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Cholesterol oxidation on fluorocarbon emulsion surface leads to the formation of 7-peroxycholesterol

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Formation of biologically active oxidized derivatives of cholesterol as a result of its oxidation on the surface of fluorocarbon emulsions was studied. A single product of cholesterol oxidation, 7-peroxycholesterol, was found. It was shown that 7-peroxycholesterol and its derivative 7-keto-cholesterol inhibit the rosette formation between human T-lymphocytes and sheep erythrocytes. These substances exert a strong cytostatic action on the growth of procaryotic and eucaryotic cell cultures. Thus, oxidative modification of blood plasma components on the surface of fluorocarbon emulsion particles with the formation of highly active compounds must be taken into account when using the fluorocarbon emulsions in medicine.

Fluorocarbon emulsion; Cholesterol; Oxidized cholesterol

1. INTRODUCTION

A biological activity of fluorocarbon emulsions is a serious problem in view of their wide use in medicine. This class of chemical substances is traditionally considered chemically inert. However, this is not always right. For example, induction of liver monoxygenase systems by fluorocarbon was shown [1]. It is known that the phase separation border provides favorable conditions for some chemical reactions. It is also necessary to take into account that these emulsions are, as a rule, saturated with oxygen [2]. When fluorocarbon emulsion is infused i.v. to animals, an intense adsorption of blood plasma proteins and lipids on the surface of the emulsion particles takes place [3]. One can expect that cholesterol and other lipids of the blood plasma lipoproteins can also be adsorbed on the surface of emulsion particles. Oxidation processes can modify these substances into chemicals with high biological activity [4,5]. Oxy- and keto-derivatives of cholesterol (for C_6, C_7, C_{16}, C_{20}, C_{22}, C_{24}, C_{25} carbon atoms) were shown to have the highest activity.

In our work, the possibility of cholesterol oxidative modification on the surface of fluorocarbon emulsions in the presence of dissolved oxygen was investigated. As a model we used fluorocarbon emulsion stabilized with phospholipid/cholesterol mixture.

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2. MATERIALS AND METHODS

Perfluorodecalin (PFD) and perfluoro-*n*-methylcyclohexylpiperidine (PFMCP) were obtained from the Institute of Element Organic Chemicals of the Academy of Sciences of the USSR. Egg phospholipids (Kharkov Plant of Bacterial Preparations, USSR), soy bean phosphatidylcholine (Nattermann, FRG), dicethylphosphate (Serva, FRG) were used. Cholesterol (Sigma, USA) was twice recrystallized from hot ethanol and its purity was tested by thin layer chromatography. All solvents of analytical purity were used after distillation. Tris(hydroxymethyl)aminomethane (Reanal, Hungary) was used as a buffer without purification.

Fluorocarbon emulsion contained 10% by volume PFD/PFMCP mixture (2:1, v/v) in distilled water. Mixture of egg phospholipids/cholesterol (final concentration 2.5% by weight) was used as an emulgator. Ethanol solutions (5% concentration) of egg phospholipids and cholesterol were mixed in ratio phospholipids/cholesterol 4:1 by weight. Ethanol was removed by evaporation with the argon stream at 60°C. The dry lipid mixture was suspended in distilled water by shaking. The final concentration of lipids was 4%. Lipid suspension was mixed with the fluorocarbon phase and the emulsion was prepared with high pressure extrusion homogenizer 'Donor-1' (Special Constructor Bureau, Academy of Sciences of USSR, Chernogolovka). Emulsion was obtained under the pressure of 400 atmospheres at 26°C during 90 min and its volume was 300 ml. The mean value of emulsion particles diameter measured by nanosizer 'Coulter model H4' (Coultronics, France) was 230 ± 54 nm.

Cholesterol oxidation products were analyzed by thin layer chromatography (TLC) method using 'Silufol' plates (Cavalier, Czechoslovakia) and solvent system chloroform/methanol/water (75:15:1 by volume). The plates were developed with 10% solution of sulfuric acid or 1% solution of dihydrochloride *N,N,N',N'*-tetramethyl phenylendiamine and were scanned with a densitometer (model N 1039, Helena Laboratories, USA) for the estimation of 7-peroxycholesterol output. Chromato-mass analysis was made with the gas-chromatograph Hitachi 8H (Japan).

For testing the cytostatic action of oxidized derivatives of cholesterol, obtained from fluorocarbon emulsion, we investigated

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their influence on the growth of procaryotic and eucaryotic cell cultures [6].

The Chinese hamster cells B11-diiFAF28 were cultured in Eagle's growth medium containing 10% of bovine serum. After 3–4 days of culturing, cells were suspended in the growth medium and sowed into Petri dishes (~100 cells per dish). After 2 h of culturing the medium was replaced with Eagle's medium containing lipid preparations (1 mg lipids per ml). The corresponding quantity of media which had been used for lipid preparations was added to the control dishes. After 2.5 h of cell incubation at 37°C in 5% CO_2 atmosphere the medium was replaced with standard growth medium. Cells were then incubated during 8 days at 37°C (5% CO_2). Obtained colonies were fixed with 70% ethanol and stained with 0.1% Methylene blue solution. In each dish we registered the number of colonies and their dimensions which correspond with the number of cells in colonies.

The *Acholeplasma laidlawii* cells were cultured in a modified Edward medium containing 0.5% of glucose, 2% of bovine serum and penicillin (100000 U/ml). A 24-h-old culture (stationary growth phase) was resowed in a fresh culture medium in a 1:100 ratio, and liposomes containing cholesterol and its oxidized derivatives were added. At fixed time periods samples were taken and their optical density was measured at 640 nm. Soy bean phosphatidylcholine, cholesterol and dicethylphosphate were used for liposomes prepared with the modified method [7]. The lipid mixture (phosphatidylcholine/cholesterol/dicethylphosphate 1:1:0.3 by weight) was dissolved in chloroform:methanol (2:1 v/v) mixture and dried by the rotor evaporator for 40 min. The aqueous medium containing 0.9% NaCl, 5 mM Tris-HCl, pH 7.4 was added to the dry lipid preparation and the sample was shaken for 60 min at 37°C. Suspension was sonified at 10 min by ultrasonic dispergator 'Sonic-300' (Fisher, USA) with intensive cooling. Then the suspension was centrifuged for 30 min at 20000 × *g* and the supernatant was removed and filtrated through a filter (Nucleopore, USA) with pore diameter 0.2 μm. Liposomes containing oxidized cholesterol products were obtained in a similar way but 25 wt% of cholesterol were replaced by the oxidized forms. The TLC analysis showed that the control liposomes (with cholesterol only) did not contain oxidized products.

The action of cholesterol oxidative products on the activity of immunocompetent cells was tested by the method of T-lymphocyte rosette formation with sheep erythrocytes [8]. Oxycholesterols were incorporated in lipid micelle preparations. Lipid micelles were formed from the mixtures phosphatidylcholine/triolein/oxidized cholesterol (or cholesterol) in the mole ratio 1:1:1. Lipid mixture was dispersed in the media containing 0.9% NaCl, 5 mM Tris-HCl, pH 7.4 by an ultrasonic treatment for 10 min under cooling. Lipid micelles suspensions (0.5 ml, 10 mg lipids/ml) were added to 5 ml of donor blood. The final concentration of oxidized cholesterol was about 0.2 mg/ml. After incubation at 37°C for 1 h the preparations were remixed and after the following 15 min top part of the sample was used for the rosette formation reaction.

3. RESULTS AND DISCUSSION

The lipid oxidation in fluorocarbon emulsions was carried out by oxygen bubbling through the emulsions during 10 min two times per day and incubation at 37°C for 5 days. Emulsions were centrifuged at 25000 × *g* for 30 min. Sediments were treated with 20 ml of diethyl ether. An ether phase was separated and dried with the rotor evaporator under the vacuum. Phospholipids were precipitated by cold acetone and sterol solution was separated from precipitate with the Buchner funnel and then concentrated with the rotor evaporator.

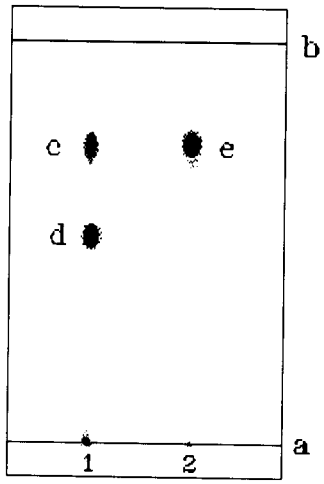

Fig. 1. TLC analysis of cholesterol oxidation products. 1, Experimental sample; 2, cholesterol standard; a, start line; b, solvent front line; c, e, cholesterol spots; d, 7-peroxycholesterol spot.

The chemical composition of the resulting solutions was analyzed by TLC method (Fig. 1). The plates developing with sulfuric acid show a blue spot (at 65°C, R_f = 0.54) and a bright red spot (at 70°C, R_f = 0.72). The blue color of the spot shows that it contains either 7-peroxycholesterol or 7-hydroxycholesterol [8]. When the plate is stained with dihydrochloride-*N,N,N',N'*-tetramethylphenylenediamine solution (a reagent for peroxide substances) a violet spot (R_f = 0.54) appears that shows the presence of 7-peroxycholesterol.

The structure of the cholesterol oxidation product was tested by chromato-mass analysis which has confirmed the TLC data. Thus we can conclude that 7-peroxycholesterol was found as a product of cholesterol oxidation reaction on the surface of fluorocarbon emulsion. The output of 7-peroxycholesterol was about 60% of the initial cholesterol content (calculated from quantitative TLC

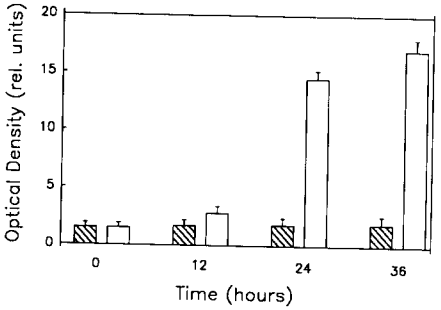

Fig. 2. Effect of 7-ketocholesterol containing liposomes on the growth of *Acholeplasma laidlawii* cells culture. Abscissa, culture growth period; ordinate, optical density of cells suspension, measured at 640 nm; open columns, control (buffer); hatched columns, 7-ketocholesterol containing liposomes. Results are means ± SD.

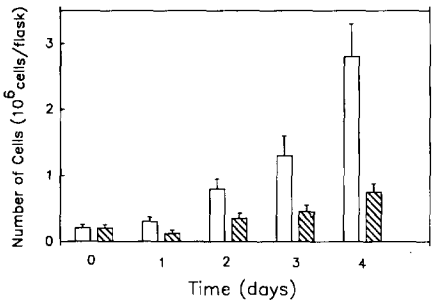

Fig. 3. Effect of 7-ketocholesterol containing liposomes on the growth of Chinese hamster B11-diiFAF28 cell culture. Abscissa, culture growth period; ordinate, number of cells (10^6 cells/flask); open columns, control (buffer); hatched columns, 7-ketocholesterol containing liposomes. Results are means ± SD.

Table I

Action of oxycholesterol containing micelles on the rosettes formation by human T-lymphocytes

Added preparation	Number of rosettes per 100 lymphocytes	Inhibition of rosette formation (%)
Buffer	44 ± 2	0
PC:Chol:TG	42 ± 2	3 ± 5
PC:7-OOH-Chol:TG	24 ± 2	46 ± 4
PC:7-OH-Chol:TG	32 ± 1	27 ± 3
PC:7-Keto-Chol:TG	40 ± 1	8 ± 3

Results are presented as $\bar{x}$ ± SE. PC, phosphatidylcholine; Chol, cholesterol; TG, triglyceride; 7-OOH-Chol, 7-peroxycholesterol; 7-OH-Chol, 7-oxycholesterol; 7-Keto-Chol, 7-ketocholesterol. The final concentration of oxidized cholesterol was about 0.2 mg/ml

data). It is known that 7-peroxycholesterol is an initial product of cholesterol oxidation pathway [9] that can be transformed to some other biological active cholesterol derivatives. We have obtained 7-ketocholesterol and 7-hydroxycholesterol from 7-peroxycholesterol by its thermal dehydration and borohydride reduction, respectively [10].

Figs 2 and 3 show the effects of liposomes containing cholesterol oxidation products on the growth of procaryotic *A. laidlawii* and eucaryotic Chinese hamster B11-diiFAF28 cells. Strong inhibition effects of oxidized cholesterol on the cell growth were observed for both types of cells.

Biological activity of obtained cholesterol C_7 oxidation products was also tested by their inhibition of rosette formation between human T-lymphocytes and sheep erythrocytes. Immunosuppressive effect of cholesterol oxides is shown in Table I. Oxidative derivatives of cholesterol were introduced in lipid micelles. The significant inhibition of the rosette formation (up to 46% of control) indicates the possibility of some negative effects of perfluorocarbon emulsion on a T-lymphocyte system in an organism when using for blood substitution.

The following conclusions can be made from our investigations: (i) the obtained results show the effective oxidation processes on the perfluorocarbon emulsion surface giving the cholesterol products oxidized at C_7 position. (ii) These C_7 oxidized products have a high immunosuppressive effect on T-lymphocytes. (iii) These facts must be taken into account when perfluorocarbon emulsions are used in practical medicine.

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