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Research Article

Noninvasive Assessment of Lipid Disposition in Treated and Untreated Atherosclerotic Rabbits

Laura E. DeForge,[1] Susan W. Schwendner,[1] Mark R. DeGalan,[1,3] Daniel S. McConnell,[2] and Raymond E. Counsell[1,2,4]

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In an effort to visualize whole body cholesteryl ester (CE) deposition using the nuclear medicine imaging technique of gamma camera scintigraphy, ^{125}I-cholesteryl iopanoate (^{125}I-CI), a nonhydrolyzable CE analogue, was used as a marker for CE deposition in atherosclerotic New Zealand white rabbits. Groups of animals were fed either a cholesterol-enriched diet (2%, w/w) or the same diet supplemented with the hypolipidemic drugs colestipol (1%, w/w) and/or clofibrate (0.3%, w/w). Injections of ^{125}I-CI were administered biweekly. At the end of 15 weeks, animals were scintigraphically scanned and sacrificed for tissue analysis. The results demonstrated that while drug treatment had no significant effect on plasma lipid levels, it substantially lessened atherosclerotic involvement in the thoracic-abdominal aorta. These differences in aortic lipid accumulation were reflected in the whole-body scans which showed a reduction in tissue accumulation of ^{125}I-CI in the drug-treated groups. Gamma camera scintigraphy thus represents a rapid means of visualizing tissue CE accumulation which could facilitate the evaluation of lipid-lowering drug efficacy and possible antiatherosclerotic effect.

KEY WORDS: colestipol; clofibrate; noninvasive imaging; atherosclerosis; rabbit; nonhydrolyzable cholesteryl ester.

INTRODUCTION

Hypercholesteremia is a primary risk factor for atherosclerosis, which can lead to coronary heart disease and cerebrovascular insufficiency. In an effort to reduce the morbidity and mortality associated with the development of advanced lesions, several different classes of lipid-lowering drugs have been developed. These drugs have been shown to reduce the plasma levels of lipids and lipoproteins through one or more of a variety of mechanisms (1). In addition, a number of other agents such as estrogen (2), chlorpromazine (3), calcium antagonists (4,5), β-blockers (6), and antiinflammatory agents (7) have been shown to be beneficial in reducing atherogenesis in animal models without significantly affecting plasma lipid levels.

Because of the complexity of actions of various drugs, drug combinations, and agents of unknown potential in reducing atherogenesis, a simple means of evaluating an effect on whole body cholesterol disposition would be valuable in assessing drug efficacy. One potential approach under investigation in our laboratory involves the use of gamma camera scintigraphy, a nuclear medicine imaging technique, in the extracorporeal detection of radioactivity in animals injected with a radiolabeled cholesteryl ester (CE) derivative. This procedure necessitates the use of a CE derivative radiolabeled with a gamma-emitting isotope such as ^{125}I, ^{123}I, ^{131}I, or ^{99m}Tc. ^{125}I-Cholesteryl iopanoate (^{125}I-CI), a CE analogue that has been used in several studies in our laboratory, is well suited for studies of this nature because the ester linkage is resistant to hydrolysis (8,9), thus allowing the compound to accumulate in tissues once taken up. Moreover, as compared to other more commonly used methods of assessing the tissue deposition of radioactivity, scintigraphic imaging permits the acquisition of images showing the whole-body distribution of radioactivity within a relatively short period of time. In addition, sacrifice of the animal is not necessary, an important consideration when using larger, more expensive animals.

Using ^{125}I-CI as a marker for CE, the dual purpose of this study was to determine whether the hypolipidemic drugs colestipol and clofibrate could slow the progression of atherosclerosis in cholesterol-fed New Zealand white rabbits and, more importantly, to determine whether differences in whole-body accumulation of ^{125}I-CI could be detected in the treated and untreated groups using gamma camera scintigraphy.

[1] Department of Pharmacology, University of Michigan, Ann Arbor, Michigan 48109.
[2] Interdepartmental Program in Medicinal Chemistry, University of Michigan, Ann Arbor, Michigan 48109.
[3] Present address: University of Miami School of Medicine, Miami, Florida 33101.
[4] To whom correspondence should be addressed at M6322 Medical Science Building I, Department of Pharmacology, University of Michigan Medical School, Ann Arbor, Michigan 48109-0626.

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MATERIALS AND METHODS

Preparation of ^{125}I-Cholesteryl Iopanoate

^{125}I-CI was synthesized and radioiodinated as previously described (8) and had an average specific activity of 0.19 Ci/mmol. Radiochemical purity was checked by thin-layer chromatography (TLC) using Eastman silica gel chromatographic sheets developed in a hexane/ethyl acetate (5:2) solvent system. The TLC strips were then scanned with a Berthold 6000 radiochromatogram scanner. The compound was formulated for administration to animals in a Tween-20/physiological saline vehicle with the Tween constituting <3% of the total volume. Radiochemical purity of the formulated compound was assessed prior to injection as described above.

Study Design

Female New Zealand white rabbits (Shankin's Rabbitry, Warren, MI), weighing 2.6 ± 0.3 kg (mean ± SD) at the beginning of the study, were divided into five treatment groups. One group was fed normal rabbit chow (N; n = 3), and the other four groups (n = 6 in each group to start) were fed chow containing 2% cholesterol (Purina Test Diets, Richmond, IN). Of these animals, one group remained untreated (C; n = 5 at the completion of the study), and the others received diets supplemented with colestipol [CP; 1% (w/w) in diet; n = 5 upon completion], clofibrate [CF; 0.3% (w/w) in diet; n = 3 upon completion], or a combination of the two drugs (CPCF; n = 6 upon completion). Five animals were euthanized due to broken backs which occurred during handling (n = 2), ear infections (n = 2), and pneumonia (n = 1). The colestipol was obtained from the Upjohn Company (Kalamazoo, MI), and the clofibrate was made from commercially available clofibric acid by esterification with ethanol according to the reported procedure (10). The rabbits were given 100 g of the designated diet daily over a 15-week period, and its consumption was monitored.

Beginning 1 week after the start of the diet regimens, animals were injected twice weekly via the marginal ear vein with 10 μCi of ^{125}I-CI (solubilized in saline as described above) in a volume of approximately 0.2 ml. The last injection was given 13 days prior to the end of the study to allow the ^{125}I-CI to be cleared from the blood.

Fifteen weeks after the start of the study, animals were anesthetized with intramuscular injections of xylazine (8 mg/kg) and ketamine (50 mg/kg) and scintigraphically scanned using a gamma camera (Ohio Nuclear Mobile Camera) with a high-sensitivity collimator. Animals were placed in a prone position with the camera centered over the back such that liver radioactivity was visible in the top portion of the resulting scan. Acquisition time for all ^{125}I-CI scans was 30 min. A few animals were also injected with ^{99m}Tc-sulfur colloid in order to visualize the bone marrow. Animals were then sacrificed by exsanguination via cardiac puncture under sodium pentobarbital anesthesia, and blood and tissues (including aorta, adrenal, liver, thyroid, bile, and gall bladder) were removed for analysis.

Tissue Analysis

Blood was collected into Vacutainer tubes (Becton-Dickinson, Rutherford, NJ) containing ethylenediaminetetraacetic acid (EDTA) and centrifuged at low speed to obtain plasma. Using enzyme assay kits, the plasma was analyzed for total cholesterol (CooperBiomedical, Diagnostic Division, Freehold, NJ) and triglyceride (Sigma Diagnostics, St. Louis, MO).

In addition, the aortas (from the arch to the femoral bifurcation) were removed, rinsed in saline, and dissected free of fat and connective tissue. The arteries were then opened longitudinally and covered with clear plastic wrap, and the perimeter, lesions, and ostia were traced onto plastic sheets. The area involved in lesions was subsequently determined by planimetry. The aortas were cut into 1-cm segments and counted for radioactivity in a Searle 1185 gamma counter (counting efficiency approximately 87%). These sections were then homogenized, and lipid extractions (11) were performed on the pooled aortic sections constituting the arch (the first 2 cm of the vessel) and the thoracic-abdominal aorta of each animal. The total and free cholesterol contents of these extracts were determined using a fluorometric assay (12).

In order to ascertain the stability of the ^{125}I-CI, total lipid was extracted from samples of plasma, liver, and adrenal into chloroform–methanol (2:1) (11). The percentage radioactivity recovered in the organic phase was calculated, and the percentage of this material which comigrated with a ^{125}I-CI standard was determined by TLC analysis in a hexane/ethyl acetate (5:2) solvent system as described above.

RESULTS

The weights of the animals as well as the daily chow consumption of each animal were monitored throughout the study. Although the weights of all groups of animals were similar at the outset, the animals in the cholesterol-fed groups were heavier than the N-group animals at the end of the study [3.4 ± 0.2 kg versus 3.0 ± 0.2 kg, respectively (mean ± SD)]. The chow consumption was not significantly different among any of the groups and averaged 98 g/day/rabbit.

The plasma cholesterol and triglyceride levels were markedly elevated in all animals receiving the cholesterol-supplemented diets (Fig. 1). Triglyceride levels were two to three times that of group N, and the cholesterol levels were 25- to 30-fold increased. As compared to group C, however, the drug treatments did not significantly alter these parameters.

The atherosclerotic involvement of the aortas was assessed by measuring the percentage surface area involved in lesions, the cholesteryl ester content of the artery, and the aortic accumulation of radioactivity [expressed as percentage injected dose (adjusted for decay) per gram of tissue multiplied by the kilogram weight of the animal]. The results are separated into values for the arch (Fig. 2) and the remainder of the aorta, the thoracic and abdominal regions (Fig. 3). This division was made since the first 2 cm of the aorta was observed to be almost uniformly atherosclerotic in all the cholesterol-fed rabbits, a finding consistent with the greater permeability of this region of the vessel to blood-borne substances such as Evans blue dye, albumin, and cholesterol (13). As compared to group N, the arch of all cho-

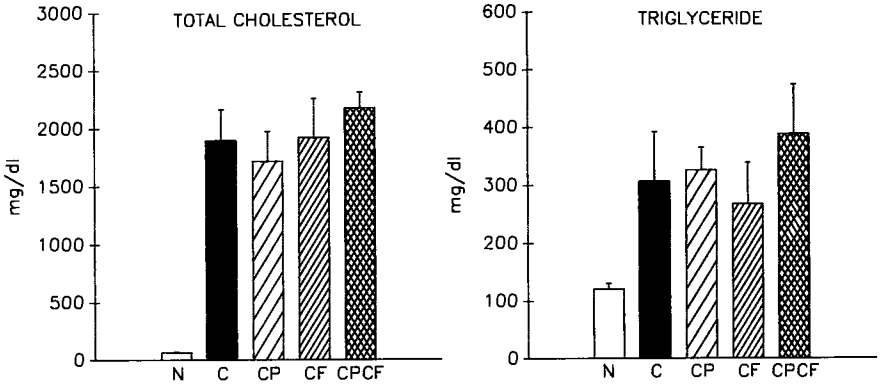

Fig. 1. Plasma total cholesterol and triglyceride levels of rabbits after 15 weeks on the following diets: N, normal rabbit chow (n = 3); C, rabbit chow plus 2% (w/w) cholesterol (n = 5); CP, 2% cholesterol chow supplemented with 1% (w/w) colestipol (n = 5); CF, 2% cholesterol chow supplemented with 0.3% (w/w) clofibrate (n = 3); and CPCF, 2% cholesterol chow supplemented with both colestipol (1%, w/w) and clofibrate (0.3%, w/w) (n = 6). The values (mean ± SE) were determined using enzymatic assay kits.

lesterol-fed groups showed significant involvement in atherosclerosis as demonstrated by all three parameters measured; none of the values for the drug-treated groups were significantly different from those of group C (Fig. 2). In the thoracic and abdominal regions of the aorta, however, drug treatment substantially reduced the severity of atherosclerosis as compared to group C. The decrease was of a similar magnitude in all three drug-treated groups, with a 56–61% decrease in surface area involvement, a 63–73% decrease in accumulation of cholesteryl ester, and a 51–62% decrease in radioactivity content. These differences attained statistical significance in several cases (Fig. 3).

The ability of the aortic accumulation of ^{125}I-CI to provide an indication of the severity of atherosclerosis is shown in Fig. 4. Here the radioactivity content of the thoracic-abdominal aorta (expressed as % kg dose/g) is plotted against both the percentage lesioned surface area and the thoracic-abdominal cholesteryl ester content. The correlation between these two sets of variables was highly significant [P < 0.01 (14)], with r = 0.82 and 0.84 for radioactivity versus surface area involvement and radioactivity versus cholesteryl ester content, respectively.

In order to assess the stability of ^{125}I-CI in various tissues, lipid extractions were performed on adrenal, liver, and plasma samples from each animal, with essentially complete recovery of tissue radioactivity. The percentage of this radioactivity recovered in the organic phase was 88.0 ± 1.4, 85.4 ± 2.4, and 74.4 ± 2.5% for adrenal, liver, and plasma, respectively (mean ± SE for all groups). TLC analysis of this material demonstrated that the percent comigrating with a ^{125}I-CI standard was 80.8 ± 3.1, 86.7 ± 2.6, and 47.4 ± 4.4% for the same three tissues. Given the fact that the ^{125}I-CI had been injected over a period of 14 weeks prior to sacrifice, the compound was relatively stable once taken up into tissues. Aortic samples were similarly analyzed in a previous study (9), showing that the radiochemical integrity of ^{125}I-CI in this tissue was comparable to that observed in adrenal and liver samples. The higher percentage of degradation products observed in the plasma was not unexpected since the 13 days between the last ^{125}I-CI injection and sacrifice would have

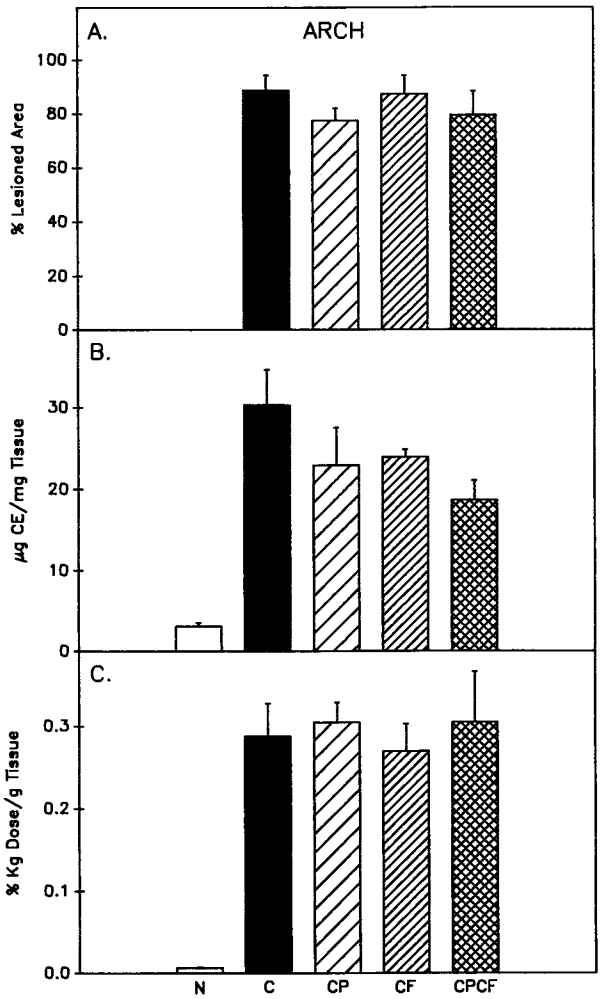

Fig. 2. Atherosclerotic involvement in the aortic arch of rabbits from the five treatment groups described in the legend to Fig. 1. A shows the percentage lesioned surface area, B shows the micrograms of cholesteryl ester per milligram of tissue, and C shows the radioactivity content expressed as percentage injected dose (adjusted for the kilogram weight of the animal) per gram of tissue. The values represent the mean ± SE.

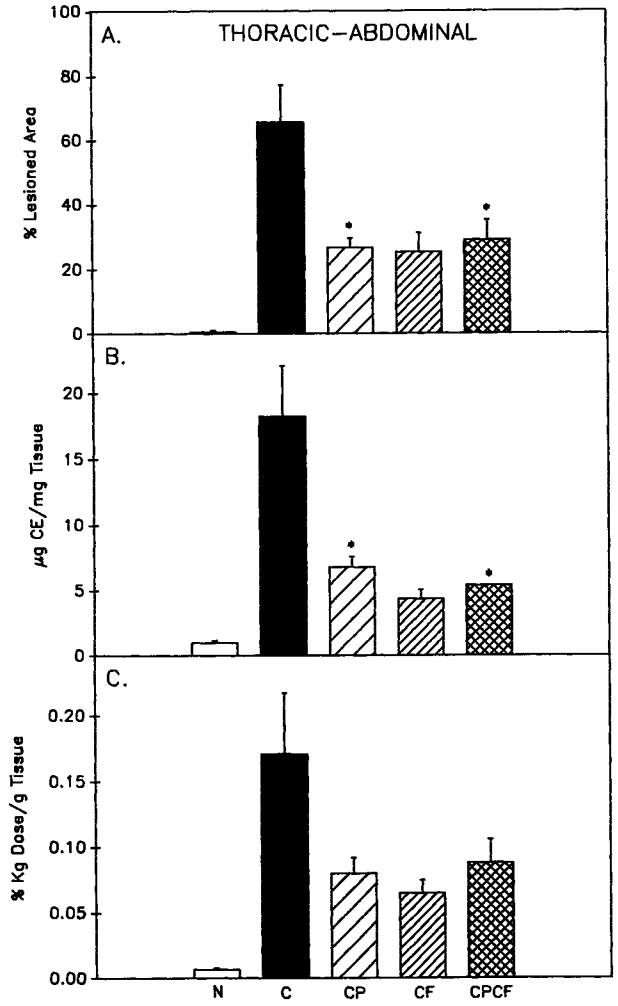

Fig. 3. Atherosclerotic involvement in the thoracic and abdominal regions of the aorta of rabbits from the five treatment groups described in the legend to Fig. 1. A shows the percent lesioned surface area, B shows the micrograms of cholesteryl ester per milligram of tissue, and C shows the radioactivity content expressed as percentage injected dose (adjusted for the kilogram weight of the animal) per gram of tissue. The asterisks denote a significant difference ($P < 0.05$) from group C as determined by Student's t test. The values represent the mean ± SE.

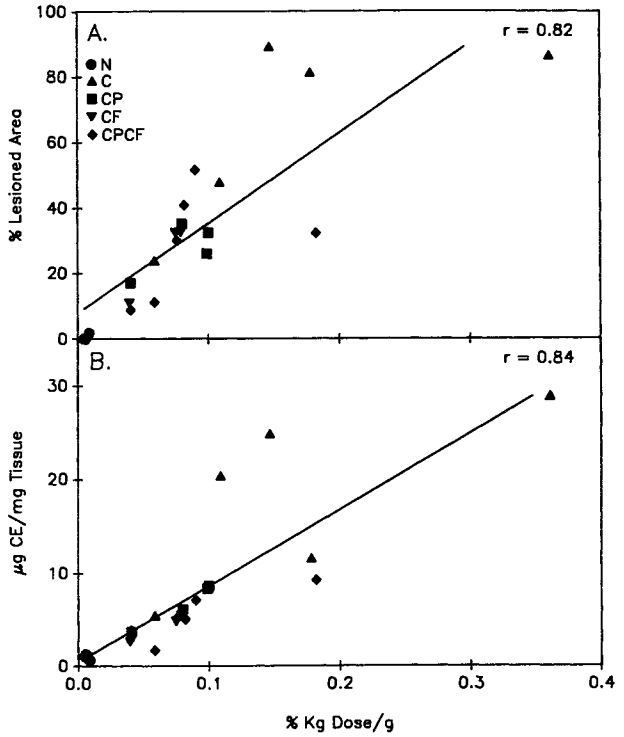

Fig. 4. Correlation of the radioactivity content of the thoracic-abdominal region of the aorta (expressed as % kg dose/g) with both (A) the percentage lesioned surface area and (B) the micrograms of cholesteryl ester per milligram of tissue. The five treatment groups are the same as those described in the legend to Fig. 1.

allowed for efflux of any degradation products from the liver as well as clearance of intact compound from the plasma. Moreover, for the cholesterol-fed groups, the plasma radioactivity content was an average of 40-fold lower than that of the adrenal and liver. ^{125}I-CI was also quite stable to metabolic deiodination as indicated by the low levels of thyroid radioactivity. Expressed as percentage dose per organ, this value was 0.125 ± 0.064 (mean ± SD for all animals). The effective half-life (the biologic half-life adjusted for the decay of the radioisotope) of ^{125}I is 42 days (15); this suggests that the low thyroid radioactivity is largely a reflection of the stability of ^{125}I-CI and not a consequence of efflux of radioactive degradation products from this tissue during the interval between the last injection of ^{125}I-CI and sacrifice.

Scintigraphic images representative of three of the groups of animals are shown in Fig. 5. These scans were taken with the animals in a prone position and the camera placed over the back. Liver radioactivity can be seen at the top of the images, and the proximal portion of the hindlimbs is visible near the bottom. In the control animal (group N), the liver accounts for virtually all the radioactivity apparent in the image, with some splenic radioactivity visible to the left (Fig. 5A). The scan of an untreated, cholesterol-fed animal (group C), in contrast, shows a greater whole-body accumulation of radioactivity characterized by an additional margin of diffuse radioactivity surrounding the liver and spleen as well as a marked accumulation of radioactivity in the midline and pelvic region of the animal (Fig. 5B). Some faint radioactivity is also apparent bilaterally in the lower abdomen above the pelvic bones, and although the source of this radioactivity was not determined, it may correspond to abdominal fat. The image of the colestipol-treated animal (group CP) shows a distribution of radioactivity intermediate between that seen for group N and that for group C (Fig. 5C). In an effort to clarify the localization of the non-liver radioactivity observed in the group C animals, a rabbit from this group was injected with ^{99m}Tc-sulfur colloid to obtain a bone marrow scan. The spleen, spine, and pelvic bones are readily apparent in the resulting image (Fig. 5D).

DISCUSSION

The results of plasma lipid analyses demonstrated that drug treatment did not significantly lower the plasma levels of cholesterol or triglyceride (Fig. 1). In spite of this, the aortas of the drug-treated animals displayed a marked reduc-

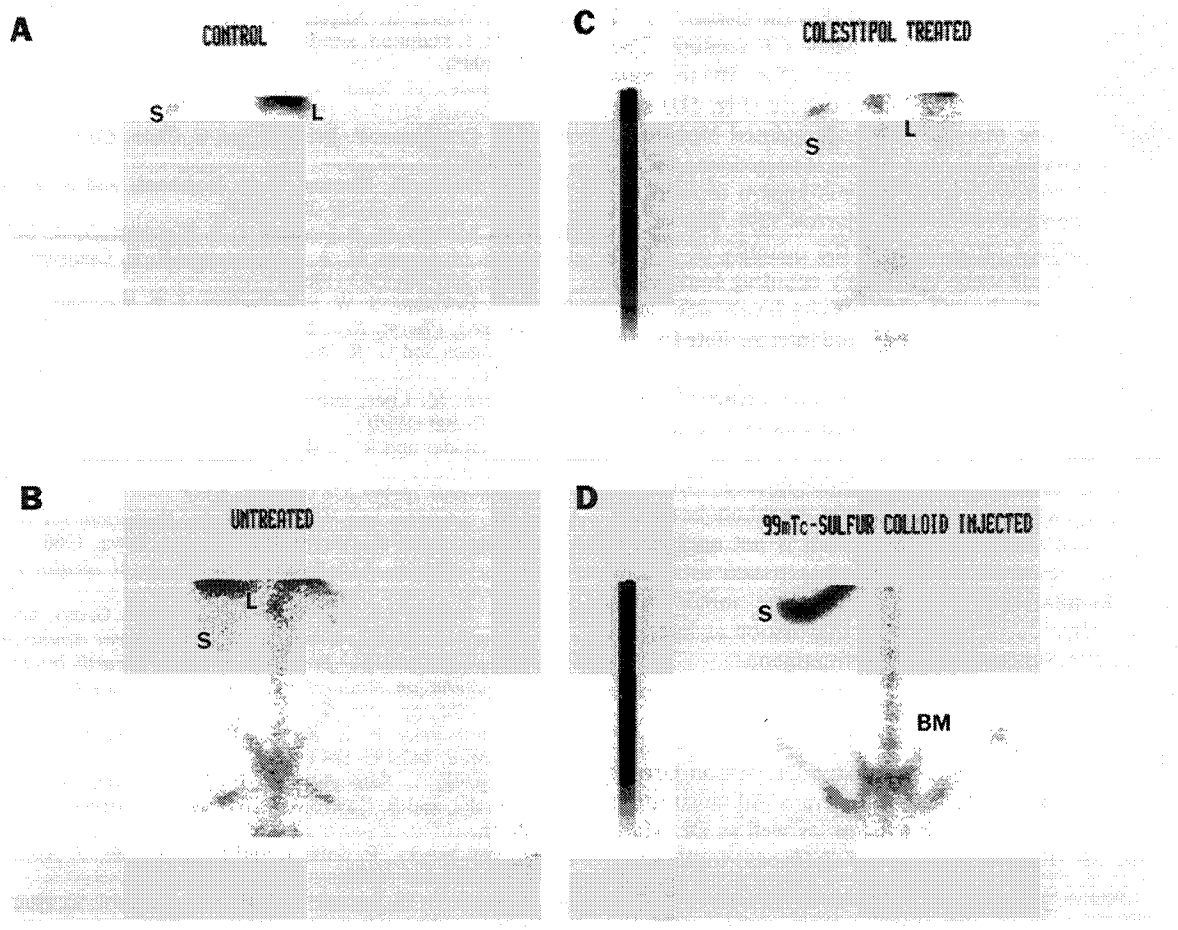

Fig. 5. Scintigraphic scans representative of three of the groups of animals. The images are of (A) an animal from the control group (group N); (B) an animal from the cholesterol-fed, untreated group (group C); (C) an animal from the cholesterol-fed/colestipol-treated group (group CP); and (D) an animal from group C injected with ^{99m}Tc-sulfur colloid. Liver, L; spleen, S; bone marrow, BM.

tion in atherosclerotic involvement. Although lesion development was not affected in the aortic arch (Fig. 2), the abdominal-thoracic region of all drug-treated groups displayed substantial decreases in surface area involved in lesions, in aortic cholesteryl ester content, and in aortic ^{125}I-CI as compared to the cholesterol-fed group (Fig. 3). Similar findings involving a reduction in atherogenesis without a concomitant decrease in plasma lipids have been encountered in other studies using colestipol (16,17) and clofibrate (18,19) as well as a variety of other agents including estrogen (2), chlorpromazine (3), calcium antagonists (4,5), β-blockers (6), and antiinflammatory agents (7). This discrepancy between the actual extent of atherosclerosis and the plasma lipid levels underscores the importance of developing alternative methods of evaluating drug efficacy and atherosclerotic risk.

While the cause of the observed ineffectiveness of the drugs in lowering plasma cholesterol levels in this animal model is unclear, some authors have suggested that an overly high dietary cholesterol:drug ratio may overwhelm the lipid-lowering capacity of the drugs (17,20). However, both bile acid sequestrants and clofibrate and its analogues have numerous effects on lipid metabolism which may alter the course of atherogenesis even in the face of hypercholesteremia. These effects include alterations in apoprotein synthesis (21,22), changes in LDL receptor expression (23), and increases in the activity of enzymes such as acyl coenzyme A cholesterol acyltransferase (ACAT) (24,25).

While other studies have shown a benefit in the combined usage of bile acid sequestrants and clofibrate and its analogues (26,27), the various aortic parameters and the serum lipid levels of the group given the drug combination were not significantly different from those of the groups given colestipol or clofibrate alone. Although it has been shown in humans that coadministration of separate doses of clofibrate and colestipol does not affect the bioavailability of clofibrate (28), it is possible that clofibric acid generated from clofibrate could interact with the anion exchange resin, colestipol, following oral administration. One further explanation for the lack of an additive effect of the two drugs is that they are acting through some as yet uncharacterized common mechanism which is already maximally stimulated upon dosage with each drug alone.

As demonstrated by scintigraphic scanning, hypolipidemic drug treatment caused alterations in the whole-body disposition of cholesteryl ester. This conclusion is supported by the observations that the tissue-associated radioactivity

was predominantly intact ^{125}I-CI and that the tissue ^{125}I-CI content was highly correlated with tissue CE content. The similarity of the scans of group C animals (Fig. 5B) to scans of animals injected with ^{99m}Tc-sulfur colloid (Fig. 5D) suggested that the bone marrow had accumulated significant levels of radioactivity. This was not unexpected since feeding rabbits cholesterol-enriched diets is known to increase the cholesterol content of the bone marrow (29). The scintigraphically visualized differences in the quantity of ^{125}I-CI localized in this tissue were verified by counting samples of bone marrow from the femur; radioactivity levels were lowest for group N, highest for group C, and intermediate for the drug-treated groups.

In conclusion, ^{125}I-CI is well suited as a tracer for CE deposition since it is resistant to hydrolysis *in vivo* and its accumulation is proportional to the tissue CE content. In addition, scintigraphic imaging of animals injected with this compound permits rapid visualization of the whole-body CE disposition. Since sacrifice of the animal is not necessary, use of the isotopes with shorter half-lives (such as ^{123}I or ^{131}I) would facilitate rescanning of the same animal, thus allowing reevaluation of cholesterol disposition at later intervals in an experimental course of treatment.

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