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Antibodies against electronegative LDL inhibit atherosclerosis in LDLr^{-/-} mice

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In order to determine the effect of antibodies against electronegative low-density lipoprotein LDL(-) on atherogenesis, five groups of LDL low receptor-deficient (LDLr^{-/-}) mice (6 per group) were immunized with the following antibodies (100 µg each): mouse anti-LDL(-) monoclonal IgG2b, rabbit anti-LDL(-) polyclonal IgG or its Fab fragments and mouse irrelevant monoclonal IgG and non-immunized controls. Antibodies were administered intravenously one week before starting the hypercholesterolemic diet (1.25% cholesterol) and then every week for 21 days. The passive immunization with anti-LDL(-) monoclonal IgG2b, polyclonal antibody and its derived Fab significantly reduced the cross-sectional area of atherosclerotic lesions at the aortic root of LDLr^{-/-} mice (28.8 ± 9.7 , 67.3 ± 17.02 , 56.9 ± 8.02 µm² (mean ± SD), respectively) compared to control (124.9 ± 13.2 µm²). Vascular cell adhesion molecule-1 protein expression, quantified by the KS300 image-analyzing software, on endothelium and the number of macrophages in the intima was also decreased in aortas of mice treated with anti-LDL(-) monoclonal antibody (3.5 ± 0.70 per field x 10) compared to controls (21.5 ± 3.5 per field x 10). Furthermore, immunization with the monoclonal antibody decreased the concentration of LDL(-) in blood plasma (immunized: 1.0 ± 1.4 ; control: 20.5 ± 3.5 RLU), the amount of cholesterol oxides in plasma (immunized: 4.7 ± 2.7 ; control: 15.0 ± 2.0 pg COx/mg cholesterol) and liver (immunized: 2.3 ± 1.5 ; control: 30.0 ± 26.0 pg COx/mg cholesterol), and the hepatic content of lipid hydroperoxides (immunized: 0.30 ± 0.020 ; control: 0.38 ± 0.15 ng/mg protein). In conclusion, antibodies against electronegative LDL administered intravenously may play a protective role in atherosclerosis.

Key words: Minimally modified LDL; Passive immunization; Monoclonal antibody; Atherogenesis

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Introduction

Modification of low-density lipoprotein (LDL) plays a key role in atherosclerosis. A minimally modified circulating electronegative LDL subfraction, denoted LDL(-), is increased in hypercholesterolemia and diabetes and has been implicated in atherosclerosis progression (1,2). LDL(-) particles have lower antioxidant content, increased lipid peroxidation products, as well as conformational al-

terations of apoB (3,4). LDL(-) is cytotoxic to cultured endothelial cells (5), promotes increased leukocyte recruitment for inducing the release of IL-8 and monocyte chemotactic protein 1 (6), impairs angiogenesis (7), and has less affinity for the LDL receptor (3,8). Intravenous injection of immunoglobulins is used as a therapy for autoimmune and systemic inflammatory diseases (9,10). In contrast, it has been suggested that antibodies reactive to oxidized LDL (ox-LDL) may contribute to the pathogen-

esis of atherosclerosis (11). However, anti-ox-LDL IgM antibodies may protect against atherosclerosis possibly because they can scavenge the ox-LDL particles (12). We report here that the passive immunization with xenogeneic polyclonal antibodies, xenogeneic Fab fragments, and allogeneic monoclonal antibodies IgG reactive to LDL(-) significantly reduced atherosclerosis in LDLr^{-/-} mice.

Material and Methods

Polyclonal antibodies reactive to LDL(-)

New Zealand rabbits were immunized by intramuscular injection every 2 weeks for 3 months with 50 µg LDL(-) incorporated in Freund's complete adjuvant for the first injection and in incomplete Freund's adjuvant for subsequent injections. LDL(-) was purified by fast protein liquid chromatography (13). After purification, 1.0 mM EDTA, 5.0 mM phenylmethylsulfonyl fluoride, 10.0 µM benzamidine, 10.0 µg/mL aprotinin and 100.0 µM butylated hydroxytoluene were added to the LDL(-) preparation and was maintained at 4°C. The production of anti-LDL(-) polyclonal antibody was monitored by ELISA. Antibodies were purified from rabbit serum by affinity chromatography in a protein G column (Hi Trap Protein-G, Pharmacia, Biotech, USA) (14). ELISAs were carried out (15,16) using a SpectroCount analyzer (Canberra Company, USA). Plates (EIA/RIA, Costar, USA) were coated with 1 µg/mL purified native LDL and the anti-LDL(-) monoclonal antibody was added after previously blocking the microplate wells with defatted milk. The antibodies were cleaved by papain to obtain the Fab fragments (17). Fragmentation was confirmed by 10% SDS-PAGE on vertical slab gels according to Laemmli (18) with silver nitrate staining (data not shown).

Purification of monoclonal antibody

The IgG2b anti-LDL(-) monoclonal antibody (MAb 3D1036) was obtained in our laboratory as described (3). Briefly, the antibodies were obtained from the ascitic fluid induced in BALB/c mice (6 to 10 weeks old) injected with Pristane (Sigma, USA) before the inoculation of the 3D1036 hybridoma cells secreting the anti-LDL(-) MAb. Ten days after this inoculation, the MAb was purified from the ascitic fluid by affinity chromatography in a protein G column (Hi Trap Protein-G, Pharmacia, Biotech, Sweden). The MAb 3D1036 recognizes an epitope present in minimally modified LDL particles as previously reported by Damasceno et al. (2).

Passive immunization

Female C57BL/6 LDL receptor knockout mice (3 months old) were purchased from Jackson Laboratories

(USA). The following groups, with 6 animals each, were studied: group 1 (controls), treated with PBS; group 2, treated with 100 µg of an irrelevant MAb (IgG2b anti-18 kDa peptides of *Taenia crassiceps*); group 3, treated with rabbit IgG polyclonal antibody (100 µg) against LDL(-); group 4, treated with Fab fragments (100 µg) derived from the rabbit polyclonal antibody reactive to LDL(-); group 5, treated with IgG2b monoclonal antibody (100 µg) against LDL(-). Antibodies were administered intravenously once one week before starting the 1.25% cholesterol-enriched diet and then every week for 21 days.

All procedures were in accordance with recommendations of the Institutional Committee for Ethics in Animal Experimentation (CEE/IB, UNICAMP, protocol #521-1, Brazil), in agreement with the guidelines of the Brazilian College for Animal Experimentation.

Tissue preparation and histomorphometry of aortic root lesions

The heart was perfused with PBS and then with 10% PBS-buffered formaldehyde. The heart and aorta were excised and fixed in 10% formaldehyde for at least 2 days, and then embedded in 5, 10, and 25% gelatin and frozen in mounting medium (Tissue-Tek OTC compound, Sakura Finetek, USA). The aortic root in the heart was sectioned proximally to distally in 10 µm starting from the semilunar valves. The area of the atherosclerotic lesion was reported as the sum of the lesion in 6 equidistant sections (80 µm) from each other in an aortic root length of 400 µm. Results from 6 mice/group are reported as mean µm² ± SD. To quantify the cross-sectional area of the oil red O-stained lesions in the aortic root, processing and staining were carried out as described (19), using the Image Pro Plus software (version 3.0) for image analysis (Media Cybernetics, USA). All analyses were double-blind and carried out independently by two observers.

Immunohistochemical and histochemical analyses

To evaluate the vascular cell adhesion molecule-1 (VCAM-1), the remaining thoracic and abdominal aortas were mounted with Tissue-Tek OTC compound, frozen in isopentane and embedded in paraffin. VCAM-1 was detected with an anti-VCAM antibody (Santa Cruz Biotechnology, H-276, USA) (20). VCAM-1 staining was quantified using an image-analyzing software (KS300, Kontron, Germany). The histochemical analysis of the aorta was done in 5-µm thick tissue sections stained with hematoxylin/eosin. The number of foam cells was estimated as described (21).

Detection of LDL(-) in blood plasma and autoantibodies

At the end of the experiment, blood was collected from

the ocular-plexus and plasma was obtained by centrifugation. LDL(-) was determined by ELISA with Fab fragments derived from rabbit anti-LDL(-) polyclonal IgG as the capture antibody. A biotinylated anti-LDL(-) monoclonal antibody was added as secondary antibody and streptavidin-horseradish peroxidase conjugate was used for quantitation. The positive reaction was evaluated by chemiluminescence and the concentration of LDL(-) was determined using a standard curve made with purified LDL(-) (22).

Anti-LDL(-) autoantibodies were determined by direct ELISA. Plasma samples (50 μ L/well) previously diluted in PBS (1:50) were assayed. An anti-mouse IgG/horseradish peroxidase conjugate (Bio-Rad, USA) was used for detection and quantitation using standard procedures. The reaction was evaluated colorimetrically at 492 nm using a SpectroCount analyzer (Canberra Company). All experiments were carried out in triplicate.

Analysis of lipid peroxidation

Lipid hydroperoxides were extracted from the liver and the cholesterol oxides (COx) were extracted from both liver and plasma of LDLr-/- mice as described by Pereira et al. (23).

Statistical analysis

ANOVA and the Tukey-Kramer test were used to analyze the data statistically. All values are reported as mean $\pm$ SD. Statistical significance was considered to be $P < 0.05$.

Results

Atherosclerotic lesions

The passive immunization of LDLr-/- mice with the anti-LDL(-) monoclonal (IgG2b isotype) and IgG polyclonal (whole or its Fab fragments) antibodies decreased the cross-sectional area of atherosclerotic lesions at the aortic root compared with controls (Figure 1). Immunization with the anti-LDL(-) MAb did not change the total plasma cholesterol concentration compared to the control group (immunized: 46.6 ± 18.7 ; control: 60.5 ± 13.6 mg/mL total cholesterol). The protective effect of the anti-LDL(-) monoclonal IgG2b was greater than that of the polyclonal IgG and irrelevant monoclonal antibody for reducing both the cross-sectional area (Figure 1A) and the number of foam cells in the atherosclerotic lesions (Figure 1B). Figure 2 shows the oil red-stained sections of atherosclerotic lesions at the aortic root of mice immunized with the same antibodies as shown in Figure 1.

Expression of vascular cell adhesion molecule-1

Mice receiving the Fab fragments derived from the

rabbit anti-LDL(-) polyclonal antibody showed the largest decrease of VCAM-1 expression in aorta sections than those receiving the whole antibody and non-immunized control mice (Figure 3).

Concentration of free LDL(-) and autoantibodies

The passive immunization of LDLr-/- mice with anti-LDL(-) monoclonal IgG2b decreased the circulating levels of free LDL(-) and also increased free anti-LDL(-) IgG antibodies in relation to the non-immunized controls and irrelevant antibody (Figure 4A and B). In contrast, immunization with the anti-LDL(-) polyclonal IgG enhanced the levels of free LDL(-) and decreased the levels of free anti-LDL(-) IgG antibodies in blood plasma compared with the non-immunized controls. The administration of Fab fragments derived from the anti-LDL(-) polyclonal IgG antibody did not affect any of the parameters evaluated when compared with the control non-immunized mice.

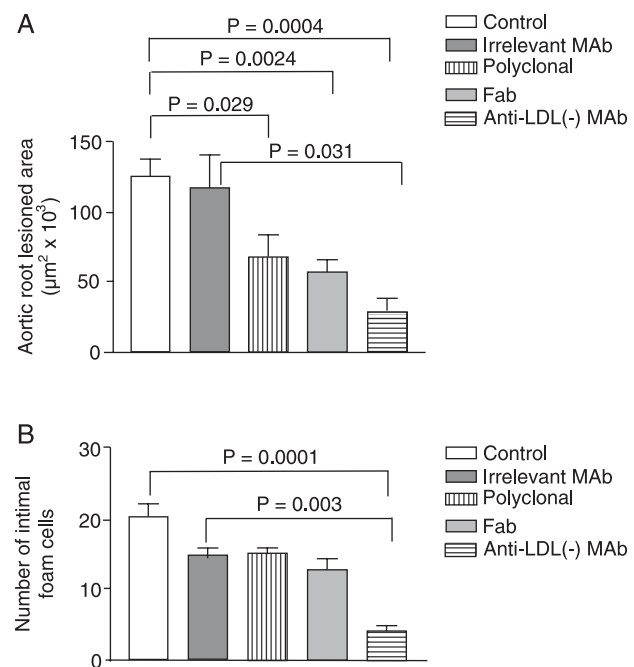


Figure 1. Atherosclerotic lesions of low-density lipoprotein receptor-deficient (LDLr-/-) mice treated with antibodies against LDL(-). **A**, Cross-sectional area of atherosclerotic lesions at the aortic root of LDLr-/- mice; **B**, number of foam cells in the subintimal layer of atherosclerotic lesions of LDLr-/- mice treated or not with anti-LDL(-) monoclonal antibody (MAb, 100 μ g), irrelevant Mab (100 μ g), rabbit polyclonal anti-LDL(-) antibody (100 μ g) and Fab fragments from rabbit anti-LDL(-) polyclonal antibody (100 μ g). $P < 0.05$, ANOVA followed by the Tukey-Kramer test.

Figure 2. Atherosclerotic lesions in aortic valve of low-density lipoprotein receptor-deficient (LDLr^{-/-}) mice. Aorta sections of **A**, control mice; **B**, mice treated with anti-LDL(-) polyclonal antibody (100 µg); **C**, mice treated with Fab fragments from anti-LDL(-) polyclonal antibody (100 µg); **D**, mice treated with anti-LDL(-) monoclonal antibody (100 µg). The sections were stained with oil red O and the arrows indicate the build-up of plaque within the aorta. Magnification: 4X and 40X.

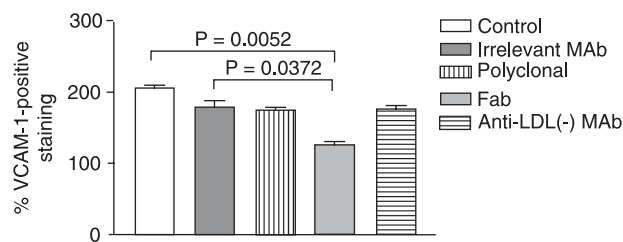
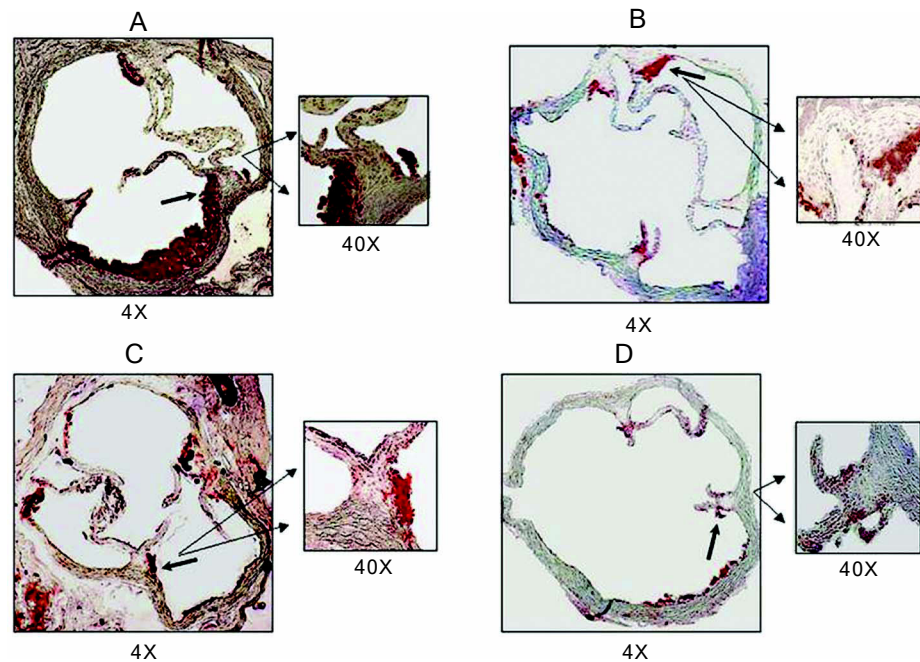


Figure 3. Immunohistochemistry staining of vascular cell adhesion molecule-1 (VCAM-1) in sections of the aorta of low-density lipoprotein receptor-deficient (LDLr^{-/-}) mice not treated or treated with irrelevant monoclonal antibody (MAb, 100 µg), anti-LDL(-) MAb (100 µg), rabbit anti-LDL(-) polyclonal antibody (100 µg) or Fab fragments from rabbit anti-LDL(-) polyclonal antibody (100 µg). Percent of VCAM-1-positive staining on aorta sections was evaluated using image-analyzing software (KS300, Kontron). $P < 0.05$, ANOVA followed by the Tukey-Kramer test.

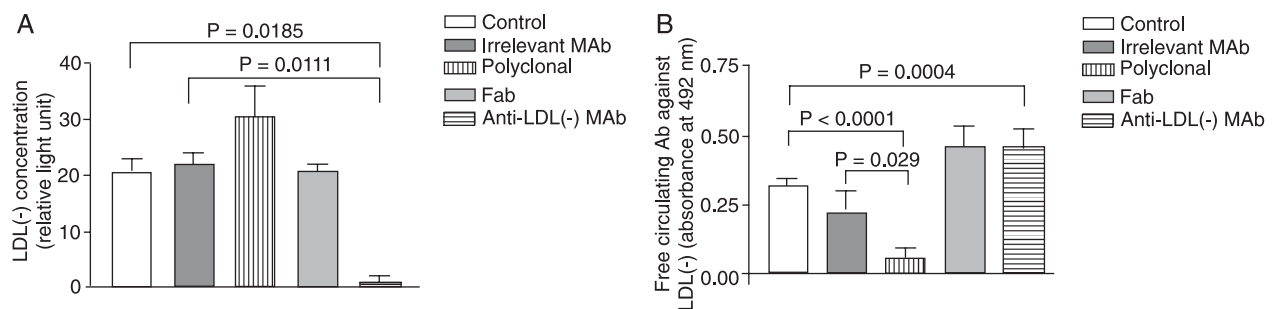


Figure 4. Determination of electronegative low-density lipoprotein [LDL(-)] and its autoantibodies in blood plasma of low-density lipoprotein receptor-deficient (LDLr^{-/-}) mice not treated or treated with irrelevant monoclonal antibody (MAb, 100 µg), anti-LDL(-) MAb (100 µg), rabbit anti-LDL(-) polyclonal antibody (100 µg) or Fab fragments from rabbit anti-LDL(-) polyclonal antibody (100 µg). **A**, Concentration of LDL(-). **B**, Levels of free circulating antibodies (Ab) reactive to LDL(-). $P < 0.05$, ANOVA followed by the Tukey-Kramer test.

Lipid peroxidation

Passive immunization of LDLr^{-/-} mice with the anti-LDL(-) IgG2b MAb reduced the concentrations of COx in plasma (immunized: 4.7 ± 2.7 ; irrelevant control 14.7 ± 2.1 ; control: 15.0 ± 2.0 pg COx/mg cholesterol) and liver by 70 and 92%, respectively (immunized: 2.3 ± 1.5 ; irrelevant control 31 ± 26 ; control: 30.0 ± 26.0 pg COx/mg cholesterol), as well as 21% of hepatic lipid hydroperoxides (immunized: 0.30 ± 0.020 ; irrelevant control: 0.40 ± 0.16 ; control: 0.38 ± 0.15 ng/mg protein) compared with non-immunized control mice.

Discussion

The most important finding of this study was that passive immunization of LDLr^{-/-} mice with monoclonal and polyclonal antibodies against LDL(-) decreases the cross-sectional area of lipid-stained atherosclerosis plaques in the aortic root. Our data showed that the monoclonal antibody (IgG2b) was more atheroprotective even when compared with the polyclonal antibody. This may be related to the recognition of the rabbit-derived polyclonal antibody as non-self by murine immune system.

LDLr^{-/-} mice treated with anti-LDL(-) monoclonal antibody had lower levels of circulating free LDL(-) than the non-immunized mice or mice immunized with irrelevant monoclonal antibody. Thus, considering that passive immunization with IgG2b anti-LDL(-) monoclonal antibody had a protective effect on atherosclerotic plaque development, it is possible that the decreased levels of free LDL(-) in blood plasma were due to the formation of immune complexes between LDL(-) and the monoclonal antibody injected in mice. We also observed an inverse relationship between circulating free LDL(-) and free anti-LDL(-) antibodies in mice treated with anti-LDL(-) monoclonal antibody. This high level of free IgG antibodies reactive to LDL(-) probably reflects endogenous autoantibodies plus the exogenous injected antibody since an anti-mouse IgG-conjugate was used in ELISA. This validated the passive immunization with this monoclonal antibody, since the mean half-life of mouse IgG is 14 days and a considerable amount of free anti-LDL(-) monoclonal antibody would be expected to remain in the circulation 7 days after the last injection. In contrast, for mice treated with rabbit anti-LDL(-) polyclonal antibodies, an immune response of these animals against the Fc portion of rabbit IgG could contribute to induce the generation of mouse antibodies against rabbit IgG-LDL(-) immune complexes. Thus, these high levels of antibodies could affect the detection of free anti-LDL(-) antibodies by ELISA in the plasma of these mice. The administration of

Fab fragments derived from the rabbit anti-LDL(-) polyclonal antibody did not affect the concentration of free autoantibodies reactive to LDL(-). This was probably because Fab fragments retain the ability to bind to LDL(-) epitopes while they lose the binding region to Fc of mouse immunoglobulins. Thus, the free mouse autoantibodies remain in circulation. The presence of anti-LDL(-) autoantibodies in non-immunized LDLr^{-/-} mice indicates that the endogenous humoral immune response was not atheroprotective since in these animals the atherosclerotic lesions were more accentuated compared with immunized mice. Another possible explanation is that the titer of endogenous antibodies was not enough and once 400 µg of additional anti-LDL(-) was added by adoptive transfer an atheroprotective effect was attained.

In vivo studies have suggested that macrophage death in early lesions decreases macrophage burden and slows lesion progression (24,25). The live macrophages may promote late lesional complications by secreting inflammatory mediators (26). In the present study, mice treated with anti-LDL(-) monoclonal IgG2b had less foam cells in the subintimal layer of atherosclerotic lesions compared with control mice. These observations are consistent with other studies showing that immunization with human IgG1 against malondialdehyde (MDA)-peptide sequences reduced atherosclerosis in apoE^{-/-} mice associated with a decreased number of macrophages in atherosclerotic plaques (27). Probably the monoclonal antibody anti-LDL(-) neutralized circulating LDL(-) and avoided its effects on endothelium including the release of monocyte chemotactic factors, the entrance of these cells into the artery wall and their differentiation to macrophages.

The removal of LDL(-) by anti-LDL(-) monoclonal IgG2b decreased the formation of lipid peroxidation products in plasma and liver of LDLr^{-/-} mice, suggesting an additional atheroprotective effect of this passive immunization. Moreover, the decreased concentration of cholesterol oxides in plasma of anti-LDL(-) monoclonal antibody-treated mice may also be a consequence of LDL(-) removal from blood circulation since these minimally modified particles have an increased content of lipid peroxidation products (5).

The expression of adhesion molecules in arterial endothelial cells occurs during the early phases of diet-induced atherogenesis (28,29). In the present study, VCAM-1 expression was reduced after treating LDLr^{-/-} mice with the Fab fragment derived from the anti-LDL(-) polyclonal antibody. One reason is that endothelial cells express Fc gamma receptors (30) and, thus, the absence of the Fc portion in the Fab fragment would not induce the activation of endothelial cells leading to decreased VCAM expression.

We have shown for the first time that antibodies against LDL(-) administered intravenously can play a protective role in atherosclerosis. Although the mechanisms involved in this atheroprotection have not yet been elucidated, our results point to the importance of immune responses against oxidized LDL in atherosclerosis and the possibility that the antibodies against modified LDL could be used to assess disease activity, as well as a therapeutic strategy for the prevention of atherosclerosis.

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