A cDNA-dependent scintillation proximity assay for quantifying apolipoprotein A-I

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Abstract We have developed a cDNA-dependent scintillation proximity assay (SPA) for rabbit apolipoprotein A-I that follows a classic radioimmunoassay scheme, in that antiserum and radiolabeled ligand are used in a process to quantify a source containing unlabeled ligand. To synthesize radiolabeled ligand we isolated a full-length rabbit apolipoprotein A-I (apoA-I) cDNA, transcribed the corresponding RNA in vitro, and synthesized radiolabled apoA-I by including tritiated leucine in an in vitro translation reaction. Assay conditions were established which allowed quantification of unlabeled apoA-I over a range of 0.2 to 4 nanograms with intra- and interassay coefficients of variation of 5% and 10%, respectively. Purified rabbit apoA-I, apoA-I in rabbit liver parenchymal cell conditioned media, and apoA-I contained in rabbit plasma all generated parallel titration curves. Quantification of rabbit plasma apoA-I was not affected when sheep anti-rabbit apoA-I serum was mixed with sheep anti-rabbit apoB or apoE serum; thus, the antibody need not be specific to quantify the ligand of interest. To show utility of the assay, apoA-I mass was quantified in in vitro and in vivo models displaying altered apoA-I levels. In each model apoA-I values from the cDNA-dependent SPA and the established methodologies of Western blotting and electroimmunodiffusion were highly correlated. The approach outlined in this report should permit rapid development of scintillation proximity assays for other proteins given the widespread availability of full-length cDNAs.—**Hanselman, J. C., D. A. Schwab, T. J. Rea, C. L. Bisgaier, and M. E. Pape.** A cDNA-dependent scintillation proximity assay for quantifying apolipoprotein A-I. *J. Lipid Res.* 1997. **38:** 2365–2373.

Supplementary key words parenchymal cells • liver • hepatocytes • immunoassay • antibody • rabbit

Lipoproteins are plasma particles that transport cholesterol and other lipids between tissues. In addition to lipid, lipoproteins are coated with amphipathic apolipoproteins which serve to *1*) solubilize the particles in the aqueous environment of plasma and maintain lipoprotein structure including size and composition, *2*) mediate binding to appropriate cellular receptors, and *3*) co-activate other plasma and tissue-bound enzymes involved in lipoprotein remodeling. Although lipoproteins are a heterogeneous collection of particles, two major types can be distinguished with respect to apolipoproteins: apolipoprotein B (VLDL, IDL, LDL) and apolipoprotein A-I (HDL)-containing particles. The reduction of apoB and elevation of apoA-I in plasma is desirable because this apolipoprotein profile is associated with reduced risk of coronary heart disease. Thus, accurate quantification of apolipoproteins in both plasma and cell culture systems is important for understanding factors that regulate lipid metabolism.

Over the last several decades several quantitative immunological methods have been developed to measure apolipoproteins. Early methods used diffusional techniques through agarose and include radial immunodiffusion (1) and immunoelectrophoresis (2). These techniques are still used today and results can be visually assessed. They are somewhat limited in that the methods not only require a considerable amount of antiserum but they generally suffer from a lack of sensitivity. Methods such as ELISA (3), Western blotting (4), and radioimmunoassays (RIA) (5) have overcome some of these limitations. RIA in particular proved to be a vast improvement in sensitivity while also conserving valuable antiserum. However, the method requires highly purified radiolabeled ligand or a monospecific polyclonal antiserum. In general, RIA has utilized iodinated ligands which have limited shelve life, although recent use of ^{3}H- or ^{35}S-labeled protein ligands generated by in vitro translation has overcome this limitation (6). In this report, we describe an assay that follows the basic

Abbreviations: apoA-I, apolipoprotein A-I; SPA, scintillation proximity assay.

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RIA scheme and not only takes advantage of ^{3}H-labeled protein ligand produced by in vitro translation but also the relatively new scintillation proximity technology (7, 8). Indeed, quantification of apolipoproteins by scintillation proximity assay (SPA) has not been reported.

A key feature of SPA is the use of scintillant-impregnated microporous beads that emit light when radiolabel is brought in close proximity to the scintillant. Modification of these beads has allowed the generation of numerous assays for a variety of biological ligands and enzymatic activities (9–16). In one application of the SPA the beads are coated with antibodies to the antigen of interest and then in a classic radioimmunoassay scheme, the concentration of an unknown sample is determined by displacing or competing out radiolabled ligand. As only radiolabeled ligand bound to antibody-coated beads will induce light emission, there is no need to separate bound from free ligand. Using this scintillation proximity technology, we report here a cDNA-dependent scintillation proximity assay to quantify rabbit apolipoprotein A-I.

MATERIALS AND METHODS

Materials

Polyvinyl toluene (PVT) SPA fluoromicrospheres coated with anti-sheep IgG antibodies were purchased from Amersham (Arlington Heights, IL).

Rabbit apolipoprotein isolation and production of antibodies in sheep

ApoA-I was isolated from the d 1.063–1.21 g/ml fraction of normal rabbit plasma. The fraction was dialyzed against 10 mM Tris-HCl, pH 8.6, 1 mM EDTA and subject to preparative (approximately 20 mg protein/gel) SDS-polyacrylamide gel electrophoresis under reducing conditions according to the procedure of Laemmli (17). Increased opacity of the protein bands in the unfixed gels was visualized by soaking the gel in ice-cold 0.25 M KCl (10–15 min) followed by soaking in water (18). The wide apoA-I band in the gels was conservatively excised and subject to electroelution in Laemmli gel buffer (25 mM Tris-HCl, 192 mM glycine, 0.1% SDS, pH 8.6). Purity of electroeluted apoA-I was confirmed by analytical SDS-PAGE. ApoA-I mass was quantified by the method of Lowry et al. (19). Polyclonal antiserum was prepared after four subcutaneous injections consisting of approximately 2 mg apoA-I in Freund's Complete Adjuvant (initial injection) and Incomplete Adjuvant (final three injections) (20) over a 6-month period. Sheep were bled after the third and fourth injection. The specificity of the apoA-I antisera lot used in these studies was shown to be specific when assessed by Western blotting against whole rabbit plasma.

ApoE was isolated from the delipidated fraction (d < 1.006 g/ml) prepared from cholesterol-fed rabbits. The procedures for isolation of purified apoE and antibody production in sheep were similar to the method used for apoA-I. Antibodies to rabbit apoB were made in sheep immunized with rabbit LDL (d 1.020–1.050 g/ml) and were prepared by Dr. Joseph Cornicelli (Parke-Davis/Warner-Lambert).

Cloning of full-length rabbit apoA-I cDNA

A full-length cDNA clone for rabbit apoA-I was isolated by reverse transcriptase-based polymerase chain reaction (RT-PCR). The following upstream and downstream primers, respectively, were designed based on the published sequence (21): 5′-ATGGGATCCAGA CTGCTTGGAGAGCTCCAG-3′ and 5′-ACCATCGAT $(T)_{20}$CCCCCCAAGTTGTAAACTAC-3′. The upstream primer encodes a BamHI site at the 5′-end while the downstream primer encodes a ClaI site at its 5′-end. To synthesize an apoA-I cDNA, total rabbit liver RNA and the downstream primer were subjected to reverse transcription (GeneAmp, RNA PCR Kit, Perkin-Elmer). The product was amplified using the upstream primer and Taq polymerase in a PCR protocol of 94°C for 30 sec and 67°C for 3 min (35 cycles) in the GeneAmp PCR System 9600. The amplified product (approximately 1000 bp) was digested with BamHI and ClaI, purified by agarose gel electrophoresis, cloned directionally into pBluescript II SK+ (also digested with BamHI and ClaI), and confirmed by DNA sequencing. The plasmid containing the entire cDNA coding region for rabbit apoA-I was called pApoAI-FL/Rb.

[^{3}H]apoA-I synthesis by in vitro transcription and translation

pApoAI-FL/Rb was linearized with ClaI and in vitro transcribed with a T3 Ribomax Kit as suggested by the manufacturer (Promega Biotech, Madison, WI). Rabbit apoA-I RNA was translated in vitro essentially as suggested by the manufacturer (Promega Biotech, Madison, WI). Briefly, the 250 µl reaction consisted of 175 µl of rabbit reticulocyte lysate, 125 µCi L-[4,5-^{3}H]leucine (139 Ci/mmol), 25 µg rabbit apoA-I RNA, and leucine-free translation buffer provided with the kit. The reaction was incubated for 1 h at 30°C. Percent incorporation of [^{3}H]leucine was assessed using trichloroacetic acid precipitation and scintillation counting whereby the mass of apoA-I synthesized was estimated based on the known leucine content of rabbit apoA-I (37 leucine

residues/molecule). Radiolabeled apoA-I was separated from unincorporated [^{3}H]leucine by Sephadex G50 gel chromatography (Pharmacia, Piscataway, NJ) in phosphate-buffered saline (PBS) containing 1% BSA. Fractions containing radiolabeled apoA-I were pooled and used for development of the cDNA-dependent SPA method. It should be noted that Wheat Germ Translation Extracts (Promega Biotech, Madison, WI) did not yield full-length apoA-I translation products.

Isolation and culturing of rabbit liver cells

Isolation and culturing of rabbit parenchymal and nonparenchymal cells has been described in detail elsewhere (22, 23). Briefly, nonparenchymal cells were isolated and 6×10^7 cells were plated on 10-cm dishes in 10-ml of media. After 24 h conditioned media were collected and an equal volume of 100% ethanol was added to precipitate protein. After centrifugation to obtain the protein precipitant, the pellet was resuspended in DMEM and extensively dialyzed against DMEM. Resuspension volume corresponded to 1 ml DMEM per 10-cm dish used, which was arbitrarily set as a 10× concentrate. The bioassay for apoA-I inhibitory activity was performed by plating parenchymal cells in this resuspended and dialyzed media as previously described (23).

Animals and diets

Male New Zealand White rabbits (1.5–2 kg; Kuiper, Gary, IN) were housed individually in a temperature-controlled room with a 12-h light/12-h dark cycle with lights on at 6 AM. Rabbits were meal-fed (between 6 AM and 8 AM) 100 g/day control chow (Purina 5321) or chow supplemented with 14% coconut oil for 4 weeks. At the end of the study, blood from animals fasted 24 h was collected in tubes containing 2 mM EDTA from which plasma was prepared for measurement of apoA-I. All animal procedures were performed in accordance with NIH and Warner-Lambert Parke-Davis laboratory animal treatment guidelines.

Western blotting and immunoelectrophoretic assays

For Western blotting (4), 10 µl (1/150 of media from 1×10^6 cells) of media from parenchymal cells treated with various concentrations of nonparenchymal cell conditioned media was separated on a 10% Tricine SDS-PAGE (Novex, San Diego, CA) under reducing conditions. The separated proteins were transferred to nitrocellulose using a Bio-Rad Transfer Apparatus (Bio-Rad, Hercules, CA) and a Tris-glycine buffer (12 mM Tris-base, 96 mM glycine, pH 8.5) containing 20% methanol (4). To detect apoA-I, nitrocellulose was blocked with Blotto (50 mM Tris, pH 8.0, containing 2 mM $CaCl_2$, 0.2% NP-40, 0.01% Antifoam A, and 5% (w/v) Carnation Dry Milk) for 30 min, treated with a 1:1,000 dilution of sheep anti-rabbit apoA-I serum in buffer containing 50 mM Tris, pH 8.0, with 150 mM NaCl and 0.05% Tween 20, then incubated with a 1:3,000 dilution of rabbit anti-sheep IgG conjugated with alkaline phosphatase (Pierce, Rockford, IL), and developed using NBT/BCIP as substrate.

For the immunoelectrophoretic assay, the method of Laurell (2) was essentially followed. Briefly, a solution containing 1% agarose (SeaKem ME Agarose, FMC), 2% polyethylene glycol, and 1% sheep anti-rabbit apoA-I serum was prepared in TTL buffer (80 mM Tris-base, 25 mM Tricine, containing 1.4 mM calcium lactate, pH 8.6) to cast the gel. To prepare plasma samples, 15 µl of plasma was mixed with 485 µl TTL buffer and 500 µl of 8 M urea before heating at 37°C for 60 min, after which 4 µl was loaded onto the agarose gel. After electrophoresis, the gel was stained with amido black and "rocket heights" were determined for quantification purposes. A standard rabbit plasma was used to generate a standard curve for each gel. The mass of apoA-I in the rabbit plasma standard was determined using the cDNA-dependent SPA with pure rabbit apoA-I as standard.

SPA for apoA-I

To prepare the anti-sheep IgG SPA beads for the assay, the lyophilized SPA beads were resuspended in 1× Bead Storage solution (50 mM Tris, 2 mM leucine, 1% BSA, 150 mM NaCl, 5 mM EDTA, pH 7.4) at a concentration of 15 mg/ml. Purified [^{3}H]apoA-I was diluted to 400 dpm/µl in 2× NETTALS (225 mM NaCl, 10 mM EDTA, 100 mM Tris, 2.5% Triton X-100, 2% albumin (bovine), 2 mM leucine, 1.25% SDS, pH 7.4) and sheep anti-rabbit apoA-I serum was diluted 5,000-fold in 2× NETTALS for subsequent use in the SPA. The assay was carried out in 96-well polyethylene terephthalate sample plates (Wallac, Turku, Finland) by incubating the sample containing unlabeled apoA-I and sheep anti-rabbit apoA-I serum for 1 h at room temperature (100 µl volume in 1× NETTALS) prior to adding 0.75 mg SPA beads and 20,000 dpm [^{3}H]apoA-I to a final volume of 200 µl in 1× NETTALS. After the appropriate incubation time, the sample plates were centrifuged for 15 min at 2500 rpm and each well was counted for 1 min in a Microbeta Counter (Wallac model 1450) standardized for ^{3}H counting as indicated by the manufacturer. We observed that even calibrating the microbeta reader as suggested by the manufacturer did not correct for the quenching effect of phenol red contained in most culture media. We advise additional experiments to determine the quenching effect of phenol red-containing solutions.

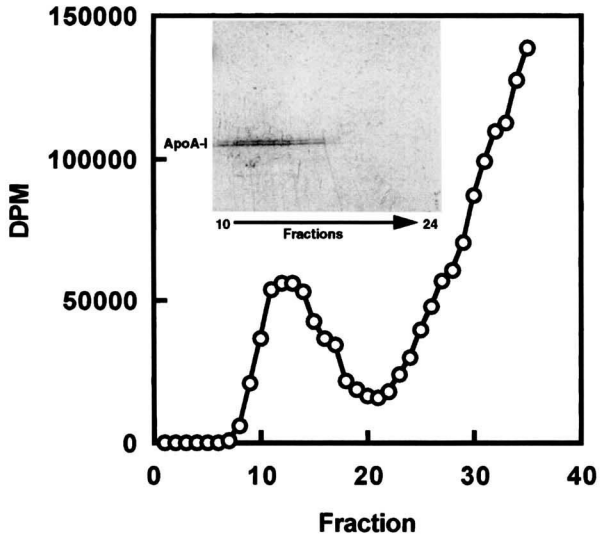

Fig. 1. Isolation of [^{3}H]apoA-I by size exclusion chromatography. An RNA containing the entire coding region of rabbit apo-I was translated in vitro in the presence of [^{3}H]leucine. Reaction products were separated on Sephadex G-50 and individual fractions were counted; the results are shown here. The first peak corresponds to synthesized protein and the second peak is unincorporated [^{3}H]leucine. The inset is a fluorograph of the radiolabeled proteins in the first peak after SDS-PAGE. The apoA-I synthesized is 28 kDa.

RESULTS

A plasmid containing the full-length apoA-I cDNA was used in an in vitro transcription reaction to generate a single RNA species of predicted size (approximately 1 kb) as assessed by agarose gel electrophoresis (data not shown). This RNA was translated in vitro in the presence of [^{3}H]leucine and this de novo synthesized apoA-I was separated from unincorporated [^{3}H]leucine by size exclusion chromatography (**Fig. 1**). SDS-PAGE followed by fluorography revealed that the in vitro translation reaction produced radiolabeled protein of MW_r 28 KDa (Fig. 1, inset) which appeared as a doublet. The apoA-I doublet is likely due to the presence of immature apoA-I (prepro or pro apoA-I) and not an alternative start site codon as the only other methionine in apoA-I is at amino acid 109. Because the translation system uses rabbit reticulocytes as the source of translation factors, it is possible that incomplete processing occurred in the lysate preparation. Indeed, the lysate probably contains many plasma factors as we found it contained rabbit apoA-I at a concentration of about 7 ng/ul. Nonetheless, after correcting for the amount of rabbit apoA-I that was endogenous to the rabbit reticulocyte lysate, the specific activity of [^{3}H]apoA-I was 5.0×10^3 dpm/ng.

To develop the cDNA-dependent SPA, several parameters were varied including the antigen/antiserum ratio, SPA-bead concentration, incubation time, and assay buffer. For determining the optimum antigen/antiserum ratio it was necessary to define conditions where [^{3}H]apoA-I was in excess of antibody and sufficient scintillation counts were generated from the [^{3}H]apoA-I/SPA bead interaction. This was important because SPAs have a maximum efficiency of 40% due to the two-phase nature of the system. **Figure 2A** shows that increasing the amount of antiserum relative to a fixed amount of [^{3}H]apoA-I (20,000 dpm representing 4 ng of apoA-I) resulted in a plateau at which point the maximum apoA-I signal could be detected. This plateau corresponds to about 4000 dpm or about 20% efficiency. We chose a [^{3}H]apoA-I mass of 4 ng (20,000 dpm) for all subsequent experiments as a reasonable detection window was established. The data (Fig. 2A) also indicated that 10 nl of antiserum bound only 75% of the [^{3}H]apoA-I available, i.e., the [^{3}H]apoA-I antigen is in excess of antibody.

To determine the amount of anti-sheep IgG SPA beads to use in the assay, increasing amounts of beads were added to a mixture containing 20,000 dpm [^{3}H]apoA-I and 10 nl antiserum and incubated for 24 h. An SPA bead amount of 0.25 mg was sufficient to bind to all the antigen/antibody complexes; we chose three times this amount for all subsequent assays to ensure complete capture of the sheep antibody (Fig. 2B).

To determine the kinetics of binding, 20,000 dpm [^{3}H]apoA-I, 10 nl antiserum, and 0.75 mg SPA-beads were incubated for various time periods before counting. Figure 2C indicates that by 3 h the ternary complex has been completely formed. For all subsequent assays we chose 3 h before counting the plates.

To determine that the increased [^{3}H]apoA-I signal was due to anti-apoA-I antibodies and not a nonspecific component in the sheep serum, various dilutions of sheep non-immune serum were incubated with 20,000 dpm [^{3}H]apoA-I and 0.75 mg SPA-beads. The signal from the non-immune serum was less than 1% of the antiserum at the plateau of the curve (Fig. 2D).

Further studies investigated the effects of detergents, protease inhibitors, and denaturants on the SPA. The buffer previously used for immunoprecipitations (23), which contained 1.25% Triton-X-100 and 0.625% SDS, appeared to give satisfactory SPA results. Nonetheless, we tested the effect of EDTA, Trition-X-100, and SDS on the SPA at concentrations up to 20 mM, 5%, and 2.5%, respectively; none of these agents interfered with the assay (data not shown). In addition, the protease inhibitors AEBSF, benzamidine, pepstatin A, and aprotinin did not interfere with the assay (data not shown). However, 1 M urea dramatically interfered with the assay probably due to protein denaturation. Guanidine-HCl

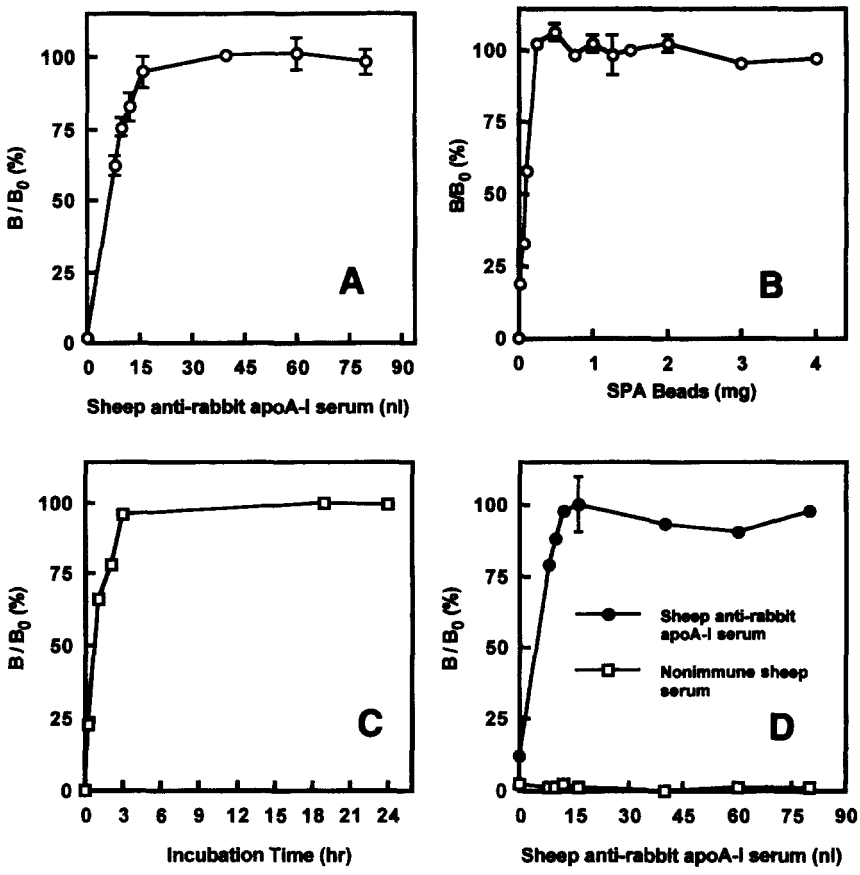

Fig. 2. (A) Determination of the optimum antigen/antiserum ratio for the apoA-I cDNA-dependent SPA. [^{3}H]apoA-I (20,000 dpm) was incubated with 1 mg of Scintillation Proximity Assay Beads and various dilutions of sheep anti-rabbit apoA-I serum for 24 h. (B) Determination of optimum amount of anti-sheep IgG SPA beads. [^{3}H]apoA-I (20,000 dpm) was incubated with 10 nl of sheep anti-rabbit apoA-I serum and various amounts of SPA beads for 24 h. (C) Determination of optimum incubation time. [^{3}H]apoA-I (20,000 dpm) was incubated with 10 nl of sheep anti-rabbit apoA-I serum and 0.75 mg of anti-sheep IgG SPA beads for various times. (D) Determination of background counts from non-immune sheep serum. [^{3}H]apoA-I (20,000 dpm) was incubated with 0.75 mg of anti-sheep IgG SPA beads and various amounts of sheep anti-rabbit apoA-I serum or sheep non-immune serum and incubated for 24 h.

up to 20 mM did not affect the assay (data not shown). Based on these data, the immunoprecipitation buffer containing 1.25% Triton-X-100 and 0.625% SDS was selected as the SPA assay buffer (see Materials and Methods).

To develop a quantitative assay, an antibody titration protocol was developed using the optimized conditions. Purified apoA-I was preincubated with antiserum for 1 h and then [^{3}H]apoA-I and SPA beads were added followed by a 24 h incubation. With this protocol, antibody binding sites not occupied by purified apoA-I in the preincubation are filled with [^{3}H]apoA-I thus producing a titration curve as shown in **Fig. 3A.** Transformation of the data into logit form (logit = log $((B/B_O)/(1 - B/B_O))$ (24) permitted the generation of a linear standard curve over 1.5 logs with a quantitative range of 0.2 to 4 ng and an r value of 0.995 (Fig. 3B). This upper limit is consistent with an initial input of 4 ng apoA-I which appeared to be a mass in slight molar excess of antibody binding sites (Fig. 2A). Additional studies showed no difference between a 1-h or 3-h pre-incubation of unlabeled apoA-I with antiserum followed by the addition of [^{3}H]apoA-I and SPA-beads (data not shown). These data led us to the standard assay protocol: *1*) a 1-h pre-incubation of unlabeled apoA-I with 10 nl sheep anti-rabbit apoA-I serum, *2*) addition of 20,000 dpm [^{3}H]apoA-I (5×10^3dpm/ng) and 0.75 mg anti-sheep IgG SPA beads, and *3*) a 3-h incubation at room temperature.

Titration curves of purified, cultured parenchymal cell, and plasma apoA-I were parallel indicating that the apoA-I from the various sources reacted in an immuno-

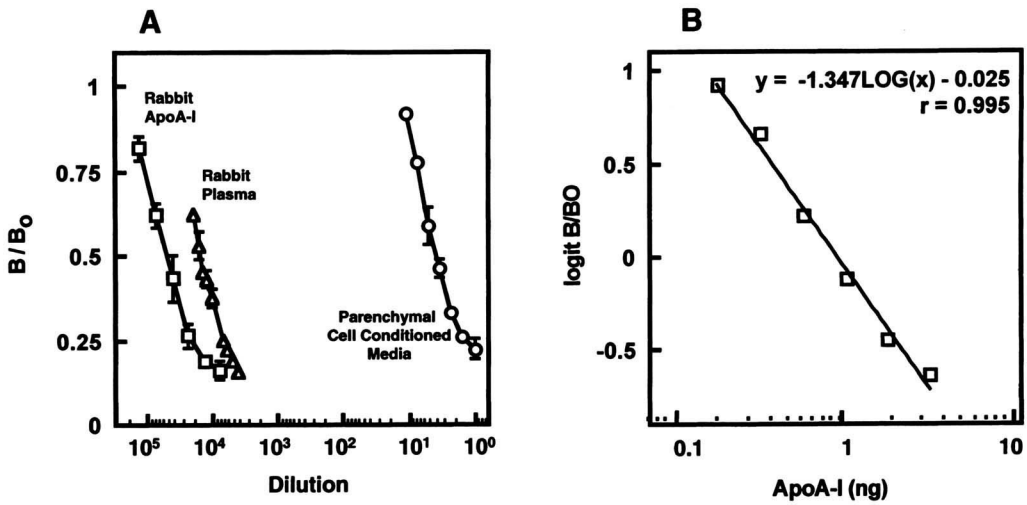

Fig. 3. (A) Parallel titration curves for pure apoA-I, apoA-I contained in rabbit plasma, and apoA-I secreted from cultured hepatocytes. The initial concentration of pure apoA-I used for that dilution was 890 μg/ml. (B) Transformation of the "pure apoA-I" data in panel A to logit form (24) established a linear relationship between apoA-I mass and radioactive counts from the assay.

logically similar fashion (Fig. 3A). Further characterization of the assay showed an inter- and intra-assay coefficient of variation of 5% and 10%, respectively (data not shown).

The apoA-I cDNA-dependent SPA was compared with established methodologies. We chose both in vitro and in vivo models known to vary apoA-I mass with specific treatments. We have described an in vitro model whereby apoA-I secretion in rabbit liver parenchymal cells is inhibited by a factor secreted by hepatic nonparenchymal cells (23). Previously, immunoprecipitation methods were used to monitor apoA-I secretion. In the current report, we applied the apoA-I cDNA-dependent SPA and conventional Western blot analysis to this model. Both methods measured a similar marked reduction in parenchymal cell secretion of apoA-I caused by the paracrine factor (**Fig. 4**).

Coconut oil-fed rabbits have elevated plasma apoA-I compared to chow-fed animals (25, 26). We used this model to generate a range of apoA-I plasma levels to

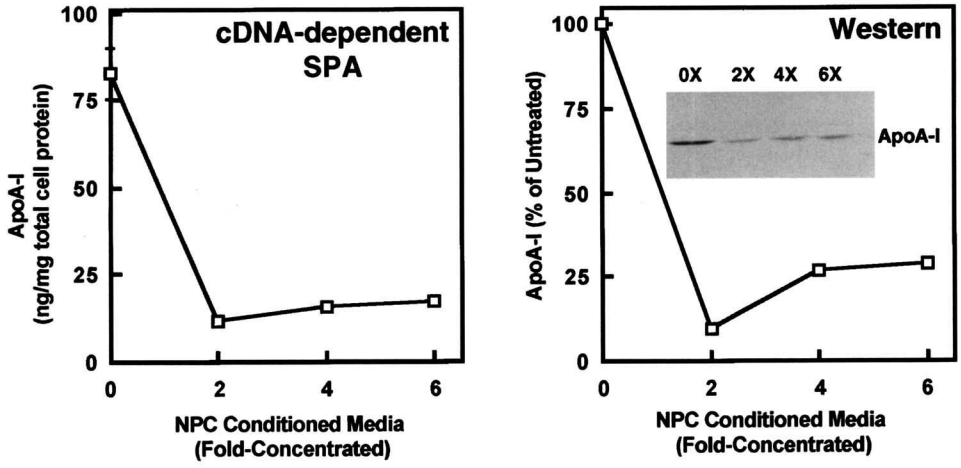

Fig. 4. Comparison of apoA-I mass determination using cDNA-dependent SPA and Western blotting. Rabbit nonparenchymal cell conditioned media was collected and used to treat parenchymal cells. After 24 h treatment media was analyzed for secreted apoA-I protein using either the cDNA-dependent SPA (left panel) or Western blotting (right panel). The Western blot was scanned using a laser densitometer to obtain relative levels of apoA-I in the samples. Note, error bars for the cDNA-dependent SPA data at 2-, 4-, and 6-fold concentrated are contained within the symbols and thus not visible.

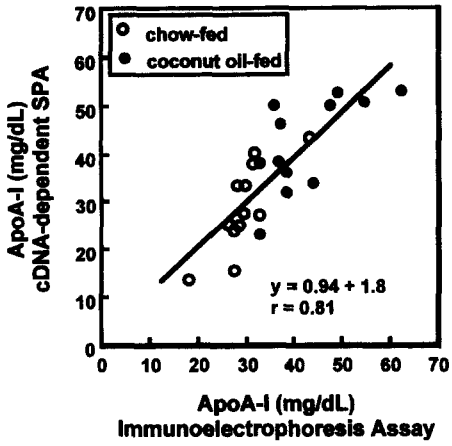

Fig. 5. Correlation between the cDNA-dependent SPA and Immunoelectrophoresis assay for apoA-I mass in rabbit plasma. Rabbits were fed a chow diet (n = 12) or a chow diet containing 14% coconut oil (n = 12) for 4 weeks. At that time plasma was collected and plasma apoA-I mass was quantified using either the cDNA-dependent SPA or immunoelectrophoresis assay.

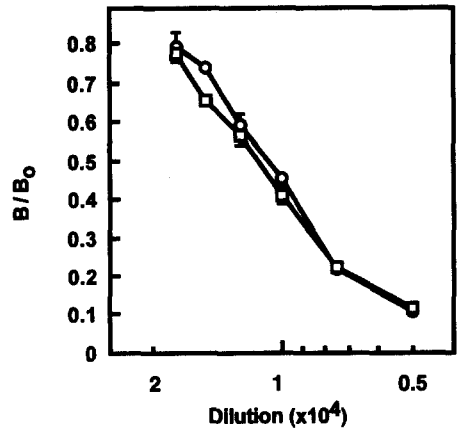

Fig. 6. The presence of contaminating antibodies does not alter the quantification of apoA-I using the cDNA-dependent SPA. Rabbit plasma was diluted and apoA-I was measured in the standard SPA protocol except the incubations contained 10 nl sheep anti-rabbit apoA-I serum and 20 nl of nonimmune serum (□) or 10 nl each of sheep anti-rabbit apoA-I serum, sheep anti-rabbit apoB serum, and sheep anti-rabbit apoE serum (○). Thus, each incubation contained a final volume of 30 nl of sheep serum. The x-axis in log scale.

compare an immunoelectrophoretic assay to the apoA-I cDNA-dependent SPA. As shown in **Fig. 5,** samples taken from 24 animals resulted in the measurement of apoA-I levels over a 6-fold range. Regression analysis indicated an *r* value of 0.81 between the two assays. Furthermore, the 43% increase in plasma apoA-I levels after coconut oil-feeding was observed using either method (**Table 1**).

The absolute mass (mg/dl) of New Zealand White rabbit plasma apoA-I in chow-fed animals using the apoA-I cDNA-dependent SPA was 28.9 ± 2.7 (n = 12) in close agreement with the value of 29 ± 4.3 (n = 10) reported by Mezdour et al. (27) using an ELISA. Both of these values are significantly lower than the 103 ± 4 mg/dl (n = 4) reported by Carlson and Knottke (26) using a radioimmunoassay. Differences in absolute apoA-I plasma values may be due to animal variability, assay variability, the source of apoA-I standards, or methods used to measure absolute mass of the apoA-I standards.

One benefit of generating a pure radiolabeled ligand is the ability of the assay to quantify a ligand of interest with antiserum containing antibodies to several proteins. To demonstrate this benefit we purposely combined sheep anti-rabbit apoA-I serum with non-immune sheep serum or sheep anti-rabbit apoB or apoE serum. It was apparent that antibodies to these other apolipoproteins had no affect on the titration curve for rabbit plasma apoA-I (**Fig. 6**). These data indicate that in vitro synthesis of a radiolabeled ligand derived from cDNA is sufficiently pure to allow quantification of ligand even though the host antiserum contains antibodies to more than one protein from the target species.

TABLE 1. Comparison of data from an apoA-I immunoelectrophoresis assay and the apoA-I cDNA-dependent SPA in chow-fed and coconut oil-fed rabbits

Treatment	n	Immunoelectrophoretic Assay	cDNA-Dependent SPA
		mg/dl	*mg/dl*
Chow-fed	12	29.6 ± 1.6	28.9 ± 2.7
Coconut oil-fed	12	42.4 ± 2.7[a]	42.2 ± 2.8[a]

Data are expressed as mean ± SEM.
[a] $P < 0.005$ compared to chow-fed.

DISCUSSION

In this report we have described a sensitive immunological method that has broad applicability to quantification of many other proteins. In addition to using scintillation proximity technology, a key feature of our approach is the synthesis of pure radiolabeled ligand by in vitro translation. In all immunological assays to quantify antigen it is necessary to have either a purified antigen or specific antibody. ELISA, immunodiffusion, immunoturbidometric, and radioimmunoassays are all dependent on a specific antibody that is generated by injecting isolated antigen into recipient animals. If a homogeneous antigen preparation cannot be obtained, each assay will be partially compromised by measuring the level of contaminating proteins. Synthesis of a radiolabeled antigen from an RNA containing the coding sequence overcomes this problem.

There are at least two instances where this cDNA-based approach would be very advantageous. First, isolation of membrane-bound proteins and subsequent generation of antibodies can prove problematic. However, if a cDNA for a membrane-bound protein is available, the radiolabeled antigen can be easily synthesized in the presence of microsomal membranes (28) and unknown nonradioactive ligand mass can be quantified in a cDNA-dependent SPA using antiserum derived from a crude membrane preparation. Scintillation technology is able to tolerate several detergents that would aid in developing assays for membrane-bound proteins. Second, the recent advances in sequencing entire genomes of organisms and the future prospect of having the entire human genome sequence necessitates methods for quantifying the protein levels for the various open reading frames that have been and will be identified. In theory, a full-length cDNA would not be required to develop the SPA. In addition to the numerous cDNAs, one can envision banks of crude antiserum to whole organisms or specific organs that could be used to quantify levels of potentially interesting proteins by cDNA-dependent SPAs. Such assays would prove valuable in evaluating the role of these proteins in various nutritional, pharmacological, hormonal, or pathological states.

Perceived limitations of the cDNA-dependent SPA approach may be that sufficient ligand cannot be synthesized in vitro to achieve a reasonable signal in the SPA. This may be overcome by producing a higher specific activity ligand by including a mixture of radiolabeled amino acids in the in vitro translation reaction. Additionally, one can alter the antigen–antibody ratio by either reducing the amount of antibody to ensure antigen excess or adding unlabeled antigen to the reaction. In either case, the unlabeled antigen need not be pure.

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