Technetium-99m Low Density Lipoproteins: Preparation and Biodistribution

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The focal uptake by human atherosclerotic lesions of ^{125}I bound to low density lipoproteins (LDL) can be demonstrated by external imaging. However, ^{125}I has poor imaging characteristics. Therefore, we have developed a technique for labeling LDL with technetium. To facilitate analysis, LDL was first labeled with ^{99m}Tc, by reduction of TcO_4^- with dithionite in the presence of the protein. The labeled LDL was stable to electrophoresis, ultracentrifugation, and passage in vivo. This technique was repeated with minor modification with ^{99m}Tc to prepare [^{99m}Tc] LDL for use as an imaging agent. Its biodistribution in 16 rabbits was similar to that of [^{125}I] LDL and it allowed high resolution external imaging of LDL uptake by tissues, including the injured, healing, arterial wall, and the adrenal cortex.

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Early lesions of atherosclerosis encroach upon the vessel wall, rather than the lumen, and are thus difficult to detect by angiography. A method for detecting lesions by their metabolic activity might allow their recognition at early stages of development. With this goal in mind, we have shown that radioiodinated low density lipoproteins (LDL) localize under the regrowing endothelial edge of the rabbit aorta after balloon de-endothelialization (*1*). A preliminary study in man with autologous LDL radiolabeled with iodine-125 (^{125}I) showed that carotid atherosclerosis could be imaged externally with the gamma scintillation camera (*2*). These observations suggested that arterial disease might be detected through the metabolic changes which occur within the vessel wall before the disease progresses to the point of hemodynamic impairment. Although the results with radioiodinated LDL were encouraging, the relatively poor imaging qualities of ^{125}I, and the limited availability of radioisotopically pure ^{123}I, (the only iodine isotope suitable for diagnostic imaging), led us to seek a better radiolabel for following the metabolism of LDL by extracorporeal imaging. Technetium-99m (^{99m}Tc) is the most widely used isotope in nuclear medicine, because its short half-life and gamma emission permit excellent external imaging with a low absorbed radioactivity dose to the patient. We report here a simple method for producing technetium-labeled LDL and show in an animal model that it allows external imaging of LDL uptake by multiple organs, including the arterial wall and the adrenal glands.

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MATERIALS AND METHODS

Preparation of low density lipoproteins

LDL (density = 1.025 to 1.050 g/ml) were isolated from normal plasma by sequential flotation in the ultracentrifuge at 100,000 *g* for 22 hr (*3*). Plasma density was adjusted by addition of a KBr solution of density 1.32 g/ml (*3*). Following 24 hr of dialysis against buffer containing 0.2*M* NaCl and 1.0 m*M* disodium ethylenediaminetetraacetic acid (EDTA), pH 8.6, the LDL was filtered through a 0.22 μ filter before use. Its identity and purity were confirmed by double immunodiffusion against rabbit anti-human antisera (*1,3*). The protein was quantitated by the method of Lowry et al. (*4*), with

a correction for the higher color yield of LDL with respect to bovine serum albumin, as described by Margolis and Langdon (*5*).

Radiolabeling procedure

The method was developed with technetium-99 (^{99}Tc), a technetium isotope with a half-life of 2.12×10^5 yr, to facilitate the conduct of the technetium chemistry. The same conditions were then applied to ^{99m}Tc (half-life 6 hr), with equal success.

A standard reaction mixture was prepared by adding an aliquot of $^{99}TcO_4^-$ (1 μCi, 0.6 μmole) to LDL (2–6 mg protein), previously dialyzed against bicarbonate buffer, pH 8 (Pilot experiments at pH up to 12 produced progressively greater aggregation of the protein and no better yield of labeled product.) Sodium dithionite (10 mg, 57.5 μmole) was then dissolved in 0.5 ml of distilled water and immediately added to the solution of $^{99}TcO_4^-$ and LDL with gentle mixing. The reactants were allowed to stand at room temperature for 10 min. The molar ratio of reductant to $^{99}TcO_4^-$ was 10^2. Control experiments were run with all the reactants except sodium dithionite, or all reactants except LDL. The experiments were then repeated with 50 mCi of ^{99m}Tc. The conditions were similar to those for ^{99}Tc labeling, except that the dithionite (1mg) was added in 0.1 ml of 1*M* glycine buffer, pH 9.8, to avoid the rapid decomposition of the dithionite which occurred in distilled water, and reaction times were varied from 10 to 45 min. The molar ratio of reductant to $^{99m}TcO_4^-$ was $> 10^4$.

Analytical methods

Native LDL, heat-denatured LDL (100° for 5 min), [^{99}Tc]LDL, [^{99m}Tc]LDL, LDL incubated with $^{99}TcO_4^-$, and $^{99}TcO_4^-$ incubated with sodium dithionite were chromatographed on a 2.5 cm × 100 cm column of 2% agarose gel equilibrated at 4°C with bicarbonate buffer, pH 8. The LDL preparations contained 2–6 mg protein in a volume of 1–3 ml.

Following gel-filtration, ^{99}Tc LDL, $^{99}TcO_4^-$, and reduced pertechnetate without LDL were analyzed by paper electrophoresis (*3*); the strips were stained for lipid and scanned for radioactivity. Gel-filtered [^{99m}Tc]LDL preparations were also analyzed by paper electrophoresis. The paper strips were marked off in 2-mm segments and then divided in half lengthwise. One half strip was stained for lipid, and the other half was cut into 2-mm pieces which were counted in a gamma counter.

Gel-filtered [^{99}Tc]LDL was also analyzed by immunoelectrophoresis (*3*). After removal of unprecipitated antigens and antisera, dried slides were radioautographed on high speed x-ray film* in a cassette† for 6 wk.

Technetium-99 LDL obtained by gel filtration was subjected to ultracentrifugation for 22 hr at 100,000 *g* and a density of 1.063 g/ml. A control tube, which contained LDL and pertechnetate, but no dithionite, was also ultracentrifuged, without prior gel filtration. Radioactivity in the supernatant (0.5 ml) and the infranatant (5.5 ml) was determined. The presence of LDL in the supernatant was verified by double immunodiffusion (*3*).

Labeled LDL preparations were extracted with chloroform-methanol 2:1 (v/v) according to the procedure of Bligh and Dyer (*6*). After vortexing and centrifugation, the chloroform layer was removed. Protein was packed by recentrifugation and the aqueous phase was removed. Radioactivity in the three phases was counted after evaporation of the solvents under nitrogen.

Behavior in vivo

Two New Zealand White rabbits (2–3 kg) were injected intravenously with gel-filtered [^{99}Tc]LDL (0.15 μCi, 1.7 mg LDL protein) which was also filtered through a 0.22-μ filter just prior to injection. Six to seven blood samples were collected at intervals over a 36-hr period. Plasma was immediately separated at 4°C and divided into aliquots for measuring radioactivity and for isolation of [^{99}Tc]LDL by ultracentrifugation at a density of 1.063 g/ml. Sixteen other rabbits were injected intravenously with [^{99m}Tc]LDL, (4–8 mCi, 1.5 mg protein). Eight of the 16 had had balloon de-endothelialization of the abdominal aorta performed 2–4 wk previously as described elsewhere (*1*). Sixteen hours after injection, the rabbits were anesthetized and imaged anteriorly and posteriorly with a standard Anger scintillation camera‡ with a parallel-hole collimator. Imaging time was 10 min; ~ 300,000 counts were obtained. Biodistribution studies were carried out following imaging.

RESULTS

When $^{99}TcO_4^-$ was reduced in the presence of LDL, and the reaction mixture gel filtered on 2% agarose, one third to one half of the radioactivity eluted as a macromolecular peak, while the rest eluted in a volume characteristic of small molecules (Fig 1A). Results were verified by chromatographing heat-denatured LDL, native LDL, and pertechnetate on the same column (not shown). Denatured LDL eluted in the void volume; native LDL eluted at the same volume as the major peak of ^{99m}Tc LDL, and pertechnetate eluted where the small molecule peak eluted in the column runs shown in Fig. 1. When pertechnetate was incubated with LDL in the absence of dithionite and the reaction mixture was chromatographed, no radioactivity was detected in the LDL peak (Figure 1B). Similar results were obtained when $^{99m}TcO_4^-$ was substituted for $^{99}TcO_4^-$.

Preparative ultracentrifugation for 22 hr at

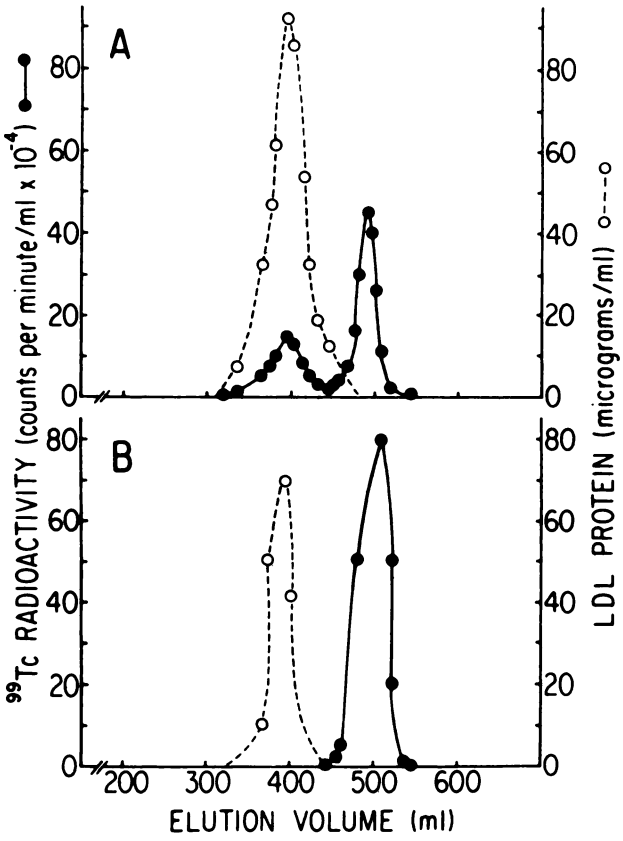

FIGURE 1
Elution patterns on 2% agarose gel of two representative experiments. A: LDL was incubated with $^{99}TcO_4^-$ and sodium dithionite (at pH 9) for 10 min (see text for details) and then chromatographed. B: Native LDL and $^{99}TcO_4^-$ were incubated together as in A., but in absence of sodium dithionite. Solid lines represent radioactivity, dotted lines, protein

100,000 *g* and a density of 1.063 g/ml was used to investigate the stability of the radionuclide-LDL bond in [^{99}Tc]LDL. At that density, LDL floats to the top of the tube in the preparative ultracentrifuge. Thus technetium tightly bound to LDL would also be found at the top of the tube, while unbound technetium would remain distributed throughout the tube after ultracentrifugation. When the [^{99}Tc]LDL obtained by column chromatography of the reaction mixture was ultracentrifuged, 84% of the total radioactivity floated with LDL in the top 0.5 ml of the 6 ml ultracentrifuge tube (mean of three experiments). When the reaction mixture incubated under the same conditions without sodium dithionate was ultracentrifuged, only 5% of the total radioactivity was recovered in the top 0.5 ml following ultracentrifugation. Neither unreacted pertechnetate nor reduced technetium species floated in the ultracentrifuge at a solvent density of 1.063 g/ml.

Immunoelectrophoresis of [^{99}Tc]LDL produced a single precipitin arc against anti-LDL antiserum; the electrophoretic mobility of the arc was similar to that of unlabeled LDL (Fig. 2). Autoradiography of the immunoelectrophoresed proteins produced a single arc, corresponding to the immunoprecipitin line of [^{99}Tc]LDL and, around the anodal half of the arc, some diffusely scattered radioactivity which had not immunoprecipitated.

Three preparations of [^{99}Tc]LDL were analyzed for the distribution of radioactivity between protein and lipid. When the protein in each sample was precipitated with trichloroacetic acid, the precipitate contained 78–90% of the total radioactivity; on chloroform extraction of the labeled protein only 2.4–4.4% of the total counts were present in the extracted lipids.

Paper electrophoresis of gel-filtered native LDL and [^{99}Tc]LDL, followed by lipid staining, gave in each case a single band with identical beta mobility. Thus, the electrophoretic properties of LDL were not altered by the labeling procedures. Control samples of ^{99}Tc reduced in the absence of LDL demonstrated a single sharp peak of radioactivity at the origin when scanned in a strip counter.

Paper electrophoresis of gel-filtered [^{99m}Tc]LDL followed by counting of 2-mm sections of the strip, and lipid staining, indicated that 60-90% of the radioactivity was associated with LDL. However, small areas of radioactivity occurred on either side of the LDL peak (Fig. 3). The relative contribution of these minor components varied with the time of incubation of LDL and pertechnetate with dithionite. The faster moving shoulder, which migrated 2–6 cm from the origin towards the anode, was about 50% higher after a 15 min incubation than after 30 or 45 min. The small peak centered at the origin was about 15 to 50% higher after 30 and 45 min, respectively, than after 15 min of dithionite incubation. Thus, the faster shoulder decreased in height with time, while the slower small peak increased. (The height of the LDL peak did not appear to vary with time.) When two rabbits were injected with [^{99m}Tc]LDL which had been incubated with dithionite for only 10 min, the uptake of radioactivity by bone was striking. In contrast, bone uptake of radioactivity was very low with the [^{99m}Tc]LDL preparations which were incubated with dithionite for 30 min; only the latter were used for biodistribution studies.

The disappearance of human [^{99}Tc]LDL from the plasma compartment of the rabbit was biexponential. The major component of the die-away curve had a half-time of about 20 hr, similar to previous results with [^{125}I]LDL (*7*). When plasma samples drawn 1, 2, 4, and 12 hr after injection of [^{99}Tc]LDL were ultracentrifuged for 22 hr at a density of 1.063 g/ml, 70-80% of the total plasma radioactivity floated in the top 0.5 ml of the tube. When the short-lived gamma-emitting isotope ^{99m}Tc was used in place of ^{99}Tc, the results were very similar to those with the longer-lived isotope. One-

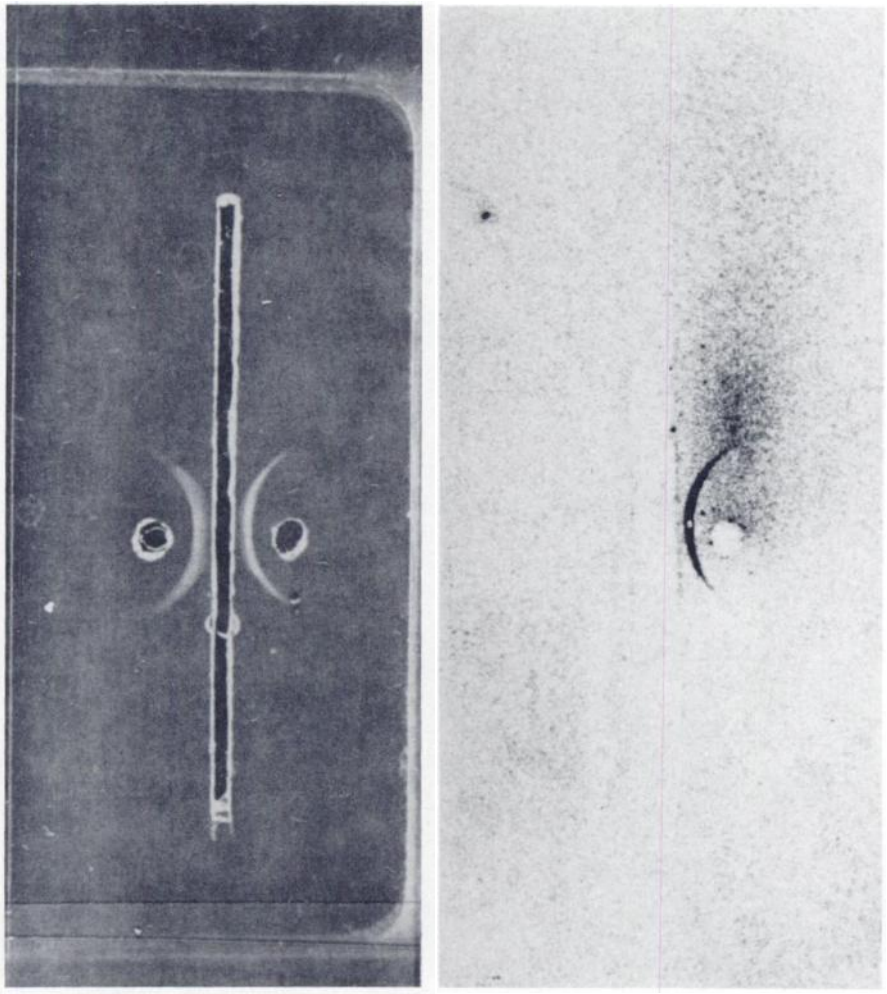

FIGURE 2
Immunoelectrophoresis (left) and radioautograph of same slide (right) of LDL and [^{99}Tc]LDL. Native LDL was in left-hand well of immunoelectrophoretic slide, and [^{99}Tc]LDL in right-hand well. Center trough was filled with anti-LDL antiserum

third to one-half of the applied radioactivity eluted from the 2% agarose column simultaneously with the LDL peak. When this peak was ultracentrifuged, 88-93% of the radioactivity floated at density 1.063 g/ml.

To measure the biodistribution of [^{99m}Tc]LDL, in preparation for human studies, 16 rabbits received i.v. injections of 4-8 mCi of gel-filtered human [^{99m}Tc]LDL which had been incubated with dithionite for 30 min. Eighteen to 21 hr later the organs were removed, rinsed free of blood, weighed and counted. Counts per gram or per organ were related to an aliquot of the injected dose which was counted simultaneously with the tissue samples (Table 1). Technetium-99m LDL was found mainly in the liver, adrenals and kidney, in a pattern similar to that seen with other radiolabeled LDL preparations (*8,9*). Calculations based on urine radioactivity at 24 hr, indicated that at least 27% of injected radioactivity was excreted in the urine in the 24-hr period.

Gamma scintillation camera scanning of the rabbits in whom the biodistribution of [^{99m}Tc]LDL was measured was performed immediately after injection, at 1 hr, 4-8 hr, and again at 18-21 hr, just before killing. The initial scintigram showed a typical blood-pool scan (Fig. 4A). Although some radioactivity appeared in the kidneys by 20 min and in the bladder at 1 hr, there was no consistent early accumulation in lungs, liver, or spleen. Rather, the blood-pool image gradually changed with time to an image of specific organ uptake of radioactivity, most prominently by the adrenals, liver, kidneys, spleen and intestine (Fig. 4B). The intensity of organ radioactivity was proportional to that obtained subsequently in the biodistribution study (Table 1).

DISCUSSION

Our earlier demonstration that radioiodinated LDL accumulated in high concentration in injured and healing arteries in animals (*1*), and could be used to image atherosclerosis in man (*2*) led us to a search for methods of labeling the lipoprotein with radioiotopes suitable for imaging. The isotope most generally useful in nuclear medicine is ^{99m}Tc, because it is readily available, and has excellent imaging characteristics, This isotope has

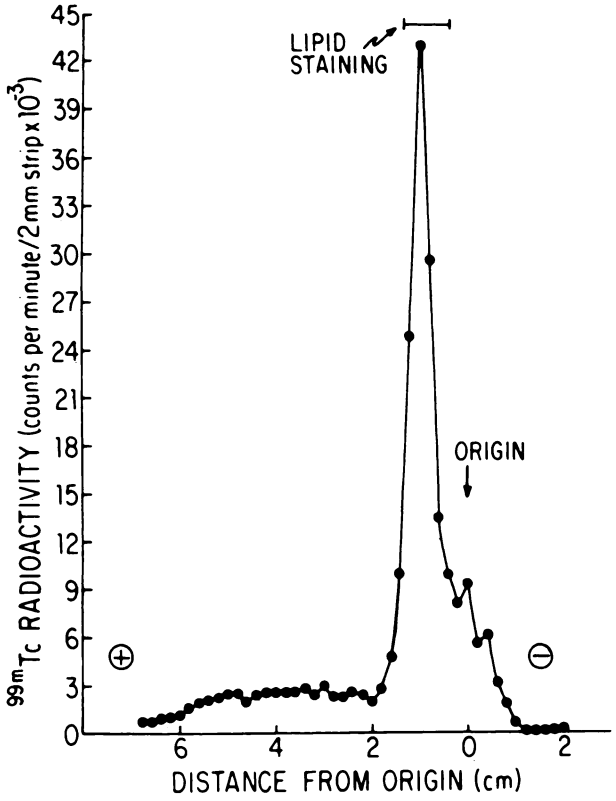

FIGURE 3
Distribution of radioactivity and lipid-staining following paper electrophoresis of gel-filtered [^{99m}Tc]LDL. Reaction mixture had been incubated for 30 min, and about 73% of radioactivity was associated with main LDL peak. (see text for details)

not previously been studied as a labeling agent for LDL.

The present studies show that ^{99}Tc and ^{99m}Tc can be linked directly to LDL in the presence of a reducing agent, and that 80% or more of the Tc-LDL bonds are strong enough to survive ultracentrifugation and electrophoresis. In the absence of a reducing agent, there is little binding of technetium to LDL, indicating that the technetium is not just nonspecifically adsorbed to the protein. Furthermore, the half-life in vivo in the rabbit of technetium-labeled LDL is similar to that of ^{125}I LDL, the standard radioactive tracer for the metabolism of intact, native LDL. This indicates that the labeling procedure does not appreciably alter the metabolism of LDL in vivo.

The conditions of labeling proteins with technetium are most important for a successful outcome and may vary from one protein to another. The relative instability of LDL, which has an isolectric pH of 5.7 (*11*) and aggregates rapidly at pH below 7, restricts the range of chemical procedures which can be used. Fortunately, the reduction of pertechnetate by dithionite at mildly alkaline pH does not aggregate or denature LDL, and is one of the most efficient methods for attaching technetium to proteins (*11*). We achieved a satisfactory preparation for imaging by incubating LDL and ^{99m}Tc for 30 min in the presence of sodium dithionite.

Our results show that [^{99m}Tc]LDL can be used to assess the organ distribution pattern of LDL in the rabbit by external imaging, and to detect and localize areas of arterial injury as well. Since we were able, in pilot studies, to image symptomatic and asymptomatic atherosclerotic lesions in man (*2*) with the far less effective imaging agent [^{125}I]LDL, the present results are an important advance towards the goal of external imaging of atherosclerotic lesions in man. In addition, since [^{99m}Tc]LDL produces excellent images of rabbit adrenals, this radiopharmaceutical gives promise of being a useful diagnostic tool not only for detecting atherosclerotic disease, but also for evaluating adrenal cortical function. Although the optimal imaging time in these studies was about three times the half-life of ^{99m}Tc and thus relatively late for this isotope, we believe that imaging with technetium-labeled lipoproteins holds promise because of the low cost and excellent imaging characteristics of technetium and because in the future labeled derivatives lipoproteins may increase the rate of tissue uptake, and thus decrease the time to effective imaging.

TABLE 1
Biodistribution of [^{99}Tc] LDL

Organ	Percent of injected radioactivity (mean ± s.e.m.) Per g	Per organ
Venous blood	0.03 ± 0.01	7.2 ± 1.3
Liver	0.19 ± 0.02	21.1 ± 1.3
Spleen	0.22 ± 0.04	0.4 ± 0.1
Adrenal (whole)*	0.81 ± 0.19	0.2 ± 0.1
(cortex)*	0.92 ± 0.09	0.3 ± 0.1
(medulla)*	0.59 ± 0.06	0.1 ± 0.1
Kidney (cortex)	0.11 ± 0.02	2.9 ± 0.4
(medulla)	0.04 ± 0.01	0.9 ± 0.2
Small bowel	0.01 ± 0.00	1.3 ± 0.2 †
Large bowel	0.05 ± 0.01	2.3 ± 0.3 †
Muscle	0.002 ± 0.0	1.6 ± 0.3 †
Aorta (thoracic)	0.02 ± 0.00	—
(abdominal)	0.03 ± 0.01	—
Bone with marrow	0.02 ± 0.00	7.7 ± 0.2
Lung	0.03 ± 0.01	0.7 ± 0.2
Gonads	0.009 ± 0.00	0.1 ± 0.0
Pancreas	0.008 ± 0.01	0.02 ± 0.00
Urine§	0.272 ± 0.11	27.2 ± 1.1

* Entire adrenal gland was weighed and counted in 12 animals, while cortex and medulla were dissected out, weighed, and counted separately in four animals.

† Percent of injected dose is expressed per 100 g for small and large bowel, while that for muscle is expressed for body muscle mass, assumed to be 45% of body weight.

§ Percent of injected dose for urine was calculated from disappearance curve from plasma and the urine radioactivity at time of sacrifice. Total urine volume per experiment was estimated to be 100 g.

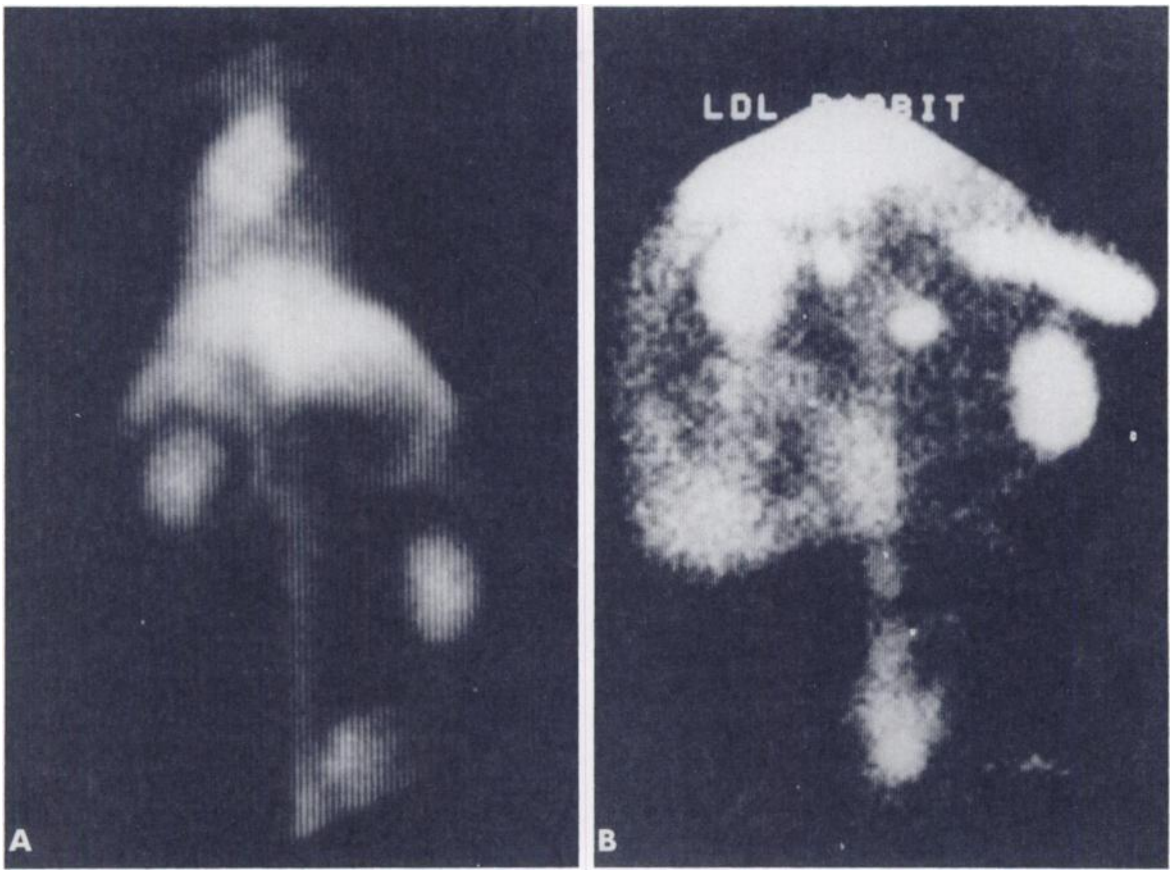

FIGURE 4
A: Anterior scintigram of rabbit 25 min after i.v. injection of 4 mCi [^{99m}Tc]LDL. Some early uptake of radioisotope by kidneys is evident, but most of radiolabel remains in blood pool. B: Anterior scintigram of rabbit 4 wk after balloon deendothelialization of abdominal aorta and 16 hr after i.v. injection of 3 mCi of [^{99m}Tc]LDL. Liver is in upper part of image (partly outside the field of view). Both kidneys and both adrenals, the spleen, large and small bowel, healing aorta, and bladder (lower part of the image) are all clearly visible. Adrenal glands (just lateral to aorta on either side) appear much larger than their true size because of their extremely high LDL uptake. 10-min image was made

FOOTNOTES

* Kodak XOMAT-AR, Eastman Kodak Co., Rochester, NY.

† Kodak X-OMATIC, Eastman Kodak Co., Rochester, NY.

‡ Technicare 420/550, Technicare Corp., Solon, OH.

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