol-Fed Atherosclerosis Model**
New Zealand White rabbits (12 per group) cared for according to institutional guidelines were vaccinated by subcutaneous injection of 200 μg of TT/CETP or 200 μg of human chorionic gonadotropin (hCG, control group) in a CFA/PBS emulsion during week 1. The groups were boosted subcutaneously during weeks 5, 8, 12, and 22 with 200 μg of the appropriate vaccine in an IFA/PBS emulsion. During week 16, the rabbits were placed on a diet supplemented with 0.25% cholesterol (TestDiet) to induce atherosclerotic lesion formation and were maintained on this diet for 16 weeks. Blood samples were collected from fasted rabbits at several time points for analysis of antibodies, CETP activity, and lipoproteins. The aortas were harvested during week 32 for atherosclerotic lesion analysis.

**Lesion Analysis**
The aorta, from the aortic valve in the heart to the bifurcation into the common iliac arteries, was removed from each rabbit, fixed in formalin, cut open to expose the lumen, and pinned flat in wax pans. The tissue was stained with Sudan IV to visualize areas of fat deposition and photographed for analysis. The area of the entire aorta and the area of Sudan IV staining were quantified by planar morphometry (The Morphometer, Woods Hole Educational Associates).

**Kidney Histopathology**
Formalin-fixed paraffin blocks of kidney tissue were sectioned with a microtome, stained with hematoxylin and eosin, and evaluated in a blinded fashion by a board-certified veterinary pathologist to determine whether the vaccine caused any pathological changes due to possible immune complex deposition (Pathology Associates, Inc).

**Results**

**Preparation and Characterization of Vaccine Antigen**
The vaccine antigen TT/CETP is a synthetic peptide composed of an N-terminal cysteine, residues 830 to 843 of tetanus toxin, and residues 461 to 476 of human CETP (Figure 1A). It was important to determine that the B-cell epitope encoded by the C-terminal 16 amino acids of human CETP assumed a conformation in TT/CETP peptide vaccine similar to that in the whole CETP molecule for the vaccine to elicit antibodies capable of binding to and inhibiting the function of native whole CETP. Surface plasmon resonance was used to measure binding of TT/CETP to TP2, a CETP-specific monoclonal antibody that binds to an epitope contained in the C-terminal 16 amino acids of CETP. As shown in Figure 1B, the synthetic peptide vaccine TT/CETP bound to the monoclonal anti-CETP antibody TP2.

**Antibody Titers and CETP Activity in TT/CETP-Vaccinated Rabbits**
To test the immunogenicity of the TT/CETP vaccine construct, 7 rabbits were vaccinated by intramuscular injection of 200 μg of TT/CETP vaccine peptide emulsified with CFA for the first immunization and with IFA for the subsequent 2 booster injections. Plasma samples were collected periodically for 1 year. Rabbit and human CETP differ by 1 amino acid in the CETP C-terminal 16-amino-acid stretch chosen for the vaccine B-cell epitope, a nonconservative E to K switch at amino acid 465 of the human sequence. To ensure that the antibodies detected were specific for rabbit CETP, rrbCETP was used in the ELISA. Figure 2 shows the geometric mean of end-point antibody titers and CETP activity for the 7 vaccinated rabbits over a 1-year period. The ELISA data indicate that the TT/CETP is immunogenic, eliciting high titers of antibodies that bind to rrbCETP. The antibody titers fall by 78% from week 16 to week 52 in the absence of boosting.

Plasma CETP activity was assayed at several time points after vaccination and was shown to be reduced compared with prevaccination levels (Figure 2). At week 31, CETP activity was reduced to 57% of prevaccination levels and recovered to 66% of prevaccination levels by week 52. At several time points, there is a statistically significant inverse correlation between antibody titer and CETP activity (P<0.014), indicating that the vaccine induces a functional immune response.

**Antibody Titers in Cholesterol-Fed Rabbits**
The TT/CETP vaccine was tested for efficacy in a cholesterol-fed rabbit model of atherosclerosis. New Zealand White rabbits (12 per group) were vaccinated as described in the experimental procedures. Rabbit antibody titers were quantified by use of an ELISA that detects antibody binding to rrbCETP. The TT/CETP vaccine again elicited high titers of antibodies that bind to rrbCETP (Figure 3A).

**Western Blot Analysis of Anti-CETP Antibodies**
We characterized the antibodies elicited by TT/CETP by Western blot analysis. Figure 3B shows 2 Western blots that demonstrate binding of TP2 (monoclonal anti-CETP) and TT/CETP-vaccinated rabbit sera to rhuCETP and rrbCETP.
Qualitatively, TP2 appears to recognize rabbit and human CETP equally well, whereas antisera from the CETP-vaccinated rabbits appears to recognize human CETP better than rabbit CETP. This differential recognition is not unexpected, because the immunogen consists of the human CETP sequence, which differs from the homologous rabbit sequence by 1 nonconservative amino acid substitution at position 465. Most importantly, however, TT/CETP-vaccinated rabbits produce antibodies that bind to rabbit CETP. Immunoglobulins purified from the plasma of TT/CETP-vaccinated rabbits also inhibit CETP activity in vitro (data not shown).

CETP Activity Analysis in Cholesterol-Fed Rabbits

To confirm the inhibition of CETP activity seen in the regular chow-fed rabbits (Figure 2) and to examine the effects of the TT/CETP vaccine under conditions of diet-induced hypercholesterolemia, we measured plasma CETP activity in control-vaccinated and TT/CETP-vaccinated rabbits placed on a diet supplemented with 0.25% cholesterol (Figure 4). The plasma CETP activity in TT/CETP-vaccinated rabbits was significantly reduced compared with the activity in control rabbits. CETP activity was reduced an average of 35% at week 10 and remained lower than in controls for the duration of the experiment. Both vaccinated and control groups showed a rise in plasma CETP activity when placed on the 0.25% cholesterol diet. This is consistent with published data indicating that rabbits exhibit a 2- to 3-fold increase in CETP activity due to hypercholesterolemia.27 However, even under these conditions, the TT/CETP-vaccinated animals had significantly lower plasma CETP activity than controls (Figure 4).

FPLC Lipoprotein Analysis

The cholesterol content of FPLC-fractionated lipoproteins in pooled plasma samples from the TT/CETP-vaccinated group (n=12) and the control-vaccinated group (n=12) at week 27 are shown in Figure 5. After 11 weeks of 0.25% cholesterol chow, the fraction of total cholesterol in HDL was 42% higher in the TT/CETP-vaccinated group than in the control group (18.6% versus 13.1%), and the fraction of total cholesterol in LDL was 24% lower in the TT/CETP-vaccinated group than in the control group (14.5% versus 19.1%). The overlaid FPLC histograms also demonstrate that the HDL-C
peak of the TT/CETP-vaccinated group is shifted to the left compared with the control group, indicating the presence of larger HDL particles in the treated animals.

**Lipoprotein Analysis**

Total cholesterol concentration was determined at several time points. On initiation of the high-cholesterol diet, the plasma total cholesterol concentrations rose significantly in both groups (data not shown). When the average plasma total cholesterol values for the TT/CETP-vaccinated and control groups are plotted as a function of time and the areas under the curves for weeks 19 to 32 determined, the TT/CETP-vaccinated group is lower during the cholesterol-feeding phase of the experiment (Figure 6). The lipid profile shows that HDL-C plasma concentration was determined at several time points with a precipitation assay. On initiation of the 0.25% cholesterol diet, the plasma HDL-C concentrations increased in both groups of rabbits; however, the plasma HDL-C concentration increased more in the TT/CETP-vaccinated group than the control group and remained higher for the duration of the experiment (data not shown). When the average plasma HDL-C values for the TT/CETP-vaccinated and control groups are plotted as a function of time and the areas under the curves for weeks 19 to 32 determined, the TT/CETP-vaccinated group had 35% more HDL-C (P<0.066) than the control-vaccinated group during the cholesterol-feeding phase of the experiment (Figure 6).

**Lesion Analysis**

To quantify the effects of the CETP vaccine on atherosclerotic lesion development, rabbits were euthanized at week 32, and the aortas were removed, stained, and analyzed by planar morphometry. The total area of the aorta and the area of aortic lesion were defined by Sudan IV staining. The area of aortic lesions in rabbits treated with the TT/CETP vaccine was 39.6% less than in rabbits immunized with control vaccine (21.4% versus 35.4%, P<0.046; Figure 7).

We tested the TT/CETP vaccine in this model of atherosclerosis 3 separate times, with similar results each time. When the data from all 3 experiments were analyzed together (n=26 for the TT/CETP-vaccinated rabbits; n=25 for the control-vaccinated rabbits), the area of aortic lesion was decreased by an average of 37.6% in the TT/CETP-vaccinated group than in the control group (P<0.006).

**Kidney Analysis**

Because of the possibility that the vaccine-induced antibodies could form immune complexes with endogenous CETP, the kidneys from both groups of animals were examined for any pathological changes by a board-certified veterinary pathologist. Histochemical analysis of kidney tissue showed no evidence of pathological changes that could be attributed to the TT/CETP peptide vaccine.

**Discussion**

The relationships between HDL-C, CETP activity, and atherosclerosis are complex and not entirely understood. CETP activity has been described as both proatherogenic and antiatherogenic. CETP appears to be proatherogenic in its function of transferring cholesteryl ester from HDL to LDL and VLDL, thus lowering the plasma concentration of HDL-C and raising the concentration of LDL-C and VLDL-C. Paradoxically, CETP has also been proposed to be antiatherogenic in its ability to facilitate the production of small, lipid-poor HDL species that may function to help move cholesterol from peripheral tissues to the liver for catabolism in a process called the reverse cholesterol transport pathway.

The epidemiological data concerning the atherogenic potential of CETP activity remain mixed. Several mutations that affect CETP expression and activity have been found, all of which lead to elevated plasma HDL-C. A recent large community-based study of 48 531 men and women in Japan demonstrated that elevated HDL-C due to CETP mutation is associated with decreased risk of coronary heart disease. However, 1 epidemiological study suggests that low CETP activity due to a particular CETP mutation is associated with increased prevalence of coronary heart disease for a subset of patients with HDL-C levels between 40 and 60 mg/dL.

Another epidemiological study performed in Japan came to the conclusion that CETP gene mutation may not represent a longevity syndrome and that marked hyperalphalipoproteinemia may not be antiatherogenic.
Polymorphisms of the CETP gene have also been studied to help understand the relationship between CETP, HDL-C, and atherosclerosis. Most of these studies also show that variations in the CETP gene affect HDL-C levels in the plasma\(^4\text{0–4}\) and, in general, support the concept that high CETP activity is related to low HDL-C plasma levels and the progression of atherosclerosis. However, some data suggest that the atherogenicity of CETP activity may be determined by the metabolic context.\(^4\text{4}\)

The data presented here indicate that sustained reduction of CETP activity can be achieved with a vaccine that elicits an antibody response to endogenous CETP. This antibody response leads to a reduction of CETP activity (Figures 2 and 4) and a corresponding increase in HDL-C (Figure 6).

Most importantly, the percentage of aorta covered with fatty-streak lesions is reduced by 39.6% in the TT/CETP-vaccinated group compared with the control group (Figure 7). The results presented here are similar to those previously reported by Sugano et al,\(^5\) who used antisense oligonucleotides to achieve CETP inhibition in rabbits, and indicate that reducing CETP is antiatherogenic in a rabbit model of atherosclerosis.

The elevation of plasma HDL-C by 35% to 42% in the TT/CETP-vaccinated rabbits is equivalent to that reported for niacin in humans, which is the most effective drug for raising HDL-C and has been shown to reduce the rates of both non-fatal and fatal myocardial infarction and total 15-year mortality.\(^4\text{5}\) The elevation of HDL-C by the TT/CETP vaccine in rabbits is far higher than reported for either statins or fibrates in people.\(^4\text{6}\) In a recent large intervention trial, gemfibrozil use was associated with a 6% increase in plasma HDL-C, a 31% decrease in triglycerides, and a 22% relative reduction in the risk of major cardiovascular events.\(^4\text{7}\)

Like most other lipid-modifying drugs, the TT/CETP vaccine appears to alter the level of more than one lipoprotein. Whether the reduced aortic lesion area seen in the TT/CETP-vaccinated rabbits is due solely to the elevation in HDL-C, to the combined effects on HDL-C and non–HDL-C, or to size and composition changes in lipoproteins remains to be elucidated.

The rabbit model of atherosclerosis has proved useful for testing and characterizing lipid-modifying therapies such as the HMG-CoA reductase inhibitors. However, the disease induced in this model differs significantly from human atherosclerosis.\(^4\text{8}\) Also, levels of several key components of the lipoprotein metabolic pathway, such as CETP and hepatic lipase, vary significantly in plasma concentration and between humans and rabbits, which makes it difficult to extrapolate these results to humans. The effect of inhibiting CETP activity on the course of human atherosclerotic disease can only be answered with clinical studies.

CETP inhibition remains a potential approach to elevate plasma HDL-C in humans, and clinical trials of several small-molecule CETP inhibitors are already under way. Statins also can reduce CETP activity, and this may contribute to their efficacy.\(^4\text{9}\) A vaccine approach to CETP inhibition may offer improved compliance compared with small-molecule inhibitors of CETP. Compliance with daily drug regimens is low, even for drugs with good side-effect profiles. In the case of statins, after 1 year, compliance is <50%.\(^5\text{0}\) Also, the CETP inhibition elicited by the TT/CETP vaccine is very specific, because the immune response is focused on a small fragment of the protein unique to CETP and its function. The TT/CETP peptide vaccine is currently in a phase 1 clinical trial to evaluate safety and immunogenicity.

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**References**


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