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Cholesterol-rich diet enhances peripheral blood mononuclear cell proliferation, vitamin E, and glutathione levels in rabbits

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The role of dietary cholesterol in the modulation of both cell-mediated immunity and oxidative status is still not completely understood. In this study the effect of high levels of dietary cholesterol on cell-mediated immunity and on antioxidant levels was investigated in rabbits. For 3 months two groups of five animals each were fed a standard diet that was either supplemented with 0.5% cholesterol or not supplemented. Vitamin E and glutathione concentrations were measured on plasma and on peripheral blood mononuclear cells (PBMC). Proliferative response through [^{3}H]-thymidine incorporation was measured on PBMC. The cholesterol rich diet increased the activation of immune cells and the levels of vitamin E and glutathione in both PBMC and plasma. The possible involvements of these two antioxidative components in regulating cholesterol-induced atherosclerosis and immune function are discussed. (J. Nutr. Biochem. 9:294–297, 1998) © *Elsevier Science Inc. 1998*

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Introduction

Previous experimental and epidemiological studies have shown that both hypercholesterolemia and oxidatively modified low density lipoprotein (LDL) are the most important risk factors in the pathogenesis of atherosclerosis.[1,2] Moreover, a chronic inflammatory fibroproliferative process has been suggested to be involved in the formation of atherosclerotic lesions. The increase of cytokines such as interleukin (IL) 2, IL-1, tumor necrosis factor alpha (TNFα), and transforming growth factor beta (TGFβ) released from blood cells has been also reported in atherosclerosis.[3] Some evidence indicates that cholesterol availability is a major determinant of the capacity of lymphocytes to proliferate[4] and a direct link between cholesterol level and T-cell activation, possibly by autoimmune responses to modified lipoproteins,[5] has been suggested. Recent data show that oxidized LDLs significantly inhibit lymphocyte proliferation in humans.[6] However, it has been shown that experimental atherosclerosis can be reversed by dietary vitamin E.[7]

Vitamin E could influence the immune system by its antioxidant function either by decreasing the concentration of reactive oxygen metabolites or by formation of arachidonic metabolites such as prostaglandin (PGE_2), because both processes have been shown to decrease immune responsiveness.[8,9] Recently, it has been suggested that glutathione (GSH) plays an important role in the function of lymphocytes, increasing IL-2 production.[10] The relationship between induced hypercholesterolemia and levels of anti-

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oxidative defense components of immune cells is still unclear. The goal of this study was to investigate the effect of feeding a diet rich in cholesterol on the in vitro activation of immune cells and on the regulation of vitamin E and GSH levels in rabbits.

Methods and materials

Male New Zealand white rabbits, obtained from Charles River (Italy), were matched by weight (2.5 kg) and housed in separate cages. After a 2 week period on a standard diet, the rabbits were divided randomly into two groups of five animals each. The first group was fed with standard rabbit chow and the second group received the same diet supplemented with 0.5% cholesterol (Sigma Chemical, Milano, Italy) for 3 months. The cholesterol diet was prepared every 3 days by dissolving cholesterol in diethyl ether and spraying the solution on the pellets. The vitamin E content in the standard rabbit chow was determined to be 40 mg/kg.

All procedures were approved by the Università della Tuscia Animal Studies Committee.

For the isolation of peripheral blood mononuclear cells (PBMC), blood samples were collected through the ear vein by heparinized vacuum tubes (Vacutainer, Becton Dickinson, CA).

Peripheral blood was diluted 1:2 with Hanks' balanced salt solution (Flow Laboratory, Scotland) in 50 ml centrifuge tubes. Histopaque-1083 (Sigma Chemical) 10 ml was layered under the diluted blood and centrifuged at 400 × g for 25 min. PBMC were removed from the interface, placed in 50 ml tubes, and washed twice in Hank's. The mononuclear cells were counted using a Neubauer Counter cell (Brand, West Germany) and diluted at optimal concentration in complete culture medium (see below).

Lymphocyte proliferation was measured by [^{3}H]-thymidine incorporation after stimulation with T-cell and B-cell mitogens [Pokeweed, (PWM) and phytohaemagglutinin (PHA)].

Triplicate cultures of PBMC in 96-well tissue culture plates (Costar, Cambridge, MA USA), at a concentration of 1.5 × 10^5 cells/well in culture medium RPMI 1640, containing 10% heat-inactivated fetal calf serum and 2% (v/v) antibiotics (10,000 U/ml penicillin, 10 mg/ml streptomycin), were treated with PWM (1:50, 1:500, 1:1000 dilutions, v/v) and PHA (1, 10, 100 μg/ml) and incubated at 37°C in 5% carbon dioxide (CO_2). After 2 days of incubation with PHA and 4 days with PWM, proliferative response was determined by adding [^{3}H]-thymidine to each culture well (0.5 μCi). After 18 h, the cultures were harvested on filter disks with a cell harvester. The filter disks were dried, transferred to scintillation vials, and counted on a liquid scintillation counter. PBMC proliferation activity is expressed as cpm of incorporated [^{3}H]-thymidine.[11]

Cholesterol concentration was determined in plasma and in lymphocytes after sonication using an enzymatic colorimetric test (Chol, Boehringer, Mannheim, Germany). Alfa-tocopherol was measured on lipid extracts by reverse phase high performance liquid chromatography (HPLC) with fluorescence detection according to Bieri et al.[12] To 100 μl of plasma or cells, the internal standard (200 ng α-tocopheryl acetate) was added followed by 200 μl of 100% ethanol. The sample was vortexed for 15 s, 1 ml of hexane with BHT was added (12.5 mg/L), and the mixture was vortexed for 60 s. The samples were centrifuged for 5 min at 1000 rpm. Following centrifugation, 800 μl of the hexane layer was removed and evaporated to dryness under a stream of N_2 and the residue was redissolved in 100 μl of ethanol. Finally, 100 μl samples were injected onto a C18 Supelco column. The solvent system used in the reverse phase HPLC method was 100% methanol at a flow rate of 1.5 ml/min. Peak was detected by fluorescence measurement at 292 nm after excitation at 340 nm.

Glutathione was measured by HPLC separation and fluorometric detection of the glutathione-orthophthalaldehyde (OPA) adduct, according to Neushwander-Tetri and Roll.[13] 100 μl of plasma or cells was mixed with 100 μl H_2O and 50 μl 0.1 M TRIS pH 8.5. After 30 min at 0°C, the proteins were precipitated by the addition of 750 μl 2.5% (w/v) 5-sulfosalicylic acid (SSA) and

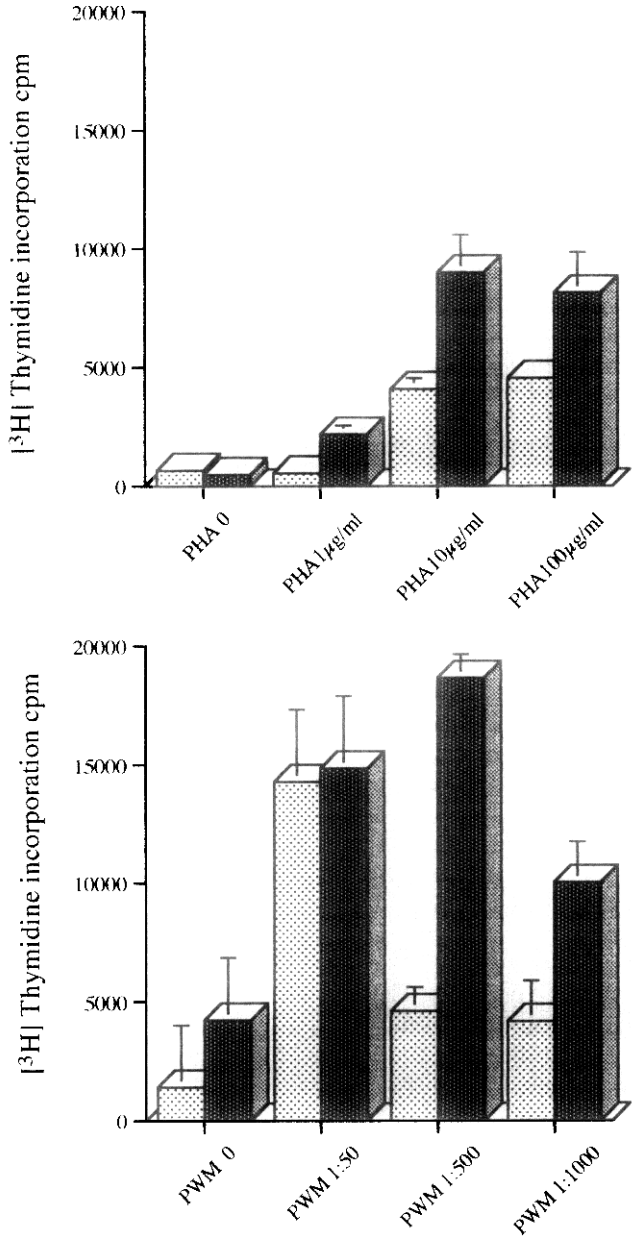

Figure 1 [^{3}H]-Thymidine incorporation of mitogen-stimulated peripheral blood mononuclear cells (PBMC) of rabbits fed a cholesterol-rich diet or a standard diet. Lymphocyte proliferation was measured by [^{3}H]-thymidine incorporation after stimulation with T-cell and B-cell mitogens [pokeweed (PWM) and phytohemagglutinin (PHA)]. Triplicate cultures of PBMC in 96-well tissue culture plates, at concentration of 1.5 × 10^5/well were treated with PWM (1:50, 1:500, 1:1000 dilutions, v/v) and solution PHA (1, 10, 100 mg/ml) and incubated at 37°C in 5% CO_2. After 2 days of incubation with PHA and 4 days with PWM, proliferative response was determined by adding [^{3}H]-thymidine to each culture well (0.5 μCi). After 18 h, the cultures were harvested on filter disks with a cell harvester. The data represent the values of one representative experiment, with S.D. of triplicate analysis (*light stippled* = control group; *dark stippled* = Cholesterol-fed group).

Table 1 Cholesterol, α-tocopherol, and glutathione (GSH) concentrations in plasma of rabbits fed a cholesterol-rich diet

Experimental group	Cholesterol (μmol/ml)	Vitamin E (nmol/ml)	GSH (nmol/ml)
Control	1.60 ± 0.13	11.6 ± 1.6	24.2 ± 3.0
Cholesterol-fed	30.07 ± 3.71	41.8 ± 4.6	46.6 ± 4.0

The values represent the n mean ± S.D. of five animals for each group. All differences between the two experimental groups are statistically significant, with $P < 0.001$ by one factor analysis of variance.

centrifugated at 13,000 × g at 5°C. The SSA supernatant was used directly for OPA derivatization. Derivatization was performed at room temperature by mixing 200 μl SSA supernatant with 200 μl of OPA solution. After 1 min the derivatized samples were neutralized and diluted by addition of 2 ml 100 mM sodium phosphate pH 7.00. This solution was chromathographed by injecting 100 μl into the column. Separation of glutathione adduct is achieved by isocratic elution over a reverse phase Whatman Partisil C18 Column with 7.5% methanol/92.5% 0.15 M sodium acetate pH 7.00 at a flow rate of 1.5 ml/min. The adduct is detected fluorometrically at 420 nm after excitation at 340 nm. The quantity of GSH in a sample was derived by comparing the derivative peak area to a standard curve generated by derivatizing known amounts of GSH.

Statistical analysis was performed using one-factor analysis of variance (ANOVA) and Scheffe's test for multiple comparison.

Results

During the 3 months of feeding the cholesterol-rich diet, no significant difference in food intake and weight gain with rabbits fed the control diet was found (data not shown).

Proliferative responses of PBMCs activated in vitro with various concentrations of PWM and PHA in one representative experiment (out of five) are reported in *Figure 1*. The values for the cholesterol-fed rabbit cells showed a consistent increase in their proliferative response in comparison to the control cells. The most pronounced enhancing effect was observed at 1:500 dilution of PWM and at concentration of 10 μg/ml for PHA. Similar trends were observed in the other four experiments.

The values of plasma cholesterol, α-tocopherol, and glutathione concentrations of the two animal groups are reported in *Table 1*. The cholesterol-fed group showed a much higher level of total cholesterol than the control group (30.07 vs. 1.60 μmol/ml; $P < 0.001$). In addition, the plasma levels of vitamin E and GSH of rabbits fed the cholesterol-rich diet were significantly higher than the control values (41.8 vs. 11.6 nmol/ml and 46.6 vs. 24.2 nmol/ml plasma, respectively; $P < 0.001$).

A significant increase in total cholesterol (49.7 vs. 20.7 nmol/10^6 cells; $P < 0.001$), vitamin E, and GSH concentrations (0.086 vs. 0.050 nmol/10^6 and 1.49 vs. 0.60 nmol/10^6, respectively; $P < 0.001$) was also found in PBMC of the high-cholesterol group (*Table 2*).

Discussion

The role of the immune system in the development of atherosclerosis has not been elucidated.[1] In particular, it is not clear whether high levels of dietary cholesterol affect cell-mediated immunity.[5,14,15] In this study, we found that a cholesterol-rich diet increased the activation of immune cells and the levels of vitamin E and GSH in both plasma and PBMC. These results raise some questions regarding the mechanisms by which the immune system may be involved in atherogenesis. The high concentrations of α-tocopherol and glutathione found in plasma and lymphocytes of cholesterol-fed rabbits suggest that these molecules may play important roles in enhancing the proliferative response of the lymphocyte activated with polyclonal activator (PHA and PWM).

Previous experiments have shown a stimulatory effect of extracellular α-tocopherol on lymphocyte activation, especially in conditions of immune depression such as advanced age or thermal stress.[8,16] The results reported here confirm this finding, suggesting that the increase of plasma level of vitamin E, probably due to the increase of LDLs and very low density lipoproteins in response to a high cholesterol diet, may provide the vitamin E necessary for human lymphocyte proliferation. The increase of vitamin E in lymphocytes corresponded to a concomitant increase of GSH. A similar increase of GSH concentration also has been found in rat hepatocytes and in pig heart after vitamin E supplementation.[17,18] Since a glutathione increase, as found after GSH supplementation, has been associated with an increase in IL-2 production in cytotoxic T cells,[10] the enhancement in GSH concentration may indicate an improvement in PBMC function.

Table 2 Cholesterol, vitamin E, and glutathione levels in peripheral blood mononuclear cells of rabbits fed a cholesterol-rich diet

Experimental group	Cholesterol (nmol/10^6 cells)	Vitamin E (nmol/10^6 cells)	GSH (nmol/10^6 cells)
Control	20.7 ± 0.5	0.050 ± 0.005	0.60 ± 0.03
Cholesterol-fed	49.1 ± 2.1	0.086 ± 0.006	1.49 ± 0.11

The values represent the mean ± S.D. of five animals for each group. All differences between the two experimental groups are statistically significant, with $P < 0.001$ by one factor analysis of variance.
GSH, growth stimulating hormone.

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