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REVIEW ARTICLE

Reality of a Vaccine in the Prevention and Treatment of Atherosclerosis

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Atherosclerosis together with multiple sclerosis, psoriasis and rheumatoid arthritis can be used as examples of chronic inflammatory diseases associated with multifactorial components that evolve over the years. Nevertheless, an important difference between these diseases relies on the fact that atherosclerosis develops from early ages where inflammation dominates the very beginning of the disease. This review highlights the inflammatory nature of atherosclerosis and the role the immune system plays in the process of atherogenesis. Although treatment of atherosclerosis has been for years based on lipid-lowering therapies reducing a series of risk factors, the degree of success has been only limited because cardiovascular complications related to the evolution of atherosclerotic lesions continue to appear in the population worldwide. In this sense, alternative treatments for atherosclerosis have come into play where both innate and adaptive immunity have been proposed to modulate atherosclerosis-associated inflammatory phenomena. When tested for their atheroprotective properties, several immunogens have been studied through passive and active immunization with good results and, therefore, the strategy through vaccination to control the disease has been made possible. Many experimental pre-clinical studies demonstrating proof of concept that vaccination using DNA and protein with an effective use of adjuvants and the optimal route of administration now provide a tangible new therapeutic approach that sets the stage for several of these vaccines to be tested in large, randomized, long-term clinical studies. A vaccine ready for human use will only be accomplished through the close association between academia, regulatory government organizations and private industry, allowing the reality of a simple and successful therapy to reduce atherosclerosis and its severe clinical complications.

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Introduction

According to the World Health Organization (WHO), coronary atherosclerotic disease is the first cause of death worldwide (1). The impact of this disease on population mortality highlights how important it is to understand its development in order to produce effective preventive

clinical measures. WHO calculates there are ~20 million deaths every year due to this disease whose treatment represents high costs for national health systems. It is estimated that, by the year 2020, ~300 million individuals will die due to acute complications of this disease.

Atherosclerosis is a multifactorial condition including factors such as hypertension, hypercholesterolemia, smoking and aging (2). Nevertheless, increasing evidence suggests that the significant damage provoked in the individual by these risk factors is not limited to the atherosclerotic process or its associated complications. Strict clinical trials have proven that the manifestations of atherosclerosis are a consequence of the “vulnerable” plaques prone to

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rupture (3). Such plaques show the highest tendency to make thrombi, the main cause of acute ischemic events in both coronary and brain vessels (4).

Although most details about the onset of the atherosclerotic plaque are still not clear, one of the postulates places oxidative stress as one of the main causes for the onset of the disease (5). Atherosclerotic lesions start as fatty streaks, mainly consisting of foam cells surrounded by a layer of endothelial cells with heterogeneous morphology (6). Streaks are generally developed in areas where the permeability of cell junctions is greater; such condition seems to facilitate the passage of macromolecules from the tunica intima to the tunica media of the vessel. In the early stages of fatty streak formation, low-density lipoproteins (LDL) are observed gathered in the subendothelial space before foam cells appear, thus being the first initiator of this pathology (7). Plaques become much more complex lesions due to the development of fibrous tissue, calcification (8), inflammation, ulceration of the arterial wall, and potential hemorrhage. Occlusion by plaque growth is an important risk factor, although it is the rupture or erosion of the plaque that originates the thrombi leading to heart infarction (9).

Lesions frequently affect the tunica intima of arteries and are characterized by the deposit and infiltration of diverse molecules and oxidized LDL (oxLDL) particles (10) leading to inflammation, accumulation and proliferation of smooth muscle cells full of lipids (mainly cholesteryl-oleate), macrophages converted into foam cells, and fibrous tissue, which narrow the arterial lumen until forming a lesion known as atheroma plaque. Many modifications have been described under these circumstances and one of the most significant in the fatty streak stage is oxidation, which may develop by exposure to reactive oxygen species (ROS) generated in different reactions of normal metabolism (11). Oxidative stress is paramount not only during the start of the lesion, but also in the progression of coronary heart disease. Diverse studies have postulated that oxLDL accelerates the growth of atherosclerotic lesions by inducing the expression of adhesion molecules, chemokines and, in the long-term, apoptosis of endothelial and smooth muscle cells (12).

Accumulation of lipoprotein particles is due not only to an increased permeability of the endothelium, but also the joining of other types of molecules to the arterial extracellular matrix (frequently glycosaminoglycans), thus increasing the time they stay within the arterial wall. Once attached to the matrix, LDL particles undergo oxidative modifications producing hydroperoxides, lysophospholipids, oxysterols, and aldehydic products from the rupture of fatty acids and phospholipids. Based on extensive experimental evidence, it has been proposed that these modifications represent one of the key pathological mechanisms in the development of atherogenesis. Lipoprotein deposits in the arterial wall also induce the accumulation

of leukocytes, a critical step in lesion formation and, hence, in the pathogenesis of the disease. Therefore, the process known as atherogenesis involves inflammatory elements from the start.

The types of inflammatory cells present in the evolving atheroma include monocyte-derived macrophages as well as lymphocytes. A number of adhesion molecules or leukocyte receptors expressed on the surface of artery endothelial cells participate in the recruitment of leukocytes towards the nascent atheroma (13). Likewise, products of oxLDL may raise the expression of leukocyte adhesion molecules through pro-inflammatory cytokines involved in leukocyte recruitment. For instance, interleukin 1 (IL-1) and tumor necrosis factor- α (TNF- α) may induce expression of P- and E-selectins on endothelial cells. Lipoprotein oxidation products also may induce the release of pro-inflammatory cytokines in vascular wall cells, providing an additional link between the arterial accumulation of lipoproteins and leukocyte recruitment. Chemoattractant cytokines such as chemokine (C-C motif) ligand 2 (CCL2), also known as monocyte chemoattractant protein-1, seem to direct the migration of leukocytes in the arterial wall.

Lipoproteins, Oxidative Stress and the Immune Response

Because the development of atherosclerosis is influenced by innate and adaptive immune responses (14), several studies have shown the activation of the immune system through the different stages in the development of atherosclerosis. A recent trial results suggest that activation of such immune responses may promote atherosclerosis by inducing and perpetuating arterial inflammation, whereas the selective activation of certain immune functions may inhibit atherosclerosis and arterial inflammation. This suggests that new approaches for the treatment and prevention of atherosclerosis are likely to appear either by selective suppression of pro-atherogenic immune responses or the selective activation of anti-atherogenic immune responses. Numerous antigens capable of triggering the immune response and affecting the development of atherosclerosis have been identified so far. These antigens together with the use of different classes of adjuvants and different routes of administration may be useful to modulate the immune response.

Trials assessing the effects of immunization on atherosclerosis have focused on two main objectives: 1) the presence of pre-existing immune responses considered part of the pathologic process such as the immune response against oxLDL epitopes and heat shock protein 60 (HSP 60); 2) targets like the cholesteryl-ester transfer protein (CETP) and TNF- α . In the first case, the objective is to stimulate immune responses that are protective per se but for which the magnitude is not strong enough unless they are increased, e.g., by a vaccine that triggers a more effective immune response or by inducing tolerance for undesired immune responses. In the second case, the objective is to produce neutralizing

antibodies that inhibit the effect of the chosen antigen. Because the identification of key antigens responsible for the activation of immune responses related to the development of atherosclerosis is a pre-requirement for any immunization therapy, finding the right adjuvant and the most appropriate administration route is also a challenge.

As for atherosclerosis, there is important evidence showing that immune activation mainly involves pro-inflammatory Th1 cells, considered responsible for disease development (15). Therefore, adjuvants that favor a change towards anti-inflammatory Th2 responses like aluminum salt-based adjuvants and incomplete Freund's adjuvant are more effective than those favoring Th1 responses. Alternatively, inhibition of Th1-mediated immune responses may be accomplished through tolerance induction by mucosal administration with or without adjuvants like the cholera toxin B subunit (CTB) (16).

There is also evidence indicating that activation of Th1 immune responses enhances the aggressiveness of the disease and counteracts many immunoregulatory mechanisms. If this concept is correct, it could be feasible to modulate the process of the disease by activating or selectively inhibiting specific immune responses. Supporting this possibility, immunization of hypercholesterolemic animals with oxLDL has proven to inhibit the process of atherosclerosis (17) providing important evidence for a phenomenon of atheroprotection through adaptive immune responses (18).

Figure 1 presents a summary of the mechanisms involved in the development of an atherosclerotic lesion

presented so far. Accumulation of LDL particles in the intima of an artery and their chemical transformation promote the recruitment of monocytes ($CD14^{hi}CD16^{-}$) and T cells that lead to adhesion molecule overexpression associated with an increase in endothelial permeability. This phenomenon, associated with a lower migration rate of antigen-presenting monocytes results in the accumulation of foam cell macrophages and activation of T helper cells that eventually lead to the secretion of proinflammatory cytokines (19). Together with these processes, resident dendritic cells ($CD11c^{+}DCs$) that mediate immunity and normally are found in the intima of arteries start to overexpress cytokines that promote the transformation of $CD4^{+}$ T cells into atherogenic Th-1 cells (20). Concomitantly, proliferation and migration of smooth muscle cells are promoted in response to growth factors and cytokine formation.

Many of the recent discoveries in relation to the pathophysiology of atherosclerosis have radically changed the traditional concepts related to the development of the disease. It is now clear that atherosclerosis corresponds to an inflammatory disease where the immune system plays an important role in its development, both during early stages as well as during the late complications of the disease (21–23). Therefore, a successful immunomodulatory therapeutic strategy has to accomplish at least one of the following points: 1) stop the growth of the atherosclerotic plaque; 2) present the capability to accomplish total or partial regression of the plaque; 3) stabilize the plaque; and 4) stimulate conditions that diminish the inflammatory process

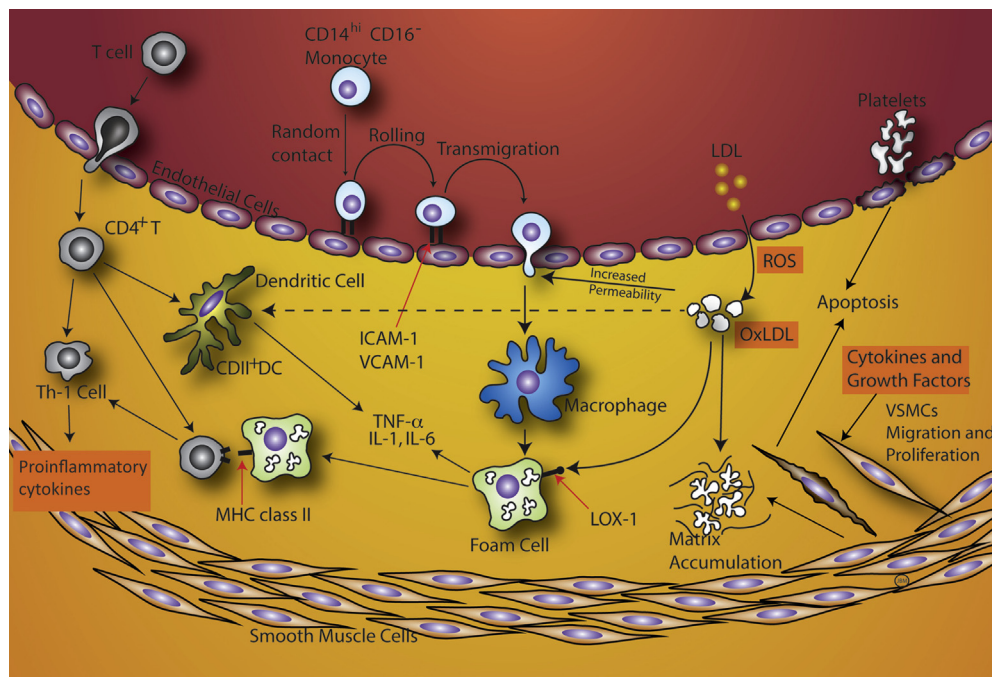


Figure 1. Schematic representation of current knowledge of biochemical processes and immune activation involved in the development of atherosclerosis.

associated with atherosclerotic plaque formation. Based on the above, several approaches have been developed and evaluated with the possibility of inhibiting atherosclerosis by active immunization or directly providing antibodies against proteins related to the development of the disease (24). Because during the course of the disease, atheroprotective immunity is accomplished, the development of immunomodulation strategies and the evolution of a vaccine in the prevention and/or treatment of atherosclerosis has been considered a feasible approach (25,26).

Development of Vaccines against Atherosclerosis

It has recently been reported that CD99, a membrane protein present in leukocytes, initially described to function in T cell activation and aggregation of lymphocytes (27–29), participates in human monocyte transmigration through cultured endothelial cells (30). Whereas the recruitment of T cells and monocytes contributes to the onset and progression of atherosclerotic plaques, the blockage of this transmigration process might constitute a protection against the development of the disease; thus, the effect of vaccination of atherosclerosis-prone mice with CD99 was determined. This vaccine constructed by cloning the extracellular domain of CD99 in pcDNA3, and administered orally, significantly reduced the formation of atherosclerotic lesions in the aortic valve and the carotid arteries when compared to mice immunized with the control vector.

Immunization of mice with vascular endothelial growth factor receptor 2 (VEGFR2) also leads to a reduction of atherosclerosis. The interference of the interaction of VEGF with its main receptor VEGFR2 also results in reduction of atherogenesis (31). Because VEGFR2 is expressed by activated endothelial cells covering the atherosclerotic plaque, the proatherogenic effect of this protein lies in the fact that the recognition between VEGF and VEGFR2 induces an inflammatory response in endothelial cells through the activation of NF- κ B (32). This activation results in high levels of expression of adhesion molecules such as VCAM 1, ICAM-1 and E-selectin, resulting in an increase in the adhesion rate of monocytes to endothelial cells (33). Therefore, a vaccination approach could attenuate both the initiation and progression of atherosclerosis by reducing the expression of adhesion molecules by endothelial cells (31).

An alternative approach has focused on the proprotein convertase subtilisin/kexin type 9 (PCSK9), a protease suggested to promote the degradation of the LDL receptor (LDLR). Experiments employing antisense technology have shown that PCSK9 inhibition resulted in an increase in LDLR in the liver (34). These experiments suggest that vaccination against PCSK9 might become a viable therapeutic strategy to reduce LDL in plasma (35). Recently, several groups have used monoclonal antibodies raised

against PCSK9 in phase II studies using alirocumab and evolocumab (36,37).

For years now, specific fragments of several apolipoproteins have also been used as an immunomodulation strategy to control the development of atherosclerosis. Among these apolipoproteins, regions between amino acids 45–76 and 12–35 of apolipoprotein C-III have been employed as immunogens in conjunction with T-helper epitopes and tetanus toxin as carrier. It has been shown that induction of an immune response or the use of antibodies against these segments results in a reduction in atherosclerotic lesions when tested in patients (38). Immunization of mice with peptides derived from apolipoprotein B-100 (apo B-100) also significantly reduced the development of atherosclerosis (39). The most effective peptides identified in these studies correspond to amino acids 661–680 (p45) and 3136–3155 (p210). Pilot vaccines containing apo B 100 p45 and p210, using aluminum salts as adjuvant and bovine serum albumin as carrier, inhibit the development of atherosclerosis in >50% of LDL receptor-deficient mice expressing human LDL apo B-100 (40). The use of peptides derived from apo B-100 conjugated with dialdehyde or malondialdehyde has also been shown to be capable of eliciting an immune response against non-native oxLDL by interacting with T cell antigen receptors (TCRs) and used as a vaccine against atherosclerosis (41–43).

Because CD4⁺ T cells have been postulated to be critical in the development of atherosclerosis, it has been thought that they may also be involved in protective immune reactions after immunization with oxLDL. This possibility was studied in apolipoprotein E knockout (apoE KO) mice and CD4/apoE double knockout mice immunized with oxLDL. Results show that the absence of CD4⁺ T cells in apoE KO mice leads to reduced atherosclerosis, indicating that CD4⁺ T cells constitute a major proatherogenic cell population. Therefore, the inhibition of CD4⁺ T responses to peptides derived from apo B-100 has led to the development of potential immunomodulatory methods to treat atherosclerosis (44).

Although the role of oxLDL in the origin and progression of atherosclerosis is still far from clear, there is evidence related to the presence of circulating antibodies against oxidized lipoproteins in association with the disease (45). Hypercholesterolemic rabbits immunized with homologous oxLDL presented an important reduced neointimal area after balloon injury despite severe hypercholesterolemia (46). With this in mind and the fact that phosphorylcholine (PC) is an important component of a series of inflammatory phospholipids such as platelet-activating factor (PAF) and oxLDL, several conjugates containing PC have been tested correlating infection and atherosclerotic lesion formation. Interestingly, it has been shown that Pneumococcal immunization decreases atherosclerosis where plasma from immunized mice shows the ability to block

the binding of oxLDL to macrophages. The production of antibodies against oxLDL suggests molecular mimicry between epitopes of oxLDL and *S. pneumoniae* and therefore the possibility that this type of immune response might produce protective effects (47). Another putative target for immunomodulation might be the lectin-like oxidized LDL receptor-1 (LOX-1) as many of the pro-atherogenic action of oxLDL occur through activation of LOX-1 (48,49). Interestingly, *C. pneumoniae*, which accelerates atherosclerosis in several animal models, has been shown to bind and activate the LOX-1 receptor on endothelial cells and macrophages (50).

Based on this experimental evidence, Table 1 presents a list of patent applications and granted patents proposing immunomodulatory procedures or vaccine formulations designed to counteract atherosclerosis.

Cholesteryl-ester Transfer Protein (CETP)

CETP is a hydrophobic glycoprotein consisting of 476 amino acids playing an important role in lipid metabolism (64). When this protein is overexpressed, it can lead to atherosclerosis by decreasing high-density lipoproteins (HDL-C) and increasing low-density lipoproteins (LDL-C) (65,66). Because the inhibition of CETP activity has been proposed to be a promising means for the attenuation of atherosclerosis, several research groups have developed peptide and recombinant protein vaccines to induce specific antibodies against CETP and reduce aortic lesions (67). The Hsp65-

CETPC vaccine was produced by fusing the heat shock protein 65 kDa (Hsp65) of *Mycobacterium tuberculosis* var. *bovis* with the linear polypeptide epitope of the cholesteryl-ester transfer protein C-terminal fragment (CETPC) and expressed as soluble protein in *Escherichia coli*. Because this vaccine was tested in the absence of adjuvants, results obtained showed that the subcutaneous administration of this vaccine in mice triggered a low immune response (66). The same group of investigators showed that immunization of rabbits using the chimeric recombinant enzyme rAnsB-TTP-CETPC containing asparaginase (AnsB), the epitope of the T helper cell containing residues 831–854 of tetanus toxin (TTP) and the B cell epitope containing residues 448–476 of human CETP (CETPC) in Freund's adjuvant, were able to overcome the problem of low immune response. Because the use of Freund's adjuvant is unsuitable for human use due to its toxicity, rAnsB-TTP-CETPC was used to immunize rabbits and stimulate production of anti-CETP antibodies employing an adjuvant based on aluminum salts, safe for human use. In this study, a high titer of anti-CETP antibodies resulted in parallel with an increase in HDL-C and a decrease in LDL-C, therefore achieving an anti-atherogenic effect (68).

The development of a DNA vaccine against CETP has also been achieved by using an eukaryotic expression construction carrier containing eight CpG motifs 5'-GACGTT-3' and the immunostimulatory sequence and core gene of the hepatitis B virus (HBc) inserted with a DNA fragment encoding 26 amino acid residues (451–476) of

Table 1. Vaccine patent applications and granted patents presented as an alternative to control the progression of atherosclerosis

Patent title	Inventors	Assignee	Ref.
Vaccine therapies and prophylactic treatments of atherosclerotic diseases	Fruchart et al., 2003 (CA2458237 A1)	GlaxoSmithKline Biologicals	(51)
Vaccine for the treatment of atherosclerosis	Fruchart et al., 2004 (EP1267908 B1)	GlaxoSmithKline Biologicals	(52)
Vaccine therapy of atherosclerosis	Metten et al., 2004 (WO 2004080375 A2)	GlaxoSmithKline Biologicals	(53)
Methods for increasing HDL cholesterol level	Kwoh et al., 2006	Avant Immunotherapeutics Inc	(54)
Immunotherapeutic treatment for inducing the regression of atherosclerotic plaques	Carlsson and Nilsson, 2007 (WO2007025781 A2)	Bioinvent Int Ab	(55)
Methods employing and compositions containing plaque associated molecules for prevention and treatment of atherosclerosis	Harats and George, 2007 (US7279459 B2)	Vascular Biogenics Ltd	(56)
Phosphorylcholine conjugates and corresponding antibodies	De Faire and Frostegard, 2011 (US8012483)	Athera Biotechnologies Ab	(57)
Peptide-based immunization therapy for treatment of atherosclerosis and development of peptide-based assay for determination of immune responses against oxidized low density lipoprotein	Nilsson and Shah, 2011 (EP2305708A1)	Forskpatent I SYD Ab	(58)
Immunomodulatory compositions, methods and systems comprising immunogenic fragments of apoB-100	Chyu and Shah, 2012 (WO2012065135 A2)	Cedars-Sinai Medical Center	(59)
Immunomodulatory methods for treatment of atherosclerosis via inhibition of CD4+ T cell response to apoB-100	Hanson, 2013 (US8609605 B2)	Cardiovax LLC	(60)
Fusion proteins and related compositions, methods and systems for treatment and/or prevention of atherosclerosis	Nilsson et al., 2013 (US 8506964)	Cardiovax LLC	(61)
Peptide epitopes of apolipoprotein B	Nilsson and Shah, 2014 (US8647628 B2)	Cardiovax LLC	(62)
Prevention and treatment of vascular disease with recombinant adeno-associated virus vectors encoding apolipoprotein A-I and apolipoprotein A-I Milano	Shah et al., 2015 (US8926958 B2)	Cedars-Sinai Medical Center	(63)

the C-terminal region human CETP. Yuan et al. developed a DNA vaccine against CETP, the pCR-X8-HBc-CETP (abbreviated as pCETP) (69). This vaccine, administered intramuscularly, inhibited the progression of atherosclerosis in cholesterol-fed rabbits by inducing the synthesis of anti-CETP antibodies. A noninvasive means of vaccination via intranasal immunization has also been shown to be successful (70). Whereas most intranasal vaccines induce poor immune responses in the absence of immunostimulants or presentation vehicles, a delivery system based on chitosan has been developed. Chitosan has been extensively studied for its compatibility, biodegradability, low cytotoxicity and property of condensing DNA, making it able to protect DNA from degradation. These studies showed that intranasal immunization with chitosan/pCETP produces a long-term systemic immune response (69). Therefore, it is suggested that intranasal vaccination can be a convenient and noninvasive route for delivery of DNA vaccines against atherosclerosis.

From the several intranasal vaccines that are being considered by the Food and Drug Administration (FDA) in the U.S., the trivalent vaccine against influenza caused by influenza virus subtypes A and B, commercially known as FluMist, has been authorized for some years now. This vaccine may be administered to persons between 2 and 49 years of age and does not require trained personnel to do so (71). These results provide evidence that intranasal vaccination is equivalent to intramuscular vaccination regarding immunogenicity and support that the intranasal route is a convenient noninvasive route for the administration of DNA therapeutic vaccines. Nevertheless, when manufacturing a nasal DNA vaccine, it must be considered that this route induces poor immunologic responses in the absence of stimulants or delivery vehicles (72).

A dimerized synthetic peptide including residues 461–476 of human CETP and residues 830–843 of tetanus toxoid TT (830–843) (CETPi-1) has been tested in parallel with a mixture that uses a single peptide replacing TT (830–843) for epitope aK-Cha-VAAWTLKAa (PADRE-CETP), both formulated with different aluminum salt adjuvants. Although differences in immunogenicity were found due to the aluminum salt used, both showed an appropriate anti-CETP antibody response (73,74). The CETPi-1 vaccine peptide corresponds to the acetate salt disulfide homodimer of a synthetic peptide of 31 residues: C-QYI KANSKFIGITE/FGFPEHLLVDFLQSL. The 16-amino acid sequence of C-terminal peptide is identical to residues 461–476 of the C-terminal sequence of human CETP protein, whereas the 14-amino acid N-terminal residues of the peptide are identical in sequence to residues 830–843 of tetanus toxin. This study was basically conducted in order to demonstrate the safety and immunogenicity of CETP in healthy adults. It was shown that a group of patients who received a single administration test and a subgroup of patients who received a booster acceptably tolerated

the vaccine at all doses. With a single injection, only one patient developed anti-CETP antibodies with the highest dose tested. Nevertheless, a second injection increased the number of patients developing a dose-related immune response. These results demonstrated that repeated administration of the vaccine is needed to induce an adequate antibody response in order to inhibit CETP activity (75).

Other efforts focused on modulating the function of CETP with a vaccine based on plasmid DNA encoding an immunogenic fusion polypeptide that includes the nucleotide sequence of at least one segment coding for a B cell epitope of CETP connected to a helper T cell epitope (76). A peptide-based vaccine for the regulation of CETP activity comprising a fragment of a T cell epitope helper connected to the fragment of a B cell epitope of CETP has also been developed (77). Similarly, a method has been proposed to increase the concentration of HDL by stimulating an immune response that inhibits CETP through immunization with the complete CETP molecule conjugated to a carrier such as Keyhole Limpet Hemocyanin or ovalbumin (54). Table 2 presents a list of patent applications and granted patents proposing immunomodulatory procedures or vaccine formulations as an effort to counteract the development of atherosclerosis.

Considering the problems shown by several of the technologies described above including the route of administration, cost and complexity of the formulation, our group has designed and tested through preclinical studies a novel intranasal anti-CETP vaccine. HB-ATV-8 vaccine is designed to inhibit the development of atherosclerosis and its complications using a synthetic peptide as immunogen (81). This peptide consists of the carboxy-end amino acids H486 to S496 of CETP conjugated into a micellar nanoparticle system formed by lipids derived from the cell membrane of archaebacteria, lysophospholipids and phospholipids (86,87).

HB-ATV-8 Vaccine

The therapeutic efficiency of vaccine HB-ATV-8 was studied in our laboratory using New Zealand rabbits following the requirements of the official Mexican regulation (NOM-062-ZOO-1999) and the Committee for Institutional Care and Use of Laboratory Animals (JMO63-15). In addition to these norms, the Guide for Care and Use of Laboratory Animals issued by the National Institutes of Health of the U.S. was also observed. The normal diet consisted of rabbit-specific food 5321 from LabDiet with the following composition: crude protein not less than 16%; crude fat not less than 2.5%; crude fiber not more than 18%; ash not more than 8% and additional minerals not more than 2.1%. In the high-cholesterol diet, a mixture of 1% cholesterol and 10% corn oil was added to the normal food. All rabbits were fed ad libitum.

After the quarantine period, administration of the high-cholesterol diet to the specific groups of experimental

Table 2. Vaccine patent applications and granted patents involving CETP presented as an alternative to control the progress of atherosclerosis

Patent title	Inventors	Assignee	Ref.
A vaccine against cholesterol to prevent hypercholesterolemia and atherosclerosis	Alving and Swartz Jr., 1992 (WO 1992010203 A1)	Alving CR	(78)
Modulation of cholesteryl ester transfer protein (CETP) activity	Ritterhaus and Thomas, 2003 (US6555113 B1)	Avant Immunotherapeutics Inc.	(77)
Plasmid-based vaccine for treating atherosclerosis	Thomas LJ, 2005 (US6846808 B1)	Avant Immunotherapeutics Inc.	(76)
Modulation of cholesteryl ester transfer protein (CETP) activity	Adari, Thomas and Ritterhaus, 2006 (US20060276400 A1)	Adari H, Thomas L, Ritterhaus CW	(79)
Peptide-based vaccine compositions to endogenous cholesteryl ester transfer protein (cetp)	Ritterhaus et al., 2008 (WO2008057529 A2)	Coley Pharm Group	(75)
Treatment of atherosclerosis	Mattner and Schmidt, 2009 (US20090104211 A1)	Affiris Forschungs-Und Entwicklungs GmbH	(80)
Immunoenzymatic quantification method for cholesterol ester transferring protein (CETP)	Alonso-García and Mas-Oliva, 2003 (US7749721 B2)	Universidad Nacional Autónoma de México	(81)
Anti-atheroma vaccine	Kakkar and Lu, 2013 (US8435538 B2)	Thrombosis Research Institute	(82)
Treatment of atherosclerosis	Brunner et al., 2013 (EP2190451 B1)	Affiris AG	(83)
Nasal vaccine against the development of atherosclerosis and fatty liver	Mas-Oliva et al., 2014 (WO2014003531 A1)	Universidad Nacional Autónoma de México	(84)
CETP fragments	Brunner et al., 2014 (US 20140147456 A1)	Affiris AG	(85)

animals was started. After 15 days, vehicle and vaccine administration to specific groups was started. For treated animals, 50 µl of the vaccine preparation was nasally administered twice weekly and treatment lasted for 3 months, after which experimental animals were sacrificed using a lethal dose of sodium pentobarbital and processed organ tissues.

The macroscopic aspect of livers from the control group in comparison to animals fed a high-cholesterol diet was found to be remarkably different because livers from this last group presented a milky aspect (84). Importantly, vaccine treatment reversed this condition (84). Microscopically, the liver of the rabbits that received a normal diet clearly show the laminar organization of normal hepatic parenchyma forming liver lobules with plates of hepatocytes radiating between the central vein and radially arranged sinusoids (Figure 2A). In contrast, the liver from rabbits fed a high-cholesterol diet for 3 months who received no vaccine showed a varied degree of micro- and macrovesicular hepatocellular damage and diffuse steatosis affecting large areas of the liver lobules and mainly hepatocytes surrounding the central vein (Figure 2B). Some of these hepatocytes show cytoplasm distention due to the presence of lipid droplets in their cytoplasm. Most hepatocytes from the lobular periphery close to the portal triads present a normal appearance although some of them show a microvacuolar cytoplasm. In some areas, lipid droplets coalesce becoming a vacuole of fat that displaces the nucleus to the periphery of the cell. In general, images show a combination of steatosis, the presence of polymorphonuclear leukocytes, monocytes or both, ballooned hepatocytes and areas of necrosis. Livers from rabbits that received the vaccine preparation simultaneously to the high-cholesterol diet during the 3-month period show centrilobular hepatocytes with some degree of vacuolization without hepatocyte ballooning,

whereas periportal and paracentral hepatocytes practically show a normal appearance (Figure 2C). Administration of the vaccine simultaneously with the initiation of the high-cholesterol diet shows a protective effect on the hepatic parenchyma. In order to evaluate the degree of fibrosis developed with a high cholesterol diet, the Masson trichrome staining technique was implemented. Histological liver sections of a control animal fed on normal diet demonstrate the scarce presence of collagen fibers around the wall of central vein but not around the sinusoids (Figure 3A). In contrast, livers from animals fed a high-cholesterol show an important development of perisinusoidal fibrosis close to the central vein and at the central vein itself (Figure 3B). These changes are more frequently observed in zone III of the hepatic acinus, corresponding to the center of the classical lobule. An association between the presence of ballooned hepatocytes and perisinusoidal fibrosis near the central vein was frequently observed (Figure 3B). On the other hand, administration of the vaccine concomitantly with the high-cholesterol diet significantly improved the histopathological appearance of the livers as shown in Figure 3C.

Figure 4 shows the histological analysis in a cross-section of the abdominal aortas of the same rabbits fed a normal diet (Figure 4A), a high-cholesterol diet without treatment (Figure 4B) or a high-cholesterol diet fed concomitantly with the intranasal administration of the vaccine (Figure 4C). Interestingly, this last group of treated experimental animals show histological characteristics remarkably similar to the control group in contrast to animals fed the high-cholesterol diet that presents significant lesions in the intima of arteries. These results, first described as part of a patent application (84), indicate the important degree of protection shown by the HB-ATV-8 vaccine, not only upon the development of lesions in the

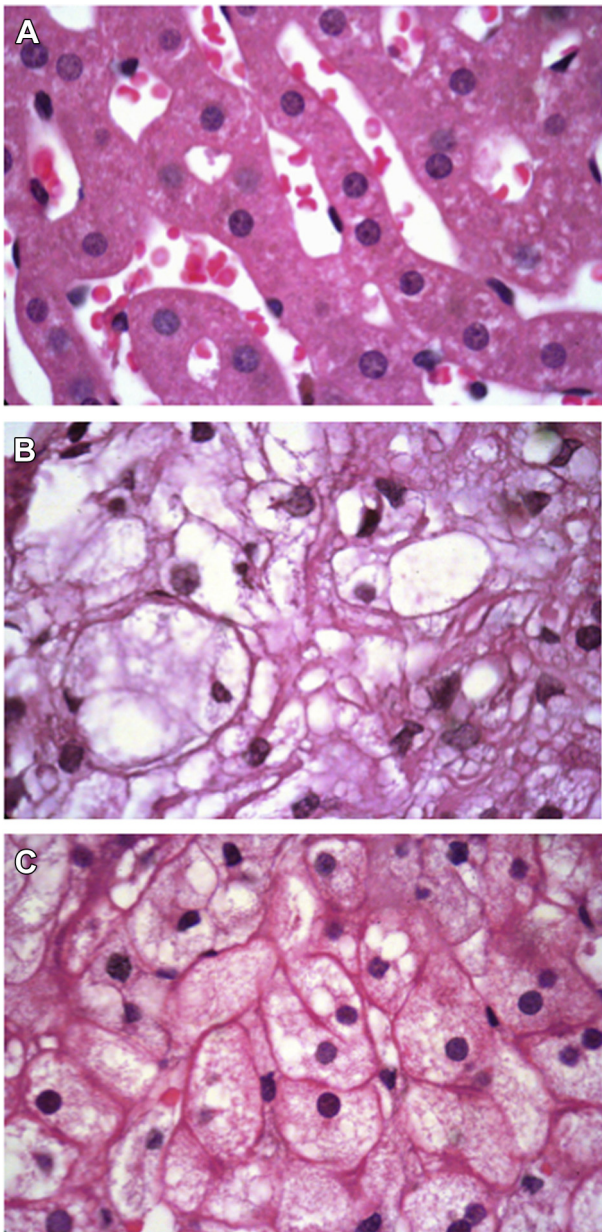


Figure 2. Hematoxylin- and eosin-stained histological sections of livers from experimental animals. (A) Control rabbits that received a normal diet. (B) Rabbits fed a high cholesterol diet for 3 months and received no treatment. (C) Rabbits fed for 3 months with a high cholesterol diet that simultaneously received the vaccine preparation. Images x1000.

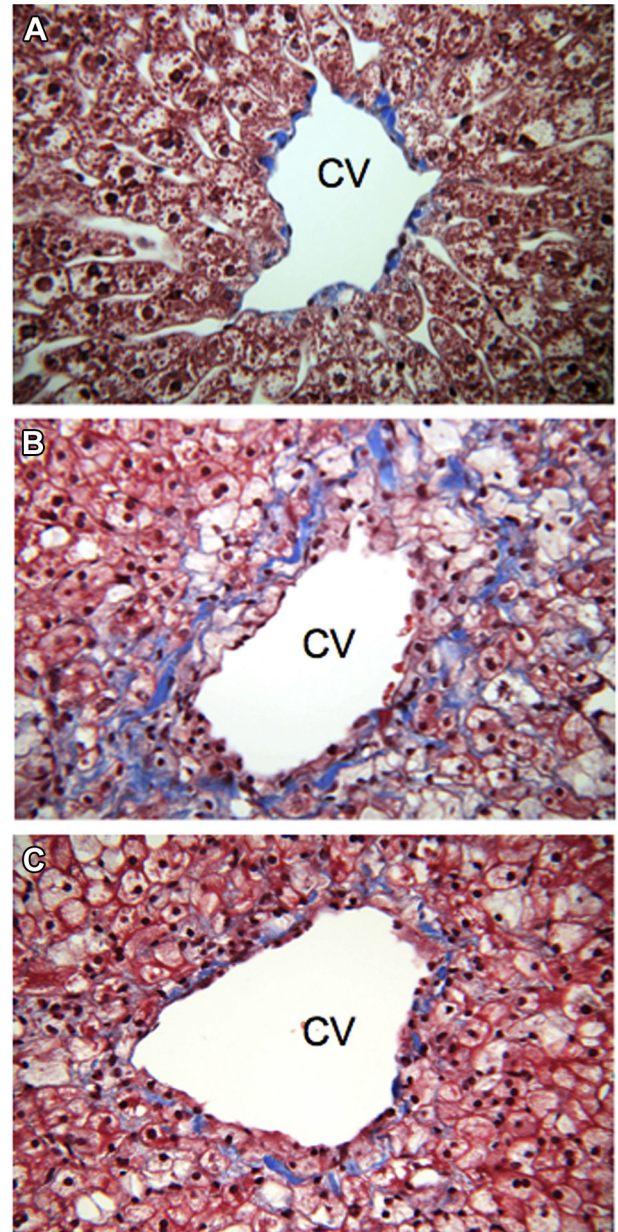


Figure 3. Masson trichome-stained histological sections of livers from experimental animals. (A) Control rabbits that received a normal diet. (B) Rabbits fed a high cholesterol diet for 3 months and received no treatment. (C) Rabbits fed for 3 months with a high cholesterol diet that simultaneously received the vaccine preparation. CV, central vein. Images x200.

intima of arteries but importantly counteracting the development of fatty liver in the group of experimental animals fed the high cholesterol diet. These encouraging results now extended to the porcine model and long-term experimentation has shown that vaccination importantly prevents the process of atherogenesis and the development of fatty liver, allowing us to further test this technological platform starting with clinical trials in the near future.

One of the novel aspects of this formulation is the use of lipids from archaeobacterial cell membranes as a vaccine

component. Use of these preparations has shown not only to function as humoral adjuvants but also to promote a strong cytotoxic T-cell immune response characterized by a long-term memory (88). Although it has been reported that the immune response obtained employing archaeobacterial lipids is similar to that obtained with the potent, yet toxic, Freund's adjuvant, preparations using lipids derived from archaeobacteria are not toxic (89–93). Use of these particular lipids in association with lysophospholipids allowed us to prepare micellar nanoparticles homogeneous

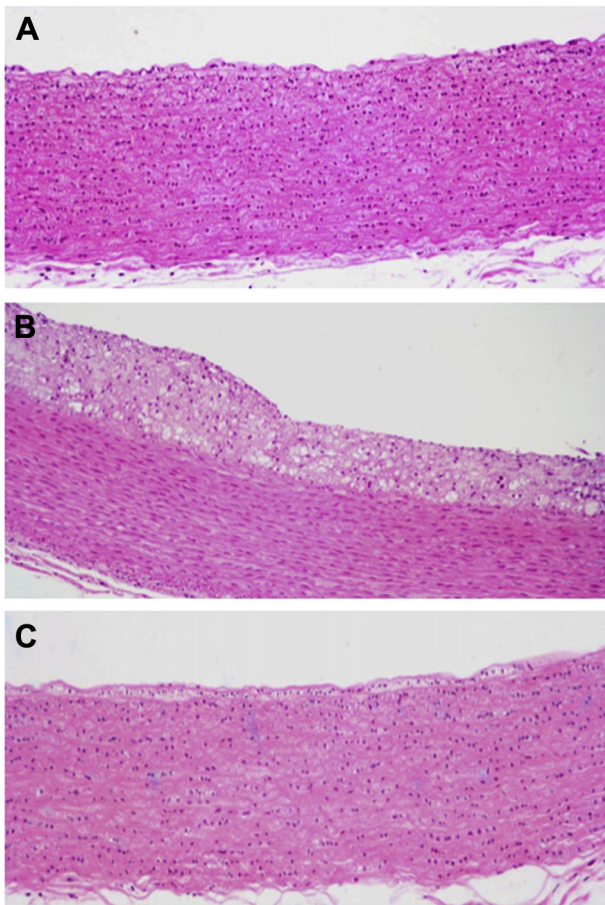


Figure 4. Hematoxylin- and eosin-stained histological sections of abdominal aorta from experimental animals. (A) Control rabbits that received a normal diet. (B) Rabbits fed a high cholesterol diet for 3 months and received no treatment. (C) Rabbits fed for 3 months with a high cholesterol diet that simultaneously received the vaccine preparation. Images x160.

in size showing good stability and, more importantly, maintenance of the native α -helical secondary structure of the antigen, a key feature required to observe positive immunological responses (86).

Perspectives

Taking into account the promising results observed with many of the immunomodulatory therapies presented above at the pre-clinical level, it is critical to use this new-found knowledge related to immunity and inflammation in atherosclerosis to guide clinical testing. These therapies have the potential to directly treat atherosclerosis by targeting proteins at the arterial wall and lipoproteins, in comparison to the current pharmacological approach mainly designed to counteract risk factors such as hypercholesterolemia, hypertriglyceridemia and hypertension. In this respect, induction of self-immune responses through the use of autoantigens represents a promising approach to understand the relationship between innate and adaptive immunity with

regard to recognition by immune receptors and the expression of protective antibodies.

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