Macrophages from Nephrotic Rats Regulate Apolipoprotein E Biosynthesis and Cholesterol Content Independently

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Abstract

The effects of the nephrotic syndrome in rats on the cholesterol content and the biosynthesis of apolipoprotein E (apoE) by resident peritoneal macrophages have been investigated. Since the nephrotic syndrome has been associated with an increased risk of coronary atherosclerosis, we hypothesized that macrophages from nephrotic rats would accumulate cholesterol and undergo transformation into foam cells, with a concomitant increase in apoE biosynthesis. The nephrotic syndrome was induced in rats with puromycin aminonucleoside. Peritoneal macrophages exposed in vivo for 7–21 d to ascites fluid derived from plasma containing sixfold elevations of lipoproteins did not accumulate unesterified or esterified cholesterol. Nevertheless, immunoprecipitation assays after incubation of the isolated cells with [^{35}S]methionine, or immunoblot analysis of the incubation medium demonstrated a 2.6-fold increase in apoE secretion compared with normal macrophages. This increase was accompanied by 5- to 10-fold increases in cellular apoE messenger RNA as determined by quantitative solution hybridization assay. Peritoneal macrophages cultured from nephrotic rats during the period of hypercholesterolemia also showed distinct and highly reproducible morphologic changes. The dissociation between apoE biosynthesis and macrophage cholesterol content provides new insight into the response of peritoneal macrophages in vivo to endogenous hyperlipemia. (*J. Clin. Invest.* 1991. 87:470–475.) Key words: apoliproprotein E • macrophages • nephrotic syndrome • hypercholesterolemia

Introduction

The triad of hypercholesterolemia, edema, and proteinuria are the characteristic signs of the nephrotic syndrome, a relatively common disorder of man. Accelerated atherosclerosis is a major complication of the hypercholesterolemia in people with the nephrotic syndrome (1, 2). A possible effect of hypercholesterolemia in the nephrotic syndrome could be to enhance the accumulation of cholesterol in tissue macrophages, since cholesterol-loaded macrophages contribute to the early lesions in atherosclerosis (3). We wished to determine whether nephrotic rat macrophages accumulate cholesterol concurrent with the development of hypercholesterolemia and the appearance of ascites.

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When loaded with cholesterol in vitro, peritoneal macrophages secrete increased amounts of apolipoprotein E (apoE),[1] a 34-kD glycoprotein that is a ligand for the low density lipoprotein receptor and other putative receptors on hepatocytes, macrophages, and other cells (4, 5). In plasma, apoE circulates as a surface component of chylomicrons, very low density lipoproteins and some subclasses of HDL (6, 7). The function of macrophage-derived apoE is not presently understood.

We reported previously that in contrast to normal rats, apoE is not detectable in the serum HDL from severely nephrotic rats, although HDL recovered from the ascites fluid of the same animals contains apoE (8). We suggested that macrophages exposed to lipoprotein-rich ascites fluid might accumulate cholesterol and in turn undergo induction of apoE biosynthesis (8). The experiments presented here show that peritoneal macrophages from nephrotic rats do not accumulate excess cholesterol although they synthesize and secrete increased amounts of apoE.

Methods

Animals and induction of nephrosis. Male Sprague-Dawley rats (225–249 g) were supplied by Ace Animals (Boyertown, PA). Nephrosis was induced by a single intraperitoneal injection of puromycin aminonucleoside (PAN; Sigma Chemical Co., St. Louis, MO) at a dose of 100 mg/kg body weight (9).

Isolation of peritoneal macrophages. Resident macrophages were harvested from unstimulated rats by peritoneal lavage with PBS containing 2 U/ml of preservative-free heparin (Sigma Chemical Co.). Heparin effectively inhibited cell clumping that occurred when the macrophages were isolated in PBS alone. Both control and nephrotic rat macrophages were isolated under identical conditions. Cells were suspended in Minimum Essential Eagle's Medium (MEM) supplemented with 20% heat-inactivated fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 μg/ml). Aliquots of cells were dispersed in 35- or 60-mm petri dishes and incubated at 37°C in 5% CO_2. The plates were washed four to six times, 2 h after plating to remove nonadherent cells. 81% of the adherent cells were macrophages, as determined by sheep red blood cell phagocytosis (10).

Measurement of cholesterol concentrations in serum and peritoneal macrophages. Serum lipids were extracted from 70–100 μl aliquots in chloroform/methanol (1:1) with 20 μg of coprostanol as an internal standard (11). After the 2-h incubation, total cellular lipid was extracted with isopropanol containing coprostanol as an internal standard. Cholesterol mass was determined by gas chromatography using a Hewlett-Packard model 5710 gas chromatogram with a 4 mm × 2 m column containing 3% silicone, OV-17. Cholesterol mass was normalized to total cell protein after dissolving the isopropanol-insoluble protein in 0.16 M Na_2CO_3, 0.1 M NaOH, 1.6% NaKtartrate, and 1% SDS (12).

Immunoprecipitation. After 2 h in culture, each macrophage monolayer was washed seven times with 1 ml of MEM and incubated for 6 h

1. *Abbreviations used in this paper:* apoE, apolipoprotein E; PAN, puromycin aminonucleoside.

(37°C, 5% CO_2) in methionine-free MEM containing 60 μCi/ml of L-[^{35}S]methionine (sp act > 1,000 Ci/mmol; ICN Pharmaceuticals, Inc., Irvine, CA) and 10 μM unlabeled methionine. The media from the cells in four to six dishes (two to three rats) were combined. Cells that had floated during the labeling were removed by centrifugation (1,000 $g \times 10$ min at 4°C), PMSF was added (to 0.1 mM), and the pooled media was concentrated in an Amicon Corp. (Danvers, MA) Centricon 10 microconcentrator with a YM10 membrane. An aliquot of this was then treated with 10% TCA-1% phosphotungstic acid (PTA), the protein precipitate resuspended in 10% TCA-1% PTA four times to remove the labeled methionine, redissolved in 0.1 N NaOH, and the amount of radioactivity determined. Aliquots of the pooled media from normal or nephrotic cells, containing the same amount of labeled protein, were incubated with a monospecific goat anti-rat apoE using purified rat plasma apoE as a carrier. The apoE was obtained from rat HDL (13). The immunoprecipitation reactions contained 150 μl of buffer (20 mM Tris, pH 7.5, 0.2% NP-40, 0.25% deoxycholate, 7 mM EDTA), 2 μg apoE, 50 μl anti-apoE, and 200–400 μl of pooled media. Aliquots of media were also incubated with nonimmune serum as a control. The precipitate was recovered using Protein A-Sepharose (Sigma Chemical Co.). The precipitate was dissolved in 10% SDS by heating at 100°C for 2 min and then chromatographed on a column of sepharose 12B using an LKB produckter (Bromma, Sweden) model 2150 high pressure liquid chromatograph to recover ^{35}S-labeled apoE. Control experiments with ^{125}I-labeled apoE indicated recovery of ~ 77% of dissolved immunoprecipitate by this technique. Results are expressed as the percentage of labeled protein recovered in apoE.

Immunoblotting. After removal of nonadherent cells, macrophage monolayers were incubated overnight in serum-free MEM. Medium was collected as described, followed by extensive dialysis (20 mM NH_4HCO_3, 10 mM PMSF) at 4°C. Pooled media samples from equivalent numbers of nephrotic and normal rat macrophages were then lyophilized and redissolved in polyacrylamide gel sample buffer (0.06 M Tris, pH 6.8, 10% glycerol, 2% SDS, 0.6% dithiothreitol). Electrophoresis was conducted in one dimension on 10% polyacrylamide slab gels containing 0.1% SDS (PAGE). Protein from the PAGE gels was transferred to nitrocellulose in buffer containing 25 mM Tris, 192 mM glycine, pH 8.3, and 20% methanol (30 V, 18 h, 4°C). The membrane was incubated in blocking buffer (5% Carnation Dry Milk, 20 mM Tris HCl, 500 mM NaCl, pH 7.5), followed by incubations with anti-apoE and secondary antibody (secondary antibody was an anti-goat IgG conjugated to horse radish peroxidase; Sigma Chemical Co.). Diaminobenzidine (DAB) was used as the chromogenic substrate (0.06% DAB, 1.8% H_2O_2).

Blot analysis of apoE mRNA. RNA was isolated as described (14) and precipitated with carrier tRNA (*Escherichia coli;* Boehringer Manheim Diagnostics, Inc., Houston, TX). The redissolved precipitate was electrophoresed in 1% formaldehyde-agarose using MOPS buffer (15). Gels were transferred to Nytran (Schleicher & Schuell, Inc., Keene, NH) in 20× SSC (1× SSC contains 0.15 M NaCl and 15 mM NaCitrate). The membranes were prehybridized and hybridized following protocols supplied by the manufacturer, using a probe for rat apoE cDNA labeled to high specific activity with ^{32}P by the random primer method (16). After washing off unbound probe at high stringency, autoradiograms were exposed for 1–5 d.

Solution hybridization. RNA isolation was carried out by the method of Chirgwin (17). RNA was isolated from both normal and nephrotic rat macrophages, 8 d after injection with puromycin aminonucleoside (the time point of peak hypercholesterolemia). A DNA probe excess solution hybridization assay for rat apoE was designed with an apoE cDNA probe synthesized from a fragment of rat apoE corresponding to nucleotides 273 to 497 (18, 19). RNA from macrophages was hybridized to completion with labeled apoE cDNA and the resultant double-stranded molecules were precipitated in 8.2% TCA after digestion of nonhybridized RNA and apoE cDNA with S1 nuclease. The precipitated hybrids were collected on glass filters, washed with TCA, and quantified by scintillation spectrometry. Values for apoE mRNA mass normalized to total RNA were obtained by comparison to a standard curve constructed with template DNA.

Results

Time course of nephrotic hypercholesterolemia. Serum cholesterol was measured up to 21 d after induction of nephrosis. By day 8, serum cholesterol levels in nephrotic rats were sixfold higher than in control rats. Fig. 1 shows that the hypercholesterolemia persisted for more than 14 d after the initial injection, with a return to normal by 21 d. The maximum serum cholesterol on day 8 was 501±208 mg/dl (mean±SD, $n = 5$) for nephrotic rats and 90±4 mg/dl (mean±SD, $n = 3$) for normals. The percentage of cholesterol ester in both the control and nephrotic rat serum was 76±3% (mean±SD, $n = 8$). Unlike the hyperlipemia induced by feeding atherogenic diets in the rat, levels of all classes of lipoproteins rise in the experimental nephrotic syndrome, including high density lipoproteins (9, 20). In this study, we specifically examined the VLDL isolated by ultracentrifugation from nephrotic rat serum by agarose gel electrophoresis, and it was found to comigrate with normal VLDL, indicating the absence of cholesterol-rich β-VLDL.

Macrophages from nephrotic rats do not accumulate cholesterol. The cholesterol content of macrophages from normal and nephrotic rat peritoneal fluid is shown in Fig. 1. Even at the peak of hypercholesterolemia and ascites fluid accumulation on day 8, macrophages from nephrotic rats maintained normal cellular cholesterol levels. The elevation in the ratio of nephrotic to normal cholesterol depicted in Fig. 1 at day 9 is not statistically significant ($P > 0.05$). Values for this time point were as follows: nephrotic macrophage cholesterol/mg protein 33.1±10.4 (mean±SD, $n = 5$), and for normal rat macrophages 21.5±3.6 ($n = 3$). Statistical analysis of the data for days 7, 8, 9, 14, and 21 showed no significant differences between normal and nephrotic rat macrophage cholesterol. To determine whether incubation of macrophages in 20% FBS before washing allowed cholesterol loss from nephrotic macrophages, cells were isolated in medium with no added FBS. Normal rat macrophages contained 36±1.39 μg cholesterol per mg cell protein

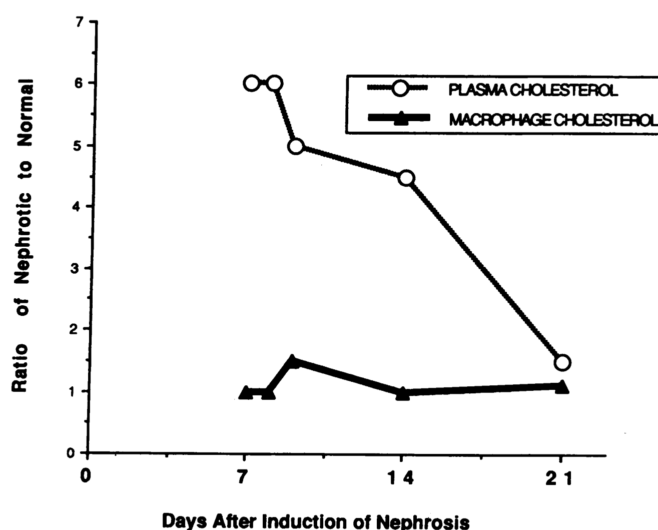


Figure 1. Ratios of nephrotic to normal plasma and macrophage cholesterol. No significant difference was found comparing nephrotic ($n = 17$) with normal ($n = 8$) rat peritoneal macrophage cholesterol.

(mean±SD, $n = 5$), which was significantly ($P < 0.01$) more than the cholesterol content of the nephrotic rat macrophages (31.4±2.48). Macrophages from both normal and nephrotic rats had no detectable cholesteryl ester.

Normal rat peritoneal fluid lipoproteins were also isolated after peritoneal lavage in PBS by ultracentrifugation ($d < 1.21$). Apoprotein concentrations were determined after delipidation. The lipoprotein levels of nephrotic rat peritoneal fluid was at least twofold higher than that of normals. HDL was the major lipoprotein present in normal rat peritoneal fluid; however nephrotic fluid contained VLDL, IDL, and LDL as previously reported (8). Direct quantitative comparison of the peritoneal contents of normal versus nephrotic rats was not possible due to the variable volume of ascites fluid, although increases in ascites fluid lipoprotein concentrations corresponded to increases in plasma cholesterol. Thus, macrophages from nephrotic rats did not accumulate cholesterol even though they were bathed in a hyperlipemic fluid.

Metabolic labeling and immunoblotting show induction of apoE biosynthesis in the nephrotic rat macrophage. Although nephrotic rat macrophages did not contain an increased intracellular cholesterol concentration, we sought to determine if the synthesis of apoE was stimulated. As shown in Fig. 2, although total protein synthesis did not differ in normal ($n = 8$) as compared with nephrotic ($n = 5$) rat macrophages, macrophages from nephrotic rats secreted an average of 2.6 times more apoE than macrophages from normal rats. ApoE represented 0.28±0.08% (mean±SD, $n = 8$) of secreted ^{35}S-protein in normal rat macrophages and 0.71±0.29% (mean±SD, $n = 5$) in nephrotic rat macrophages. Macrophages from nephrotic rats secreted significantly more apoE than normal rat macrophages ($P < 0.005$). The apoE secretory values shown in Fig. 2 for normal rat macrophages are similar to those reported by Driscoll and Getz using similar labeling conditions (21).

Immunoblot analysis of apoE from normal and nephrotic

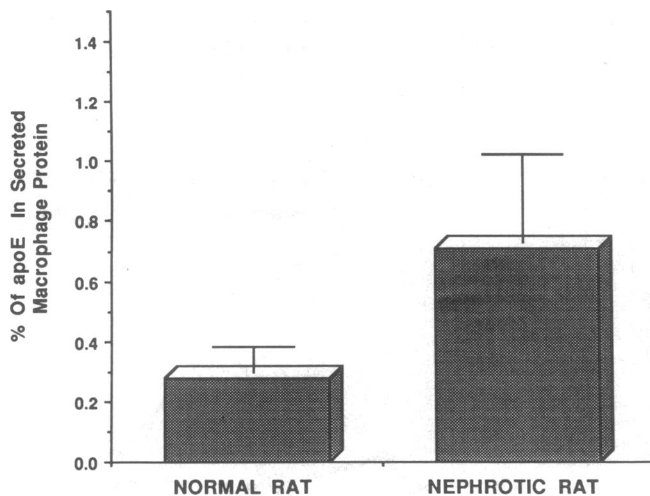


Figure 2. Secretion of [^{35}S]methionine-labeled apoE by normal and nephrotic rat peritoneal macrophages isolated 8 d after injection with puromycin aminonucleoside. Macrophages were incubated with L-[^{35}S]-met (60 μCi/ml) and secreted apoE was precipitated with a monospecific anti-apoE antibody. Results are expressed as the amount of apoE secreted as a percentage of total secreted protein. Error bars are±SD; $n = 8$ for normal rat, and $n = 5$ for nephrotic rat macrophages.

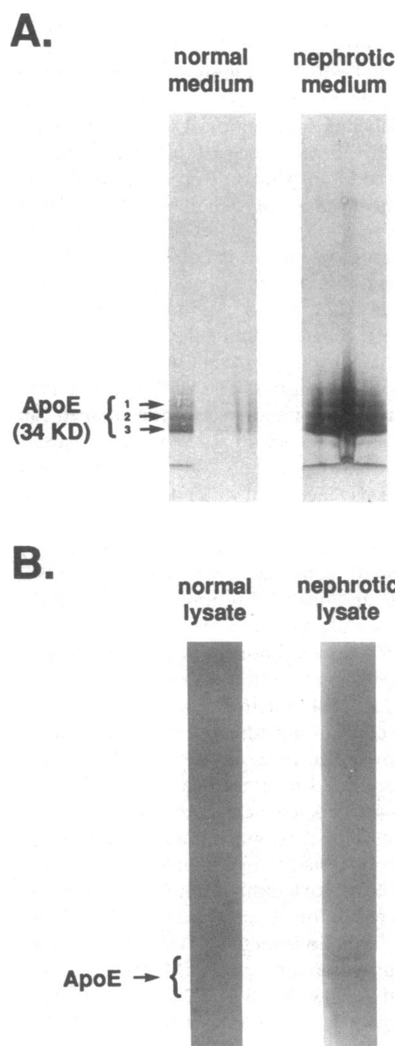


Figure 3. Western blot of secreted protein and cell lysate protein. Secreted protein (*A*) and cell lysate protein (*B*) were harvested from normal and nephrotic rat macrophages (isolated 8 d after puromycin aminonucleoside injection). Protein was dialyzed and blotted to nitrocellulose after electrophoresis through a 10% polyacrylamide gel. The antibody reacted with a single protein band that comigrated with a rat plasma apoE standard.

rats also showed that the macrophages from nephrotic rats secreted increased amounts of immunoreactive apoE. When incubated with the monospecific anti-apoE antibody, the three previously noted apoE isoform bands (22) were detected and were seen to migrate identically with purified rat plasma apoE (Fig. 3). The lowest molecular weight isoform was predominant in both secreted protein from macrophages and apoE from rat plasma. Zannis et al. (22) identified a similar molecular weight polymorphism using two-dimensional gel separation of ^{35}S-labeled apoE secreted by human blood monocytes. Besides the well documented glycosylation of apoE as a basis of its isoform heterogeneity (23, 24), sulfation of apoE could also influence the apparent molecular weight of this protein (25).

No immunoreactive apoE was detectable in normal rat macrophage extracts, testing up to 60 μg of cell lysate protein. In contrast, apoE bands were demonstrable in 24 μg of lysates containing protein from nephrotic rat macrophages. This confirms the results of the immunoprecipitation assays. ApoE biosynthesis was therefore increased in the absence of increased levels of intracellular cholesterol. To determine the site of control of apoE biosynthesis in the stimulated macrophages, we analyzed the steady-state apoE mRNA levels by the Northern blot and solution hybridization techniques.

ApoE mRNA levels increase in nephrotic rat macrophages.

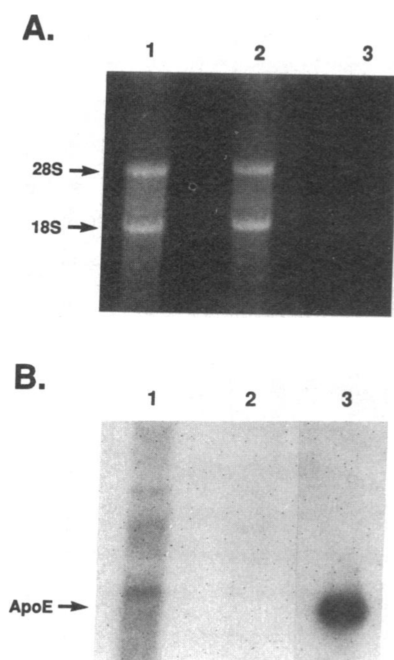


Figure 4. Northern blot analysis of normal rat macrophage RNA from cells incubated overnight with acetylated LDL (lane *1*) or medium alone (lane *2*). The ethidium stained RNA (*A*) and the corresponding RNA blotted to a Nytran membrane and hybridized to a ^{32}P-labeled apoE probe (*B*) are shown. Lane *3* contains 20 μg of RNA from normal rat liver, lanes *1* and *2* contain the RNA harvested from ~ 15 × 10^6 macrophages.

Our approach was to first establish by Northern blots that macrophages from Sprague-Dawley rats expressed apoE mRNA and that apoE expression was responsive to increases in intracellular cholesterol levels. Fig. 4 shows ethidium bromide–stained total RNA and the corresponding Northern blot of this RNA after hybridization with a rat apoE cDNA-labeled probe. By this technique, macrophage apoE mRNA was detected only in cells that had been cholesterol-loaded by incubation with 50 μg/ml acetylated LDL, consistent with previously reported results (26). In cells incubated overnight with 200 μg of acetylated LDL/ml medium, cholesterol levels rose from 34±4 μg cholesterol/mg cell protein (mean±SD, n = 3) to 226±10 μg/mg (mean±SD, n = 3).

Solution hybridization was used to quantitate apoE mRNA levels in macrophages from the nephrotic and normal rats and to determine whether differences in apoE biosynthesis were associated with changes in steady-state apoE mRNA levels. Expression of apoE mRNA by normal Sprague-Dawley rat macrophages was low. Comparison between macrophage and liver apoE mRNA showed that normal rat peritoneal macrophages contained only 0.3 pg apoE mRNA/μg total RNA, whereas rat liver contained 76 pg apoE mRNA/μg total RNA. In the nephrotic rats, apoE mRNA abundance increased to between 2–4% of the liver levels. Expressed in mass units, in normal rat macrophages apoE mRNA represented ~ 0.3 pg mRNA/μg total RNA (n = 2), whereas in the nephrotic animals apoE mRNA was ~ 2.7±1.2 pg mRNA/μg RNA (n = 6, mean±SD).

Morphologic changes in nephrotic rat macrophages correspond to the induction of apoE biosynthesis. Dramatic morphologic changes were consistently found during periods of increased apoE biosynthesis. The cells from severely nephrotic animals were elongated, while those from normal animals were cobblestone shaped (Fig. 5). In animals that were recovering from nephrosis, as judged by serum cholesterol levels, the cells reverted to the cobblestone shape, and secretion of apoE was not increased. By the classical sheep RBC test (10), used to identify macrophage phagocytosis, adherent cells of both morphologies were positive.

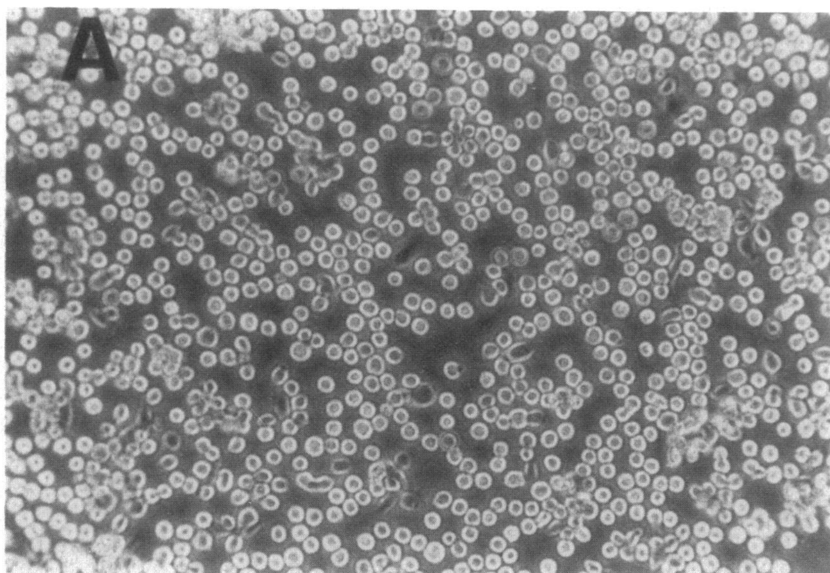
Discussion

Resident macrophages, recovered from the ascites fluid of nephrotic rats, retain normal cholesterol concentrations while simultaneously secreting protein that is enriched in apoE. Immunoreactive apoE secreted by the macrophages from both normal and nephrotic rats was identical in gel migration to that of plasma apoE from normal rats. Rat macrophages also contained apoE mRNA that had an apparent length of ~ 1,200 bases. Although apoE mRNA was not detectable when RNA from ~ 2 × 10^7 macrophages from several rats was isolated, when an equivalent number of macrophages were incubated with 50 μg/ml of acetylated LDL, a strong signal was obtained in Northern blotting. These results were consistent with the solution hybridization measurements, since little detectable apoE mRNA was found in 28 μg of RNA from normal Sprague-Dawley rat macrophages.

Normal rat macrophages responded to cholesterol loading by increasing the abundance of apoE mRNA (Fig. 4), an effect originally observed in mouse peritoneal macrophages (4, 5). The absence of excess cholesterol in nephrotic rat macrophages suggests that an alternate stimulus possibly related to PAN administration, or to the unknown stimulus to hepatic protein synthesis, induces apoE biosynthesis in these cells. In any event, the lack of cellular cholesterol accumulation in the nephrotic macrophages was not due to an artifact in macrophage isolation.

An alternative hypothesis is that increased cellular cholesterol flux maintained normal cholesterol content in the nephrotic macrophages. In this respect, it should be noted that plasma HDL levels in nephrotic rats are elevated (9). Increased uptake of lower density lipoproteins by peritoneal macrophages, which might lead to raised cellular cholesterol content, could therefore have been counterbalanced by an increased efflux rate. The fact that nephrotic hyperlipemia is primarily due to increased hepatic synthesis (27) unaccompanied by β-VLDL formation, makes it less likely that in vivo cell loading with cholesterol actually occurred. A transient rise in cholesterol content, which we may have missed, could have stimulated apoE synthesis with the excess cholesterol leaving the cell as part of an apoE-phospholipid complex. However, in cells loaded with acetylated LDL, apoE leaves the cell independent of cholesterol (28). Therefore, it is unlikely that the elevated secretion of apoE by nephrotic rat macrophages allowed a concomitant efflux of cholesterol. The clear morphologic difference between nephrotic and normal macrophages makes it more likely that a stimulus other than cholesterol was involved. It is possible, for example, that nephrotic rats have increased levels of interleukins or other cytokines that might activate the protein kinase pathways.

In addition to regulation by cholesterol, biosynthesis of apoE by tissue macrophages can be modulated by a variety of factors. For example, endotoxin, BCG, and pyran copolymer all depress apoE synthesis in peritoneal macrophages, although the action of the latter two agents is not specific for apoE (29, 30). ApoE mRNA levels are reduced in rat Kupffer cells by in vivo treatment with endotoxin (19). Conditions that stimulate apoE synthesis in neuronal tissue have also been reported; these include optic nerve degeneration (31) and axonal repair (32, 33), although specific stimulatory agents have not been identified. Using phagocytosis as a marker for macrophage activation, Diamond et al. (34) found that peritoneal macro-



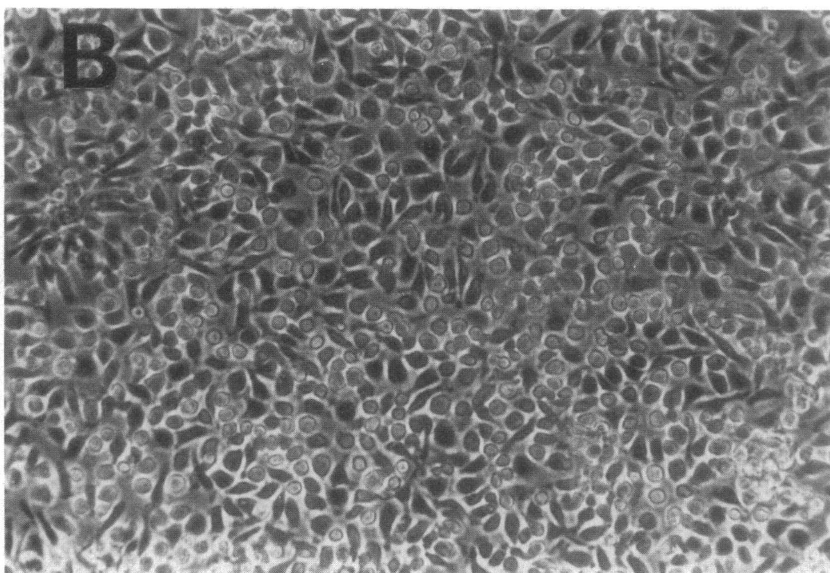


Figure 5. Photomicrograph of resident peritoneal macrophages from normal (*A*) and nephrotic (*B*) rat macrophages (8 d after puromycin aminonucleoside injection).

phages from nephrotic rats were activated 14 d after inducing nephrotic syndrome with PAN. Since cholesterol loading inhibits phagocytosis (35), the cells in the Diamond study most likely had normal cholesterol levels. Taken together, the data from nephrotic rat macrophages suggest that these cells undergo a distinct type of activation that includes increased phagocytosis and increased secretion of apoE.

In the F9 rat embryonal carcinoma cell, apoE synthesis is modulated during cellular differentiation and development (36). Normal and phorbol ester–induced differentiation caused similar increases in apoE synthesis in the mouse monocyte (37) and in the human monocyte cell (38) (in these studies [36, 37, 38], cellular cholesterol content was not measured). Thus, phenomena associated with activation of peritoneal macrophages that may be similar to events occurring in cell differentiation and development possibly result in the increased biosynthesis of apoE we observed. Furthermore, since cholesterol can modulate the protein kinase second messenger system, a common pathway for induction of apoE synthesis and macrophage activation could involve independent effects by cholesterol or other molecules on both protein kinases A and C (39). Whether these activated macrophages are involved in atherogenesis or in the renal glomerular damage itself awaits further investigation.

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