

Atheroprotective immunization with MDA-modified apo B-100 peptide sequences is associated with activation of Th2 specific antibody expression

GUNILLA NORDIN FREDRIKSON^{1,2}, LINDA ANDERSSON¹, INGRID SÖDERBERG¹, PAUL DIMAYUGA³, KUANG-YUH CHYU³, PREDIMAN K. SHAH³ & JAN NILSSON¹

¹Department of Medicine, Malmö University Hospital, Lund University, Malmö, Sweden, ²Department of Biomedical Laboratory Science, Malmö University, Malmö, Sweden, and ³Atherosclerosis Research Center, Cedars-Sinai Medical Center, UCLA School of Medicine, Los Angeles, CA, USA

(Received 18 August 2004; accepted 10 January 2005)

Abstract

Objective: The objective of this study was to evaluate if immunization with MDA-modified human apo B-100 fragments is associated with a shift in the Th1/Th2 balance.

Methods and Results: Apo E deficient mice were immunized with one of the peptides (P45; amino acids 688–707, P74; amino acids 1123–1142 or P240; amino acids 3613–3632) at 6, 9 and 11 weeks of age and compared to controls given carrier alone. Immunization with P45 and P74 reduced atherosclerosis in the aorta of 25-week-old mice by 48% ($p = 0.02$) and 31% ($p = 0.06$) and macrophage content in atherosclerotic plaques by 33% ($p = 0.02$) and 39% ($p = 0.02$), respectively. The levels of Th2-specific IgG1 against each peptide increased more than 50-fold in response to immunization, whereas the levels of specific IgM and Th1-associated IgG2a were only marginally affected. However, there was an increase in the plaque expression of both Th1 and Th2 cytokines as assessed by real time PCR. Immunization with P240, a non-homologous peptide used as control, induced a 10-fold increase of specific IgG1 but did not influence atherosclerosis or plaque content.

Conclusions: Immunization with MDA apo B-100 fragments induce a shift from Th1 to a Th2 specific oxidized LDL antibody expression, but without a concomitant downregulation of plaque IFN- γ expression.

Keywords: *Antibodies, apolipoproteins, atherosclerosis, immunization, peptides*

Introduction

Arterial accumulation of low density lipoprotein (LDL)-derived lipids is one of the most important factors causing development of atherosclerosis [1,2]. Some lipoprotein particles become oxidized in the arterial extracellular matrix leading to the formation of highly reactive peroxides and aldehydes. These substances are believed to injure vascular cells and activate an inflammatory response that plays a key role in lesion formation [1–4].

Oxidized LDL also contains epitopes recognized by circulating T cells as well as T cells in atherosclerotic plaques [5,6]. Moreover, immune recognition is

associated with formation of oxidized LDL autoantibodies [7]. Autoantibodies against oxidized LDL are common in humans and are most frequently of IgM type [8,9]. Some oxidized LDL IgM autoantibodies also cross-react with structures on apoptotic cells, certain microorganisms, and oxidized phospholipids suggesting that they have multiple functions [10–12]. Several studies have reported increased levels of oxidized LDL autoantibodies in patients with cardiovascular disease and animal models of atherosclerosis, but their exact role in cardiovascular disease remains to be fully understood [8,13–17].

Immunization of mice and rabbits with oxidized LDL has been shown to result in an inhibition of

Correspondence: G. N. Fredrikson, Wallenberg Laboratory, 1st floor, Malmö University Hospital, 205 02 Malmö, Sweden. Tel.: 46 40 337674. Fax: 46 40 332550. E-mail: gunilla.nordin_fredrikson@medforsk.mas.lu.se

atherosclerosis indicating that at least some of the immune responses against oxidized LDL are atheroprotective [18–22]. Immunization induces a T cell dependent elevation of specific IgG that represent one possible mediator of the atheroprotective effect [22]. The observation that B cell transfer reduces lesion development in splenectomized apolipoprotein E deficient (apo E0) mice favors the existence of atheroprotective autoantibodies [23]. An alternative possibility is that immunization activates a shift in the balance between Th1 and Th2 cells. Th1 cells generally secrete proinflammatory cytokines such as IL-2 and INF- γ , whereas Th2 cells produce primarily anti-inflammatory cytokines such as IL-4, IL-5 and IL-10. Induction of severe hyperlipidemia in apo E0 mice has been shown to result in a switch in oxidized LDL autoantibodies from Th1 specific IgG2a to Th2 specific IgG1 [24].

Using ELISAs based on a library of 20 amino acid long malondialdehyde (MDA)-modified polypeptides covering the complete amino acid sequence of apolipoprotein (apo) B-100 we have identified a large number of MDA-modified apo B-100 sequences recognized by antibodies in human plasma [25]. Most of these antibodies are of IgM type and their level of expression correlates with the severity of atherosclerosis as assessed by carotid intima media thickness. However, previous immunization studies in animals suggest that atheroprotection is associated with IgG expression rather than IgM [26]. Only a small number of MDA-modified apo B-100 sequences are recognized by IgG in human plasma and even fewer demonstrates a correlation with carotid intima media thickness [25]. In the present study, we have investigated the effect of immunization with either of three peptide sequences on atherosclerosis in apo E0 mice. We have also studied if immunization with these peptide sequences is associated with a shift in the Th1/Th2 balance as assessed by the expression of specific IgG2a/IgG1 antibodies.

Materials and methods

Mice

Male apo E0 mice on C57BL/6 background were purchased from B&M (Ry, Denmark). Mice ($n = 8–10$ per group) were given a first injection with a MDA-modified peptide conjugated to the carrier or the carrier alone at 6 weeks of age and booster injections 3 and 5 weeks later. The human apo B-100 peptide sequences used composed of amino acids 688–707 (IEIGLEGKGF EPTLE ALFGK; peptide 45), amino acids 1123–1142 (VISIP RLQAE ARSEI LAHWS; peptide 74) or amino acids 3613–3632 (FPDLG QEVAL NANTK NQKIR; peptide 240). The homology between the human and mouse sequences is 95% for peptide 45, 80% for peptide 74 and 0% for peptide 240

(Accession no P04114 (human) and XP_137955 (mouse)). Each injection contained 50 μ g of the MDA-modified peptide conjugated to 50 μ g of the carrier cBSA (cationized bovine serum albumin) dissolved in 0.083 M sodium phosphate 0.9 M NaCl pH 7.2, according to the manufacturer's protocol (No. 77652, Pierce, Rockford, IL, USA), and with Alum (aluminum hydroxide, Pierce) included in all injections as adjuvant. The selection of apo B-100 peptide sequences was based on studies demonstrating the presence of high IgM levels against these peptide sequences in man [25]. The mice were fed a cholesterol diet (0.15% cholesterol, 21% fat; Lactamin AB, Kimstad, Sweden) from the age of 10 weeks. At 25 weeks of age, the mice were killed and the tissue collected as previously described [26]. The Animal Care and Use Committee approved experimental protocol used in the study.

Analysis of the descending aorta and plaque content

Staining and quantification of plaque area in *en-face* preparations of descending aorta and subvalvular plaque macrophage content were done as previously described [26]. The same procedure was utilized for the different cytokine stainings as for macrophages, with exception for the antibodies used: goat anti-mouse TNF- α , goat anti-mouse IFN- γ or goat anti-mouse IL-4 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) as respective primary antibody and biotinylated anti-goat IgG (Vector Laboratories, Burlingame, CA) as secondary antibody.

T-cell preparation from the spleen

Spleen cells from the mice were isolated as previously described ($n = 4$ per group) [27]. T-cells were negatively selected using paramagnetic beads coated with anti-mouse B220 antibody to deplete B-cells and a magnetic particle concentrator, according to manufacturer's suggestion (DynaL A.S., Norway). Pilot experiments indicated $\geq 75\%$ purity for T-cells and $\geq 90\%$ purity for B-cells using RPE conjugated anti-mouse CD3 and FITC conjugated anti-mouse CD19 monoclonal antibodies (PharMingen, Becton & Dickinson, Heidelberg, Germany) by FACS analysis (FACSort, Becton & Dickinson). The negatively selected cells in RPMI 1640 medium (Gibco/BRL) containing 10% FBS were used in ELISPOT analysis.

IFN- γ and IL-4 ELISPOT

A commercially available kit for mouse IFN- γ ELISPOT was used according to the protocol of the manufacturer (Nordic BioSite, Täby, Sweden). In the IL-4 ELISPOT a monoclonal anti-mouse IL-4 antibody was used as coating antibody (Nordic BioSite)

and a biotinylated anti-mouse IL-4 (Nordic BioSite) as detection antibody. The same procedure was used as for IFN- γ ELISPOT. About 2×10^4 T-cells/well for IFN- γ and 10^5 T-cells/well for IL-4 were incubated in sterile coated filter plates (Millipore, Bedford, MA) for 20 h at 37°C in 5% CO₂ and 95% humidity. The cells were incubated in the presence or absence of the same MDA-modified peptide (200 pg/ml) as used for the immunization of the mouse and as activators of the cells were 1 ng/ml phorbol myristate acetate (PMA; Sigma, St. Louis, MO) and 500 ng/ml Ionomycin (Sigma) used. All experiments were done in quadruplicates. Spots were visually counted by using a sidelight microscopy (Olympus Europe FZ30). Mean values were calculated by counting the spots in the wells containing peptide, PMA and Ionomycin with the background spots subtracted.

RNA isolation and cDNA synthesis

Total RNA was isolated from the whole aorta from each mouse using the protocol for FastPrep™ system (BIO 101, Carlsbad, CA) with small modifications. A speed rating of 6 for 45 s to lyse the aortas was used. The synthesis of cDNA was performed by mixing 1 μ g of total RNA with random primers (GibcoBRL, Life Technologies, Gaithersburg, MD), DTT (GibcoBRL), dNTP (Boeringer Mannheim, Mannheim, Germany), RNaseINH (Promega, Madison, WI) and Moloney Murine Leukemia Virus Reverse Transcriptase (MMLV-RT) (GibcoBRL). The tubes were incubated for 10 min at 30°C, 50 min at 42°C and 2 min at 94°C.

Real time polymerase chain reaction

SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) was used in an ABI PRISM 7700 Sequence Detection System (SDS-Perkin Elmer, Applied Biosystems) for amplification of cDNA according to the manufacturer's protocol. As controls, samples without MMLV-RT in the cDNA synthesis and wells with no template were used. All samples were run in triplicates. Amplification of TNF- α , IFN- γ , IL-4 and rRNA was carried out by using the cDNA, an appropriate concentration of each primer pair and $2 \times$ SYBR Green PCR Master Mix. The endogenous control 18S rRNA was used to normalize the samples. Calculations were performed by using the standard curve method (CV < 9%). To confirm the absence of non-specific amplification the dissociation curve program was used.

Peptide—ELISA

The same MDA-modified peptide as for the immunization was used for coating (20 μ g/ml in PBS pH 7.4) of microtiter plates (Nunc MaxiSorp, Nunc, Roskilde,

Denmark) as previously described [26]. The detection of deposit antibodies recognizing the peptide sequences involved both biotinylated goat anti-mouse IgM or IgG antibodies (Jackson Immuno-Research, West Grove, PA), as well as alkaline phosphatase conjugated rat anti-mouse IgG1 or IgG2a antibodies (Pharmingen, BD Bioscience, Erembodegem, Belgium), incubated for 2 h at room temperature. Data regarding the specificity and variability of the ELISA have been published previously [25].

Serum cholesterol and triglyceride

Total plasma cholesterol and plasma triglycerides were quantified with colorimetric assays, Infinity™ Cholesterol and Triglyceride (INT), respectively (Sigma).

Serum amyloid A (SAA)—ELISA

A commercially available ELISA kit (Biosource Int., Camarillo, CA) was used for determine the level of SAA as recommended by the manufacturer.

Statistical analysis

Data are presented as mean \pm standard deviation. Analysis of the data was done using the Mann–Whitney two-tailed test. Statistical significance was considered at the level ≤ 0.05 . Box plots demonstrate median, 25th and 75th percentiles, and with whiskers showing the highest and lowest values.

Results

Effect of immunization on cholesterol and triglyceride levels

Mice were immunized with human apo B-100 fragments or carrier alone at 6 weeks of age followed by booster injections 3 and 5 weeks later. A cholesterol-rich diet was given from 10 weeks of age and the animals sacrificed at 15 weeks later. Immunizations did not affect the body weight of the animals. Mice immunized with fragment P45 had higher serum cholesterol levels than the control mice receiving only carrier (Table I), whereas no effect on lipid levels were observed in mice immunized with fragment P74 or P240. Mice immunized with apo B-100 fragment 45 also had higher triglyceride levels than the controls (Table I).

Effect of immunization on atherosclerosis

Atherosclerosis was analyzed by Oil Red O staining of flat preparations of the descending aorta and quantified by image analysis. Atherosclerotic plaques were primarily located at aortic branching points and immunization did not alter this pattern. Immunization

Table I. Lipids, body weight, cytokines and SAA in immunized apo E0 mice at the age of 25 weeks.

	MDA-P45 (n = 9)	MDA-P74 (n = 9)	MDA-P240 (n = 10)	Controls (n = 8)
Cholesterol (mg/ml)	4.05 ± 1.21*	3.33 ± 1.19	3.99 ± 1.47	3.10 ± 1.20
Triglycerides (mg/ml)	1.63 ± 0.37*	1.20 ± 0.29	1.13 ± 0.25	1.14 ± 0.45
Body weight (g)	35.56 ± 5.36	35.60 ± 2.46	35.00 ± 5.35	36.25 ± 5.18
Elispot, IFN- γ^{\dagger} (Spots/well)	19.75 ± 1.50*	21.00 ± 12.88	3.50 ± 5.07	7.25 ± 4.35
Elispot, IL-4 † (Spots/well)	2.75 ± 3.78	1.00 ± 0.82	5.25 ± 2.36*	0.25 ± 0.50
SAA (μ g/ml)	10.02 ± 5.18	7.01 ± 3.58	7.86 ± 3.34	10.51 ± 7.57

* $P < 0.05$ vs. controls. $^{\dagger}n = 4$.

with fragment P45 reduced atherosclerosis with 48% ($p = 0.02$), fragment P74 with 31% ($p = 0.06$), whereas immunization with P240 had no effect (Figure 1).

Effect of immunization on MDA Apo B-100 fragment antibodies

Specific antibodies, mostly IgM, against all fragments used were detected in the serum of control mice. However, immunization with fragment P45 resulted in a more than 10-fold increase in specific IgG, but did not affect IgM levels against this fragment (Figure 2a). In mice immunized with fragment P74 specific IgG increased almost 20-fold, whereas IgM levels increased by about 50% (Figure 2b). However, immunization with fragment P240 decreased the IgM levels by about 5% and gave a 50-fold increase of IgG antibodies (Figure 2c).

To determine if immunizations influenced the Th1/Th2 balance we measured the expression of IgG2a (Th1) and IgG1 (Th2) antibodies against each MDA-apo B-100 fragment. Only low levels of apo B-100 fragment specific IgG2a and IgG1 were expressed in control mice. However, for all fragments the level of IgG2a was significantly higher than that of IgG1 indicating a predominance of Th1 immune responses. In mice immunized with fragment P45 the level of specific IgG1 was increased more than 100-fold as

compared to control mice, whereas a minor increase was seen in IgG2a levels (Figure 3a). Similar findings (a 50-fold increase in IgG1) were made in animals immunized with fragment P74 (Figure 3b). Following immunization with fragment P240 a 10-fold increase in specific IgG1 antibodies and a small decrease of IgG2a levels were detected (Figure 3c). These findings suggest that immunization with MDA-apo B-100 fragments using alum as adjuvant induce a shift from Th1 to Th2 specific antibody expression.

Effect of immunization on inflammatory activity

A shift in immune responses against MDA-apo B-100 fragments from Th1 to Th2 would be expected to result in decreased inflammatory activity. The local inflammatory activity in atherosclerotic plaques was studied by immunohistochemical staining of macrophages and cytokines in subvalvular lesions as well as by real-time PCR analysis of cytokine expression in the aorta. The effects on systemic inflammatory activity were assessed by determining serum levels of the inflammatory marker serum amyloid A (SAA) and the expression of INF- γ and IL-4 in splenic T cells by Elispot technique.

Macrophage immunoreactivity was reduced by 33% ($p = 0.02$) in mice immunized with fragment P45 and by 39% ($p = 0.02$) in mice immunized with

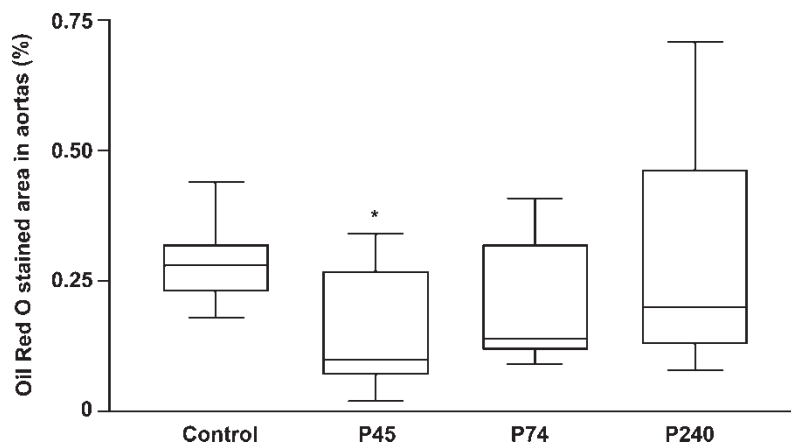


Figure 1. Stained plaque area in aortas from immunized apo E0 mice. Mice were immunized with carrier and adjuvant alone (controls) or with respective MDA-modified peptide P45, P74 or P240. Plaque areas were assessed by Oil Red O staining of *en-face* mounts of the descending aorta. Values represent stained area in percent of total area. * $P < 0.05$ vs. controls, $n = 8-10$.

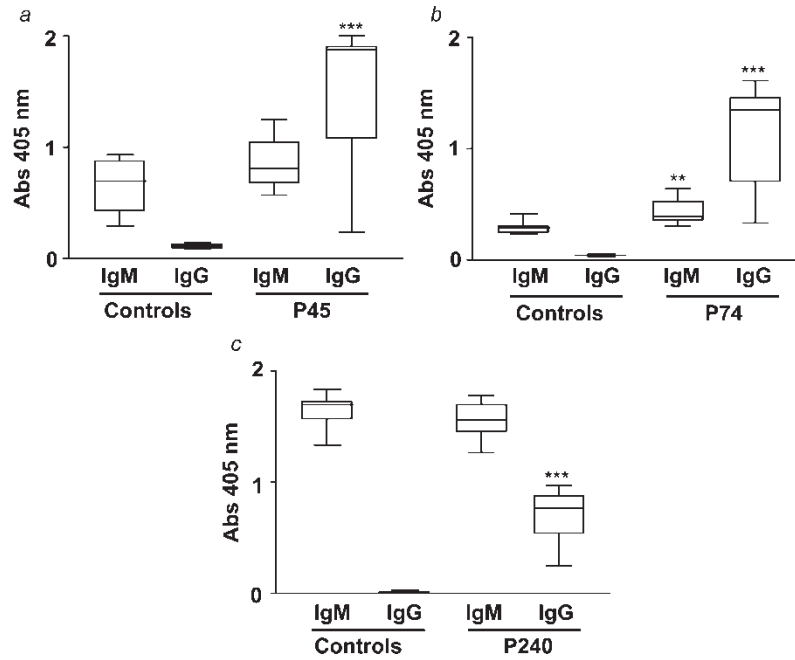


Figure 2. IgM and IgG specific antibodies in plasma from immunized apo E0 mice. Mice were immunized with carrier and adjuvant alone (controls) or with respective MDA-modified peptide P45, P74 or P240. Detection of antibodies directed to MDA-modified P45 (a), P74 (b) or P240 (c). ** $P < 0.01$ and *** $P < 0.001$ vs. controls, $n = 8-10$.

fragment P74 as compared to controls (Figure 4). No effect was seen with fragment P240.

With real-time PCR an increase of TNF- α , IFN- γ and IL-4 mRNA levels was detected in mice aortas immunized with fragment P45 compared to control mice (Table II). Also in the P74 group an increase of TNF- α was shown, but none of the cytokines were

elevated in mice immunized with fragment P240 (Table II). However, no differences were found in the plaque cytokine immunohistochemical staining between groups (data not shown).

There were no differences in serum SAA levels between controls and mice immunized with apo B-100 fragments (Table I). Elispot analysis of INF- γ

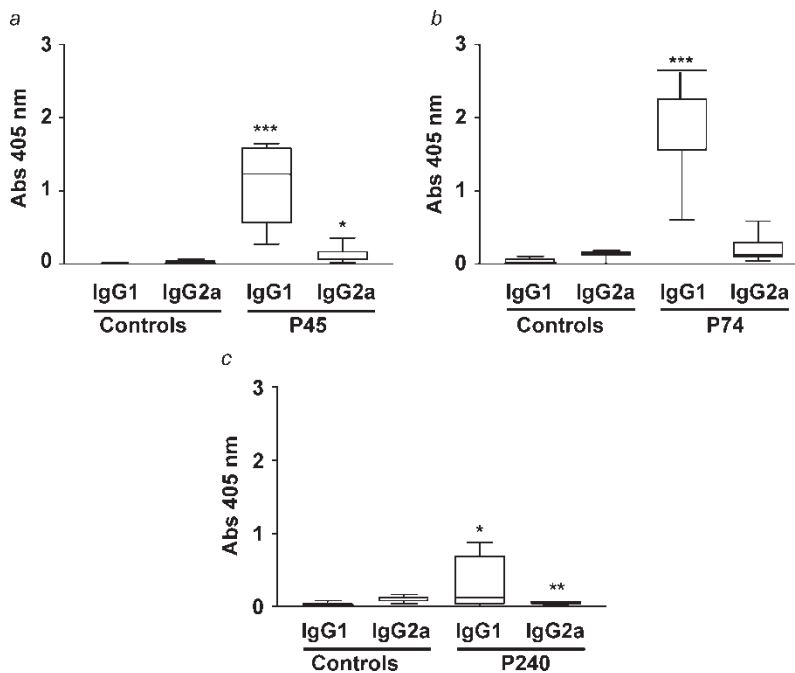


Figure 3. IgG1 and IgG2a specific antibodies in plasma from immunized apo E0 mice. Mice were immunized with carrier and adjuvant alone (controls) or with respective MDA-modified peptide P45, P74 or P240. Detection of antibodies directed to MDA-modified P45 (a), P74 (b) or P240 (c). * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ vs. controls, $n = 8-10$.

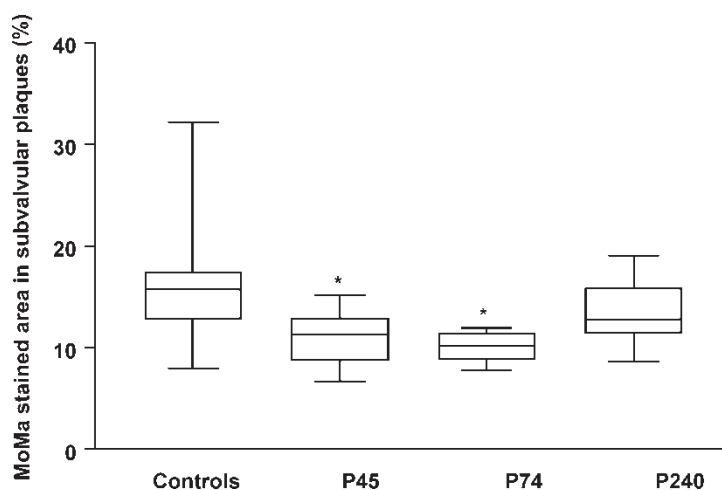


Figure 4. Investigations of macrophage content in subvalvular lesions from immunized apo E0 mice. Values represent stained area of the total plaque area. * $P < 0.05$ vs. controls, $n = 8-10$.

and IL-4 expression in the spleen was performed in four animals in each group. Immunization tended to increase the expression of both cytokines (Table I).

Discussion

The present observations confirm previous studies demonstrating that immunization with oxidized LDL and apo B-100 peptide fragments inhibits atherosclerosis [18–22,26]. They also show that immunization induces a shift from Th1 to Th2 responses as determined by the expression of specific IgG2a and IgG1 antibodies.

Zhou and co-workers have demonstrated that induction of hypercholesterolemia in apo E0 mice results in a shift in autoimmune responses against oxidized LDL from Th1 to Th2 [24]. In that study it was found that apo E0 mice with moderate hypercholesterolemia (450 mg/dl), IgG against oxidized LDL was predominantly of IgG2a type. Similar observations were also made in the moderately hypercholesterolemic adjuvans control group (300 mg/dl) in the present study. At 23 weeks of age oxidized LDL IgG1 was increased 2-fold in hypercholesterolemic apo E0 mice as compared to chow-fed in the study by Zhou et al. [24]. In the present study, immunization with apo B fragments was associated with more than a 50-fold increase in specific IgG1. Although these results do not allow a direct comparison, they indicate that a Th2 response against

epitopes in oxidized LDL is induced more effectively by immunization than in response to hypercholesterolemia. Th2 responses have been reported to be the dominating response to immunization, when using alum as adjuvant [28]. However in our study, it was obvious that also sequence homology of the peptide used for immunization is important to get a strong specific IgG1 antibody response.

Several studies support the notion that the balance between Th1 and Th2 responses may affect the progression of atherosclerosis. The arterial inflammatory response associated with the development of atherosclerosis suggests involvement of primarily Th1 cells [12,29,30]. Th1 cells produce proinflammatory cytokines such as INF- γ , IL-2 and IL-12, all of which are expressed in human atherosclerotic plaques [12,30]. Mice that are INF- γ receptor deficient develop less atherosclerosis [29], whereas administration of IL-12 accelerates atherosclerosis [31]. Th2 cells produce IL-4, IL-5, IL-10 and IL-13 that promote antibody production and inhibit the expression of proinflammatory Th1 cytokines [12,30]. Increased atherosclerosis has been observed in IL-10 deficient mice [32], whereas IL-10 overexpression has the opposite effect [33]. In addition, MDA-LDL immunization of LDL receptor deficient mice was shown to induce a dominant Th2 immune response with a higher frequency of IL-5 secreting cells specific for MDA-LDL and decreased atherosclerosis [34]. Accordingly, induction of Th2 immune responses against oxidized

Table II. Cytokine mRNA expression in aortas of immunized apo E0 mice at the age of 25 weeks.

	MDA-P45 ($n = 7$)	MDA-P74 ($n = 9$)	MDA-P240 ($n = 10$)	Controls ($n = 7$)
TNF- α	8.86 \pm 9.16*	4.73 \pm 2.12*	1.92 \pm 1.21	2.02 \pm 2.41
IFN- γ	72.62 \pm 89.22*	2.85 \pm 2.49	2.59 \pm 1.33	5.63 \pm 5.52
IL-4	0.85 \pm 1.18*	0.02 \pm 0.01	0.02 \pm 0.01	0.03 \pm 0.02

Values are given as percent of the 18S rRNA expression. * $P < 0.05$ vs. controls.

LDL antigens by immunization with apo B-100 fragments is likely to inhibit atherosclerosis in mice.

We observed a decrease in plaque macrophage immunoreactivity following immunization supporting a downregulation of Th1 activity. However, there was no reduction of INF- γ expressing cells in the spleen or in atherosclerotic plaques. This is in contrast to the findings by Zhou et al. showing induction of a Th2 response against oxidized LDL in hypercholesterolemia that is associated with a decreased expression of INF- γ in the spleen and in atherosclerotic plaques in spite of a much less prominent expression of Th2-specific IgG [24]. The reason responsible for these inconsistencies remains to be clarified. The induction of Th2 immunity against oxidized LDL antigens following immunization and diet are likely to be different in many respects, such as for example the number of antigens and mechanisms involved in antigen presentation. Accordingly, in a study by Veillard et al. [35] it was found that inflammatory cells within lesions increased together with several inflammatory mediators in apo E deficient mice on a cholesterol-rich diet, and reached a maximum after 10 weeks of diet followed by decreasing expression levels, whereas plaque size further increased. This reduction was more distinct for Th2 cells and anti-inflammatory mediators, indicating that atherosclerosis might result from an imbalance between pro- and anti-inflammatory mediators. However, the observation in the present study that immunizations reduced plaque macrophage content would be compatible with a downregulation of Th1 cytokines, as well as the increased IL-4 expression. Taken together, this suggests that sacrificing the mice at an earlier time-point may result in an even more pronounced Th2 immune response.

Immunization with MDA-modified apo B-100 fragments induced an increase in IgG but did not markedly affect IgM levels. A similar antibody response has been observed following immunization with MDA-LDL and oxidized LDL. These findings indicate that atheroprotective immune responses involve IgG but not IgM. This is in accordance with a study showing an inverse relation between IgG directed to MDA-LDL and plaque size, following immunization with MDA-LDL [22]. However, IgM antibodies against oxidized LDL phospholipids were generated after immunization with *Streptococcus pneumoniae*, and associated with a reduction of atherosclerosis suggesting that some IgM may have a protective effect [36]. In addition, both IgG and IgM antibodies have been shown to inhibit uptake of oxidized LDL in cultured macrophages and both were also found to bind to apoptotic cells and inhibit their phagocytosis by macrophages [10,37]. Thus, the functional role of IgG and IgM against epitopes in oxidized LDL remains to be fully understood. Infusion of monoclonal IgG and IgM in atherosclerotic animals should help to clarify this issue. However, the findings

that infusion of human polyclonal immunoglobulins or human IgG1 directed to MDA-modified apo B-100 peptides inhibit atherosclerosis in apo E0 mice as well as studies demonstrating that B cell transfer reduces atherosclerosis in apo E0 mice and neointima formation in RAG-1 mice suggest that antibodies have protective effects [23,38–40].

A complicating factor in the present studies is the possible influence of species differences when human apo B-100 fragments are used to immunize mice. Mice LDL are different from human LDL in the respect that most particles carry apo B-48 rather than apo B-100 [41,42]. Two of the apo B-100 fragments used in the present study are located within the apo B-48 region of apo B-100 and the third beyond this region. The complete amino acid sequence of mouse apo B-100 shows about 80% homology with the human sequence. However, the finding that mouse monoclonal autoantibodies specifically bind to epitopes in human oxidized LDL and MDA-LDL suggest that sufficient homology exists in the immune response [43]. In contrast, using a peptide sequence for the immunization with no homology to mouse apo B-100, as P240 in this study, indicated that sequence homology is important for reduction of atherosclerosis.

We have previously studied the associations between immune responses to MDA apo B-100 fragments and cardiovascular disease in man [25]. Using a nested case-control design we found that IgM against peptide fragment P45 were increased in subjects that suffered an acute myocardial infarction within 5 years. IgM against peptide fragments P45 and P240 showed significant association with carotid intima media thickness as assessed by ultrasonography and were inversely associated with the concentration of oxidized LDL in plasma. In contrast, IgM against MDA apo B-100 fragment P74 was higher in healthy controls and showed no association with carotid intima media thickness or oxidized LDL in plasma. It remains to be fully understood how the clinical observations in man and the findings in immunized mice are related to each other. It appears contradicting that antibodies that increase with disease severity, at the same time can have a protective role. One possible explanation could be that protective immune responses are activated by and in proportion to the disease process.

Immunization with peptide fragment 45 resulted in increased total cholesterol and triglyceride levels. Similar findings have been made in studies of other atheroprotective apo B-100 fragments (Chyu et al., unpublished data, 2004). The mechanism responsible for this effect remains to be clarified, but the observation suggests that the atheroprotective effect of immunization does not depend on lowering of circulating lipids.

In summary, the present studies demonstrate that activation of atheroprotective immune responses against MDA apo B-100 fragments is associated with a shift from Th1 to Th2 specific antibody expression.

The inflammatory activity in plaques is decreased, but no anti-inflammatory effect is observed at the systemic level. Th2 responses have previously been shown by Cribbs et al. to inhibit autoimmune disorders [28], an important notion for future vaccination strategies.

Acknowledgements

This study was supported by grants from the Swedish Medical Research Council, the Swedish Heart–Lung foundation, the King Gustaf V. 80th Birthday foundation, the Bergqvist foundation, the Crafoord foundation, the Swedish Society of Medicine, the Royal Physiographic Society, the Malmö University Hospital foundation, the Lars Hierta foundation, the Magnus Bergvall foundation and the Lundström foundation.

References

- Steinberg D, Witztum J. Lipoproteins, lipoprotein oxidation and atherogenesis. In: Chien K, editor. Molecular basis of cardiovascular disease. Philadelphia: W.B. Saunders Co.; 1999. p 458–475.
- Glass CK, Witztum JL. Atherosclerosis. The road ahead. *Cell* 2001;104:503–516.
- Witztum JL, Berliner JA. Oxidized phospholipids and isoprostanes in atherosclerosis. *Curr Opin Lipidol* 1998;9:441–448.
- Pentikainen MO, Oorni K, Ala-Korpela M, Kovanen PT. Modified LDL—trigger of atherosclerosis and inflammation in the arterial intima. *J Intern Med* 2000;247:359–370.
- Frostegard J, Wu R, Giscombe R, Holm G, Lefvert AK, Nilsson J. Induction of T-cell activation by oxidized low density lipoprotein. *Arterioscler Thromb* 1992;12:461–467.
- Stemme S, Faber B, Holm J, Wiklund O, Witztum JL, Hansson GK. T lymphocytes from human atherosclerotic plaques recognize oxidized low density lipoprotein. *Proc Natl Acad Sci USA* 1995;92:3893–3897.
- Palinski W, Yla-Herttuala S, Rosenfeld ME, Butler SW, Socher SA, Parthasarathy S, et al. Antisera and monoclonal antibodies specific for epitopes generated during oxidative modification of low density lipoprotein. *Arteriosclerosis* 1990;10:325–335.
- Maggi E, Chiesa R, Melissano G, Castellano R, Astore D, Grossi A, et al. LDL oxidation in patients with severe carotid atherosclerosis. A study of *in vitro* and *in vivo* oxidation markers. *Arterioscler Thromb* 1994;14:1892–1899.
- Hulthe J, Wiklund O, Hurt-Camejo E, Bondjers G. Antibodies to oxidized LDL in relation to carotid atherosclerosis, cell adhesion molecules, and phospholipase A(2). *Arterioscler Thromb Vasc Biol* 2001;21:269–274.
- Chang MK, Bergmark C, Laurila A, Horkko S, Han KH, Friedman P, et al. Monoclonal antibodies against oxidized low-density lipoprotein bind to apoptotic cells and inhibit their phagocytosis by elicited macrophages: Evidence that oxidation-specific epitopes mediate macrophage recognition. *Proc Natl Acad Sci USA* 1999;96:6353–6358.
- Shaw PX, Horkko S, Chang MK, Curtiss LK, Palinski W, Silverman GJ, et al. Natural antibodies with the T15 idiotype may act in atherosclerosis, apoptotic clearance, and protective immunity [see comments]. *J Clin Investig* 2000;105:1731–1740.
- Binder CJ, Chang MK, Shaw PX, Miller YI, Hartvigsen K, Dewan A, et al. Innate and acquired immunity in atherogenesis. *Nat Med* 2002;8:1218–1226.
- Salonen JT, Yla-Herttuala S, Yamamoto R, Butler S, Korpela H, Salonen R, et al. Autoantibody against oxidized LDL and progression of carotid atherosclerosis [see comments]. *Lancet* 1992;339(8798):883–887.
- Virella G, Virella I, Leman RB, Pryor MB, Lopes-Virella MF. Anti-oxidized low-density lipoprotein antibodies in patients with coronary heart disease and normal healthy volunteers. *Int J Clin Lab Res* 1993;23:95–101.
- Puurunen M, Manttari M, Manninen V, Tenkanen L, Alfthan G, Ehnholm C, et al. Antibody against oxidized low-density lipoprotein predicting myocardial infarction [published erratum appears in *Arch Intern Med* Apr 24;155(8) (1995) 817]. *Arch Intern Med* 1994;154(22):2605–2609.
- Bergmark C, Wu R, de Faire U, Lefvert AK, Swedenborg J. Patients with early-onset peripheral vascular disease have increased levels of autoantibodies against oxidized LDL. *Arterioscler Thromb Vasc Biol* 1995;15:441–445.
- Palinski W, Tangirala RK, Miller E, Young SG, Witztum JL. Increased autoantibody titers against epitopes of oxidized LDL in LDL receptor-deficient mice with increased atherosclerosis. *Arterioscler Thromb Vasc Biol* 1995;15:1569–1576.
- Palinski W, Miller E, Witztum JL. Immunization of low density lipoprotein (LDL) receptor-deficient rabbits with homologous malondialdehyde-modified LDL reduces atherogenesis. *Proc Natl Acad Sci USA* 1995;92:821–825.
- Ameli S, Hultgardh-Nilsson A, Regnstrom J, Calara F, Yano J, Cercek B, et al. Effect of immunization with homologous LDL and oxidized LDL on early atherosclerosis in hypercholesterolemic rabbits. *Arterioscler Thromb Vasc Biol* 1996;16:1074–1079.
- Freigang S, Horkko S, Miller E, Witztum JL, Palinski W. Immunization of LDL receptor-deficient mice with homologous malondialdehyde-modified and native LDL reduces progression of atherosclerosis by mechanisms other than induction of high titers of antibodies to oxidative neoepitopes. *Arterioscler Thromb Vasc Biol* 1998;18:1972–1982.
- George J, Afek A, Gilburd B, Levkovitz H, Shaish A, Goldberg I, et al. Hyperimmunization of apo-E-deficient mice with homologous malondialdehyde low-density lipoprotein suppresses early atherogenesis. *Atherosclerosis* 1998;138:147–152.
- Zhou X, Caligiuri G, Hamsten A, Lefvert AK, Hansson GK. LDL immunization induces T-cell-dependent antibody formation and protection against atherosclerosis. *Arterioscler Thromb Vasc Biol* 2001;21:108–114.
- Caligiuri G, Nicoletti A, Poirier B, Hansson GK. Protective immunity against atherosclerosis carried by B cells of hypercholesterolemic mice. *J Clin Investig* 2002;109:745–753.
- Zhou X, Paulsson G, Stemme S, Hansson GK. Hypercholesterolemia is associated with a T helper (Th) 1/Th2 switch of the autoimmune response in atherosclerotic apo E-knockout mice. *J Clin Investig* 1998;101:1717–1725.
- Fredrikson GN, Hedblad B, Berglund G, Alm R, Ares M, Cercek B, et al. Identification of immune responses against aldehyde-modified peptide sequences in apoB associated with cardiovascular disease. *Arterioscler Thromb Vasc Biol* 2003;23:872–878.
- Fredrikson GN, Söderberg I, Lindholm M, Dimayuga P, Chyu K-Y, Shah PK, et al. Inhibition of atherosclerosis in apoE-null mice by immunization with apoB-100 peptide sequences. *Arterioscler Thromb Vasc Biol* 2003;23:879–884.
- Barth Jr., RJ, Bock SN, Mule JJ, Rosenberg SA. Unique murine tumor-associated antigens identified by tumor infiltrating lymphocytes. *J Immunol* 1990;144:1531–1537.
- Cribbs DH, Ghochikyan A, Vasilevko V, Tran M, Petrushina I, Sadzikava N, et al. Adjuvant-dependent modulation of Th1 and Th2 responses to immunization with beta-amyloid. *Int Immunol* 2003;15:505–514.

- [29] Gupta S, Pablo AM, Jiang X, Wang N, Tall AR, Schindler C. IFN-gamma potentiates atherosclerosis in ApoE knock-out mice. *J Clin Investig* 1997;99:2752–2761.
- [30] Hansson GK, Libby P, Schonbeck U, Yan ZQ. Innate and adaptive immunity in the pathogenesis of atherosclerosis. *Circ Res* 2002;91:281–291.
- [31] Lee TS, Yen HC, Pan CC, Chau LY. The role of interleukin 12 in the development of atherosclerosis in ApoE-deficient mice. *Arterioscler Thromb Vasc Biol* 1999;19:734–742.
- [32] Mallat Z, Besnard S, Duriez M, Deleuze V, Emmanuel F, Bureau MF, et al. Protective role of interleukin-10 in atherosclerosis. *Circ Res* 1999;85(8):e17–e24.
- [33] Pinderski LJ, Fischbein MP, Subbanagounder G, Fishbein MC, Kubo N, Cheroutre H, et al. Overexpression of interleukin-10 by activated T lymphocytes inhibits atherosclerosis in LDL receptor-deficient mice by altering lymphocyte and macrophage phenotypes. *Circ Res* 2002;90:1064–1071.
- [34] Binder CJ, Hartvigsen K, Chang MK, Miller M, Broide D, Palinski W, et al. IL-5 links adaptive and natural immunity specific for epitopes of oxidized LDL and protects from atherosclerosis. *J Clin Investig* 2004;114:427–437.
- [35] Veillard NR, Steffens S, Burger F, Pelli G, Mach F. Differential expression patterns of proinflammatory and antiinflammatory mediators during atherogenesis in mice. *Arterioscler Thromb Vasc Biol* 2004;24:2339–2344.
- [36] Binder CJ, Horkko S, Dewan A, Chang MK, Kieu EP, Goodyear CS, et al. Pneumococcal vaccination decreases atherosclerotic lesion formation: Molecular mimicry between *Streptococcus pneumoniae* and oxidized LDL. *Nat Med* 2003;9:736–743.
- [37] Shaw PX, Horkko S, Tsimikas S, Chang MK, Palinski W, Silverman GJ, et al. Human-derived anti-oxidized ldl autoantibody blocks uptake of oxidized ldl by macrophages and localizes to atherosclerotic lesions *in vivo*. *Arterioscler Thromb Vasc Biol* 2001;21:1333–1339.
- [38] Nicoletti A, Kaveri S, Caligiuri G, Bariety J, Hansson GK. Immunoglobulin treatment reduces atherosclerosis in apo E knockout mice. *J Clin Investig* 1998;102:910–918.
- [39] Dimayuga P, Cercek B, Oguchi S, Fredrikson GN, Yano J, Shah PK, et al. Inhibitory effect on arterial injury-induced neointimal formation by adoptive B-cell transfer in Rag-1 knockout mice. *Arterioscler Thromb Vasc Biol* 2002;22:644–649.
- [40] Schiopu A, Bengtsson J, Soderberg I, Janciauskiene S, Lindgren S, Ares MP, et al. Recombinant human antibodies against aldehyde-modified apolipoprotein B-100 peptide sequences inhibit atherosclerosis. *Circulation* 2004;110:2047–2052.
- [41] Higuchi K, Kitagawa K, Kogishi K, Takeda T. Developmental and age-related changes in apolipoprotein B mRNA editing in mice. *J Lipid Res* 1992;33:1753–1764.
- [42] Ishibashi S, Herz J, Maeda N, Goldstein JL, Brown MS. The two-receptor model of lipoprotein clearance: Tests of the hypothesis in “knockout” mice lacking the low density lipoprotein receptor, apolipoprotein E, or both proteins. *Proc Natl Acad Sci USA* 1994;91:4431–4435.
- [43] Palinski W, Horkko S, Miller E, Steinbrecher UP, Powell HC, Curtiss LK, et al. Cloning of monoclonal autoantibodies to epitopes of oxidized lipoproteins from apolipoprotein E-deficient mice. Demonstration of epitopes of oxidized low density lipoprotein in human plasma. *J Clin Investig* 1996;98:800–814.