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EFFECTS OF AN ANTIMITOTIC AGENT (CYCLOPHOSPHAMIDE) ON PLASMA LIPOPROTEINS

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Abstract—A cyclophosphamide injection to male New Zealand white rabbits induced a pronounced hypertriglyceridemia and a hypercholesterolemia whose concentration was maximal at 16 hr. Different doses were studied.

In this hyperlipemia significant changes in plasma lipoprotein fractions appeared: the very low density lipoproteins increased and the high density lipoproteins decreased. Lipid composition showed that HDL cholesterol was very low comparatively to a high VLDL cholesterol.

The apoprotein composition of VLDL from treated rabbits was studied and compared to that of normal rabbits. After electrophoresis in urea/polyacrylamide gels, two new apoproteins which resembled those observed in irradiated rabbits appeared. The molecular weight of these proteins was about 10,000, and they focused into three bands with isoelectric points of 6.72, 6.42 and 6.10.

Total lipoprotein lipase activity in treated rabbits decreased; it was very low with 32.5 mg/kg. This lipolytic activity remains to be studied after separation of hepatic triacylglycerol lipase and lipoprotein lipase activities by chromatography.

Numerous studies deal with the action of ionizing radiations on lipid metabolism, membrane or tissue lipid compounds or circulating lipoproteins. They show that after exposure to lethal levels of whole body ionizing radiation a hyperlipidemia occurs in rabbits and other species [1–4]. This hyperlipidemia was characterized by a hypertriglyceridemia due to an inhibition of lipoprotein lipase and to modifications of the apoprotein composition of VLDL and HDL. On the contrary, studies on the pharmacobiological effects of antimitotic agents on lipid metabolism are scarce and do not concern circulating lipoproteins or plasma lipid compounds [5–8]. Few studies were carried out on the membrane fluidity and cholesterol in thymus and spleen cells from mice treated with immunomodulatory drugs [9]. This is the reason why we have tried to evaluate the radiomimetic drug effects on plasma lipoproteins. Among these drugs, cyclophosphamide is frequently used in chemotherapy. It is a nitrogen mustard which presents a cytotoxic action and interferes with normal mitosis and cell division in all rapidly proliferating tissues [10]. Some authors have investigated cyclophosphamide action on lipid plasma of rat [11], but this animal presented lipoprotein metabolism aspects different from human: in particular the clearance of "remnants" and the mechanism of cholesterol ester exchange were quite different from human metabolism [12, 13]. Besides, the results obtained in the rabbit after a provoked hypercholesterolemia were comparable to those from human pathology [14, 15]. Therefore, we have carried out experiments on lipoprotein modifications in cyclophosphamide-treated rabbits. We have studied the dose–activity relationship, together with the effect of the injected-drug dose as a function of time.

MATERIALS AND METHODS

Animals. Male New Zealand white rabbits weighing approximately 2–2.5 kg were used. They received a control diet consisting of commercial rabbit chow (purchased from U.A.R., France) for 10 days. After this period, all the animals were fasted 15 hr before treatment. Treated rabbits received only one cyclophosphamide injection (Endoxan Asta 500) in the ear marginal vein. Different doses were injected: 8.25, 16.5, 32.5, 65 mg/kg. The lethal dose for a rabbit being 130 mg/kg [16], we never passed 65 mg/kg to avoid the 100 mg/kg hepatotoxic dose. At this maximal dose, the transaminase levels were normal in our experiments. No food was given to the animals after treatment. The same animals were punctured after cyclophosphamide injection, at various times: 0 hr, 3 hr, 6 hr, 16 hr, 24 hr and 48 hr. The time 0 hr was taken just before cyclophosphamide injection; thus each rabbit was its own control. Control animals were also fasted in the same conditions. All animals had access to water. Blood was drawn by ear vein puncture or intracardiac puncture into EDTA (final concentration 1 mg/ml) at different hours after cyclophosphamide injection. Plasma was isolated by centrifugation at 1000 *g* for 15 min.

Analytical procedures. Total and esterified cholesterol concentrations were determined by enzymatic method [17] in plasma and in lipoprotein fractions. Triglycerides were measured enzymatically [18] and phospholipids were determined by the procedure of Takayama *et al.* [19]. HDL cholesterol was measured according to Grove [20] by selective lipoprotein precipitation with Na phosphotungstate and Mg^{2+}. Lipidograms were performed on large pore polyacrylamide gels (Lipofilms Sebia, France).

Lipoprotein preparation. Lipoprotein fractionation was performed in a Beckman L8–70 ultracentrifuge using a 50 Ti rotor at 4°. Fractions of the very low density lipoproteins (VLDL $d < 1.006$ g/ml) were isolated by centrifugation at 105,000 *g* (rav) for 18 hr, after removing chylomicrons by centrifugation at 15,000 *g* for 30 min. The VLDL were recentrifuged for purification at 105,000 *g* for 18 hr with a NaCl solution of density 1.006 g/ml (10 ml for 1 ml VLDL). The pure VLDL were stocked at −20°.

Total delipidation of lipoproteins. The lyophilized lipoproteins were delipidated by extraction with cold ethanol–diethyl ether according to the method of Brown *et al.* [21]. The apolipoproteins were dissolved in a 0.2 M Tris–HCl buffer, pH 8.2, containing 6 M urea and left at 4° [22]. This buffer did not allow the solubilization of the apolipoprotein B.

Polyacrylamide gel electrophoresis. The delipidated protein moieties were subjected to electrophoresis in 11.5% polyacrylamide gels at pH 8.9 in the presence of 6 M urea [23] or in 10% polyacrylamide gels at pH 8.8 in the presence of 0.1% sodium dodecyl sulfate [24]. The urea gels were fixed in 10% trichloracetic acid for 30 min, then in 10% acetic acid for 30 min. The apoproteins were stained for 10 min with a mixture containing an equal volume of 0.2% Coomassie brilliant blue in methanol/water/acetic acid, 40:53:7 (v/v/v) and 1% amidoschwarz in water/acetic acid, 90:10 (v/v). For electrophoresis in SDS, the apoproteins were reduced with 5% β-mercaptoethanol in boiling water for 2 min. These gels were fixed and stained with 0.2% Coomassie brilliant blue in methanol and acetic acid for 2 hr. The identification of gel bands was obtained by comparison of our data with those found in irradiated rabbits [4].

Analytical isoelectric focusing. Analytical isoelectric focusing was performed in 7.5% polyacrylamide gels containing 6.8 M urea and 2% ampholine, pH 4–7 [22]. It was carried out in a Shandon apparatus for 1 hr at 200 V and 18 hr at 400 V [25]. The gels were stained with 2% Coomassie brilliant blue in H_2SO_4 2N.

Lipolytic activity determination. Post-heparin blood samples were collected exactly 10 min after heparin injection (175 I.U./kg body weight). The total lipolytic activity (hepatic triacyl glycerol lipase and lipoprotein lipase) was measured using a sonicated emulsion of tri[9,10-^{3}H]oleoylglycerol, in glycerol stabilized with phosphatidylcholine [26]. One hundred μl of this substrate was incubated for 15 min at 37° with 100 μl of post-heparin serum. The assay was terminated by addition of 3.25 ml methanol/chloroform/heptan, 1.41:1.25:1 (v/v/v) [27]. The labelled free fatty acids released were extracted with 1.05 ml of 0.1 M sodium carbonate/borate buffer and radioactivity was counted. One mU of enzyme activity was defined as the amount of enzyme in 1 ml serum which releases 1 nmole fatty acid per min at 37°.

RESULTS

1. *Effect of cyclophosphamide injection on plasma lipid levels.* The plasma triglyceride concentration of treated rabbits increased with the dose and until 16 hr which was the maximal level (7.6 mmol/l) (Fig. 1). These results are greatly significant (for 65 mg/kg, N = 11, P < 0.001; for 35 mg/kg, N = 7, P < 0.001). Figure 2 represented the relation existing between the cyclophosphamide dose and the level of triglycerides. For 16.5 mg/kg, the triglyceride level was three-fold the concentration of control rabbits. This dose was interesting because it corresponded to the usual dose injected in human. The effect of cyclophosphamide was also observed at one slighter dose (8.25 mg/kg). Phospholipid levels showed a pronounced increased at 16 hr. The phospholipid of the treated group increased about 120% above the level of the control group phospholipid. Cholesterol levels also increased in the serum of treated rabbits. This effect proceeded more slowly. The highest concentration was observed at 24 hr and concerned also free cholesterol.

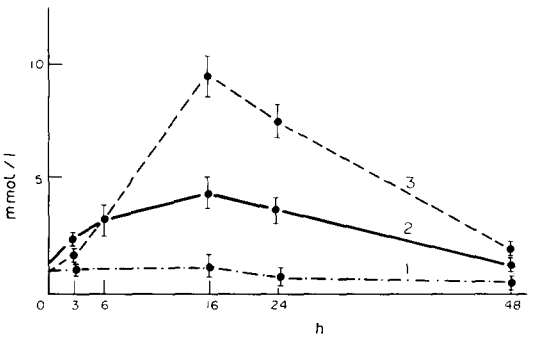

Fig. 1. Effect of cyclophosphamide on plasma triglyceride level. 1, Control rabbit plasma; 2, treated rabbit plasma, 32.5 mg/kg (N = 11, P < 0.001); 3, treated rabbit plasma 65 mg/kg (N = 7, P < 0.001).

2. *Effects of cyclophosphamide injection on lipoproteins.* Plasma lipoprotein patterns of treated rabbits were altered. Lipofilms showed the relative amounts of different lipoproteins in rabbit plasma (Fig. 3). When the cyclophosphamide dose increased, the VLDL were accumulated for 16 hr after the injection and LDL and HDL decreased. Lipid composition of these VLDL and HDL in

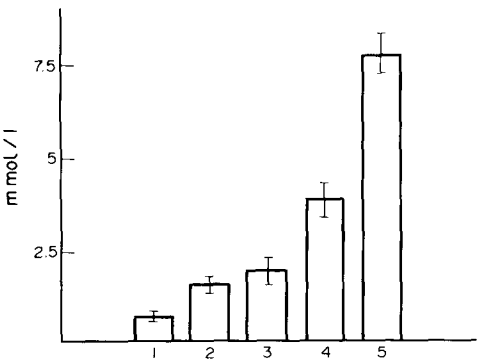

Fig. 2. Cyclophosphamide dose–triglyceride level relationship. 1, Control rabbit plasma; 2, treated rabbit plasma, 8.25 mg/kg; 3, treated rabbit plasma, 16.5 mg/kg; 4, treated rabbit plasma, 32.5 mg/kg; 5, treated rabbit plasma, 65 mg/kg.

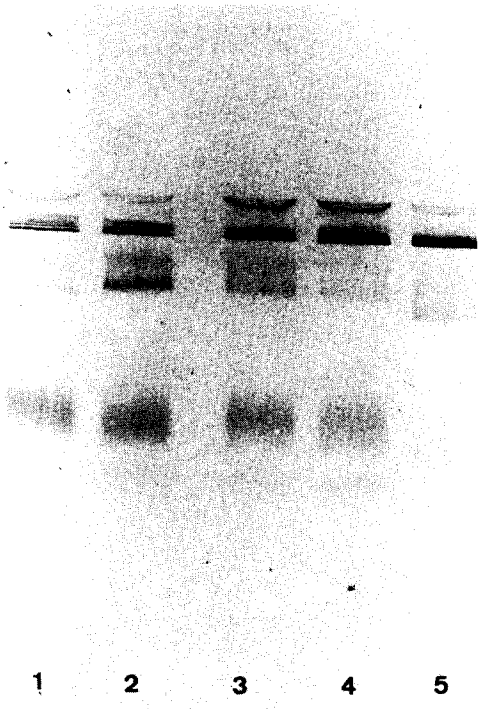

Fig. 3. Lipidograms of rabbit plasma on large pore polyacrylamide gel (Lipofilm). 1, Control rabbit; 2, treated rabbit, 8.25 mg/kg; 3, treated rabbit, 16.5 mg/kg; 4, treated rabbit, 32.5 mg/kg; 5, treated rabbit, 65 mg/kg.

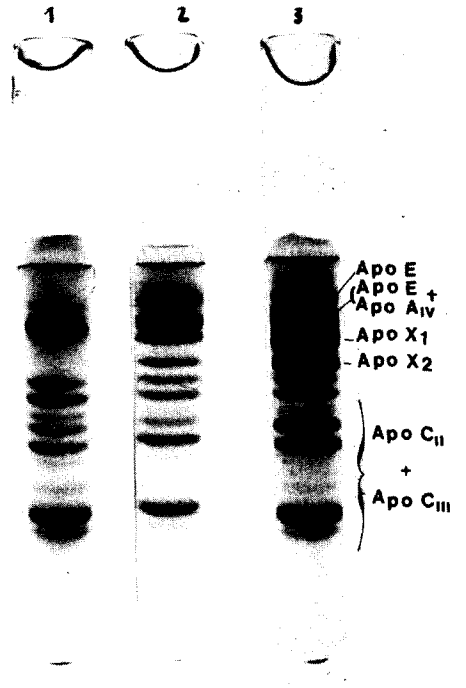

Fig. 5. Electrophoretic patterns of rabbit plasma apolipoproteins in 11.5% polyacrylamide gels at pH 8.9 containing 6 M urea. 1, Control rabbit apo-VLDL; 2, treated rabbit apo-VLDL, 32.5 mg/kg; 3, treated rabbit apo-VLDL, 65 mg/kg.

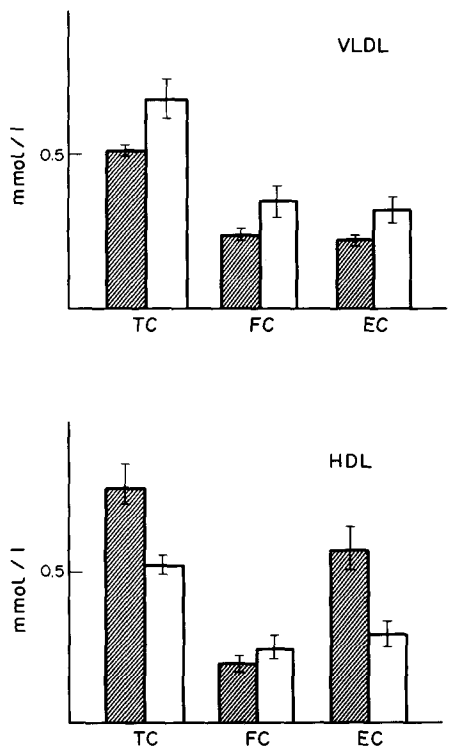

Fig. 4. Effect of cyclophosphamide on VLDL and HDL cholesterol level. ▨ Control rabbit; □ treated rabbit, 65 mg/kg; TC, total cholesterol; FC, free cholesterol; EC, esterified cholesterol.

treated rabbits showed that HDL cholesterol was very low compared to a high VLDL cholesterol. This increase of VLDL cholesterol was due to an increase of both esterified and free cholesterol (Fig. 4). On the other hand, HDL esterified cholesterol markedly decreased while the amount of HDL free cholesterol was unchanged.

3. *Effects of cyclophosphamide injection on apolipoproteins of VLDL.* Upon disc electrophoresis in polyacrylamide gels containing urea, the apolipoproteins of VLDL from normal rabbits gave several bands. In treated rabbits, two other major components appeared (Fig. 5). These new apoproteins were called X_1 and X_2. X_1 and X_2 were in high proportion in treated rabbit plasma VLDL at 65 mg/kg (Fig. 5, pattern 3). Polyacrylamide gels containing SDS were performed on rabbit apo-VLDL (Fig. 6) and showed the increase of band with an apparent molecular weight about 10,000 (Fig. 6, pattern 7). This electrophoresis showed also the existence of an apolipoprotein with an apparent molecular weight of about 43,000 which corresponded to apo A-IV. Polyacrylamide gel isoelectrofocusing patterns of treated rabbit VLDL differed also from those of the corresponding control group by the existence, in a high proportion, of three proteins with isoelectric points of 6.10, 6.42 and 6.72 (Fig. 7).

4. *Effect of cyclophosphamide injection on post-heparin plasma lipoprotein lipase activities.* The activity of total lipoprotein lipase after cyclophosphamide injection reached 30% and 80% of the control value respectively for 32.5 mg/kg and

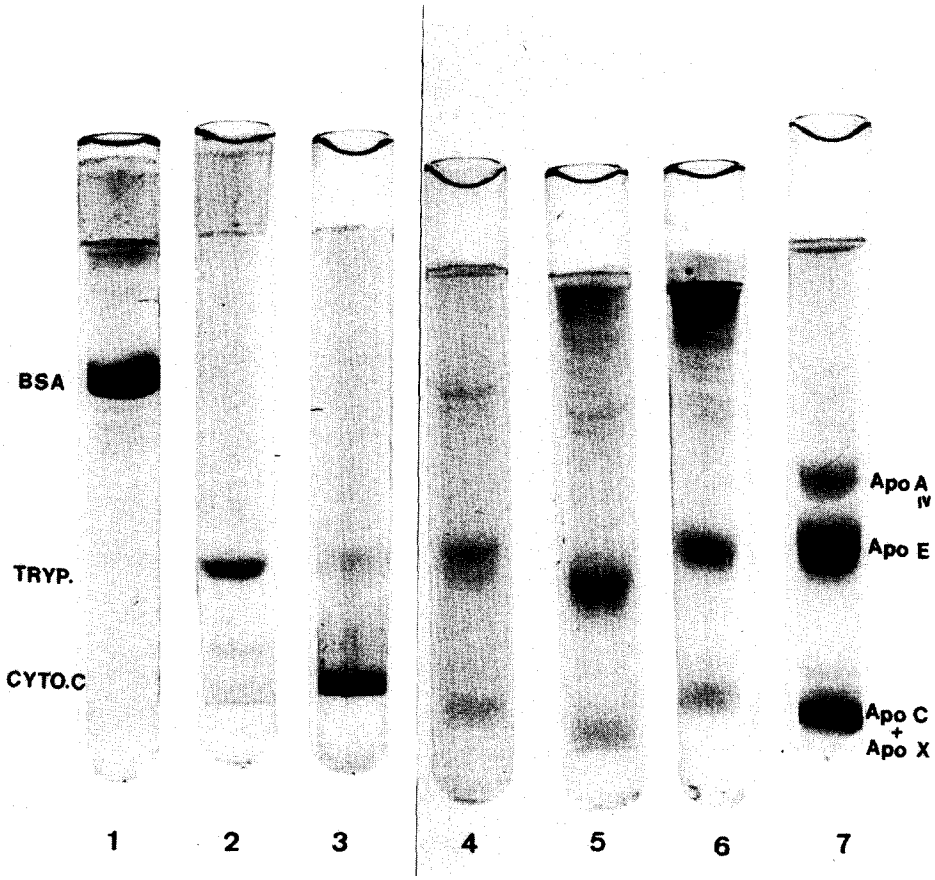

Fig. 6. Electrophoretic patterns of rabbit plasma apolipoproteins in 10% polyacrylamide gels (pH 8.8) containing 0.1% sodium dodecyl sulfate. 1, Bovine serum albumin; 2, trypsin; 3, cytochrom C; 4, control rabbit apo-VLDL; 5, treated rabbit apo-VLDL, 16.5 mg/kg; 6, treated rabbit apo-VLDL, 32.5 mg/kg; 7, treated rabbit apo-VLDL, 65 mg/kg.

16.5 mg/kg. A minimal value was obtained for 65 mg/kg. Other experiments will be performed to determine which enzymes (hepatic triacyglycerol lipase or lipoprotein lipase) are inhibited. The comparison of the patterns in Fig. 2 and Fig. 8 demonstrated that this total lipoprotein lipase activity was the lowest when triglyceride level was the highest.

DISCUSSION

Cyclophosphamide injection induces in rabbit a hypertriglyceridemia whose concentration was maximal at 16 hr and provokes modifications of lipoproteins; VLDL increase and LDL and HDL decrease (see Fig. 3). Triglyceride, cholesterol and phospholipid levels increase with the dose. For 16.5 mg/kg, the triglycerides already increase. This dose is the normal one injected in human cancer therapy. Our results show clearly that a total lipoprotein lipase post-heparin activity diminishes when the triglyceride level increases. This is a factor in the development of hypertriglyeridemia and in the VLDL accumulation after cyclophosphamide treatment in the rabbit. We have observed an inverse correlation between HDL esterified cholesterol and VLDL level in the two groups of animals. This relationship may be due to an important transfer of esterified cholesterol between HDL and VLDL [28]. This transfer would be dependent on the donor lipoprotein or acceptor lipoprotein relative concentration. The transfer activity would be higher in treated rabbits because these animals belong to a group which presents an important transfer activity [13]. Further investigations are necessary to confirm this hypothesis.

VLDL of treated rabbits are rich in new apolipoproteins which have a low molecular weight and relatively high isoelectric points (6.72, 6.42, 6.10). These apoproteins called X_1 and X_2 are similar to those observed in irradiation rabbits [4] and also to those presented in plasma HDL from diabetic and nephrotic subjects [29] and in patients receiving a perfusion of glucose [30]. The high concentration of these apolipoproteins in treated rabbit plasma VLDL might be an important factor in the development of hypertriglyceridemia. The nature of these apolipoproteins remains to be determined and their action on lipoprotein lipase enzymes will be studied in following experiments.

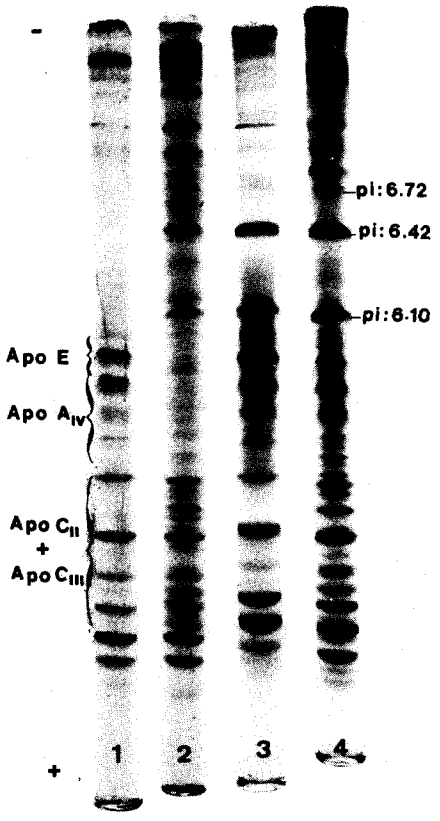

Fig. 7. Analytical isoelectric focusing in urea/polyacrylamide gels of rabbit apolipoproteins (in pH gradient 4–7). 1, Control rabbit apo-VLDL; 2, treated rabbit apo-VLDL, 16.5 mg/kg; 3, treated rabbit apo-VLDL, 32.5 mg/kg; 4, treated rabbit apo-VLDL, 65 mg/kg.

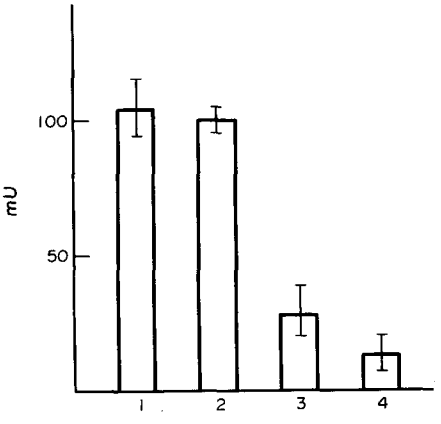

Fig. 8. Activity of total lipoprotein lipase post-heparin after cyclophosphamide treatment (1 mU of enzyme activity was defined as the amount of the enzyme in 1 ml serum which releases 1 nmol fatty acid per min at 37°). 1, Control rabbit; 2, treated rabbit 16.5 mg/kg; 3, treated rabbit 32.5 mg/kg; 4, treated rabbit 65 mg/kg.

Looking at these results, we think that cyclophosphamide has an action on plasma lipids and lipoproteins similar to that of ionizing radiations. Moreover, a study performed to clarify the role of serum lipoproteins in endotoxin-poisoned animals showed similar results [31]. It is suggested that hypertriglyceridemia, HDL decrease level, VLDL increase level and LPL inhibition were a general reaction against certain poisons and cytotoxic drugs.

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