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# Influence of Lipoproteins on Renal Cytotoxicity and Antifungal Activity of Amphotericin B

KISHOR M. WASAN,[1]† MICHAEL G. ROSENBLUM,[2] LAWRENCE CHEUNG,[2] AND GABRIEL LOPEZ-BERESTEIN[1]*

*Immunobiology and Drug Carriers Section, Department of Clinical Investigations,[1] and Department of Clinical Immunology and Biological Therapy,[2] The University of Texas M. D. Anderson Cancer Center, Houston, Texas 77030*

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**We examined the influence of high-density lipoproteins (HDLs) and low-density lipoproteins (LDLs) on the toxicity of amphotericin B (AmpB) to fungal and renal cells. *Candida albicans* was incubated for 18 h at 37°C with AmpB and deoxycholate (Fungizone) or liposomal AmpB (L-AmpB) (0.1 to 2.0 μg of AmpB per ml) in the presence or absence of HDLs or LDLs (0.5 mg of protein per ml). The MICs of AmpB and L-AmpB, whether or not HDLs or LDLs were present, were similar. LLC PK1 renal cells, derived from primary cultures of pig proximal tubular cells, were incubated for 18 h at 37°C in serum-free medium that contained AmpB and deoxycholate or L-AmpB at 20 μg of AmpB per ml, HDLs or LDLs at 0.5 mg of protein per ml, mixtures of AmpB with HDLs or LDLs, and mixtures of L-AmpB with HDLs or LDLs. HDL-associated AmpB was less toxic than AmB to LLC PK1 cells (53.0% ± 2.5% versus 81.3% ± 3.6% cytotoxicity; $P = 0.01$), while LDL-associated AmpB was as toxic as AmpB. L-AmpB, HDL-associated L-AmpB, and LDL-associated L-AmpB were less toxic to LLC PK1 cells than was AmpB (48.3% ± 1.5%, 25.5% ± 2.2%, and 52.2% ± 2.5% versus 81.3% ± 3.6% cytotoxicity; $P = 0.02$). To further understand why HDL-associated AmpB reduced renal cytotoxic effects, the LLC PK1 cells were examined for the presence of HDL and LDL receptors. LLC PK1 cells expressed high-affinity ($K_d$ = 0.0538 ng/ml; 96,000 sites per cell) and low-affinity ($K_d$ = 222.22 ng/ml; 77 sites per cell) LDL receptors but only a low-affinity HDL receptor ($K_d$ = 71.43 ng/ml; 2 sites per cell). HDL-associated AmpB and LDL-associated AmpB were less toxic than AmpB to trypsinized LLC PK1 cells (46.6% ± 10.9% and 16.8% ± 15.98% versus 74.7% ± 7.7% cytotoxicity; $P = 0.02$). HDL-associated AmB and LDL-associated L-AmpB were also less toxic than AmpB to the cells (20.4% ± 6.2% and 13.5% ± 8.6% versus 74.7% ± 7.7% cytotoxicity; $P = 0.01$). The antifungal activities of AmpB and L-AmpB were not altered in the presence of HDLs or LDLs. We conclude that the reduced nephrotoxicity associated with the use of L-AmpB is related to a decreased uptake of AmpB by renal cells when AmpB is associated with HDLs because of the low level of expression of HDL receptors in these cells.**

Amphotericin B (AmpB) remains one of the drugs of choice in the treatment of fungal infections, although its use is limited by dose-dependent nephrotoxicity (5). When AmpB is incorporated into liposomes composed of dimyristoylphosphatidylcholine (DMPC) and dimyristoylphosphatidylglycerol (DMPG), it is as effective as, but less toxic than, the commercially available form of AmpB (Fungizone; which consists of AmpB and deoxycholate) in animal models (1, 13, 14). Previous studies have demonstrated a decrease in the cytotoxicity of AmB when it is delivered in the form of liposomal AmpB (L-AmpB) to LLC PK1 cells (a pig kidney epithelial cell line) (8, 12) and primary cultures of rabbit proximal tubule cells (7). To date, the mechanisms that result in the decreased renal cytotoxic effects of L-AmpB are not fully understood. Krause and Juliano (12) have suggested that the decreased toxicity of L-AmpB compared with that of AmpB is related to a selective transfer of the drug from liposomes to fungal cell membranes but not to mammalian cell membranes.

Brajtburg and coworkers (3, 4) demonstrated that AmpB is highly bound to plasma lipoproteins (3) and that the AmpB-induced cytotoxic effects on mammalian erythrocytes but not *Candida albicans* cells decrease in the presence of either high-density lipoproteins (HDLs) or low-density lipoproteins (LDLs) (4). We have previously demonstrated that AmpB predominantly associates with HDLs in human serum after 1 h of incubation at 37°C (18) and that the amount of AmpB associated with HDLs increases when AmpB is incorporated into liposomes composed of DMPC and DMPG (7:3; wt/wt).

In the present studies, we examined the influence of AmpB's association with HDLs and LDLs on the cytotoxic effects of AmpB on *C. albicans* fungal cells and LLC PK1 renal cells to further understand the decrease in renal cell toxicity that results when AmpB associates with lipoproteins.

## MATERIALS AND METHODS

**Chemicals and lipids.** AmpB was provided by Bristol-Myers Squibb (Newark, N.J.). Chromatographically pure DMPC and DMPG were obtained from Nippon Fine Chemical (Tokyo, Japan). Purified HDLs, LDLs, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Chemical Company (St. Louis, Mo.). Organic solvents were obtained from Baker Chemical Company (Phillipsburg, N.J.).

**Cell cultures.** LLC PK1 cells were kindly provided by J. Lever, Department of Biochemistry, The University of Texas Medical School. The cells were grown at 37°C in 5% humidified $CO_2$ in a 1:1 mixture of Dulbecco's minimum essential medium and Ham's F-12 medium supplemented with 10%

* Corresponding author. Mailing address: Immunobiology and Drug Carriers Section, Box 60 The University of Texas M. D. Anderson Cancer Center, 1515 Holcombe Houston, TX 77030. Phone: (713) 792-3508. Fax: (713) 796-1731.

† Present address: Department of Cell Biology, Cleveland Clinic Foundation, Cleveland, OH 44195.

fetal calf serum, 100 U of penicillin per ml, and 100 μg of streptomycin per ml. Fresh medium was added to the cultures every 2 days and the cultures were split into 3:7 dilutions upon reaching confluence. At 24 h before use, preconfluent experimental cultures were set up in 96-well plates at a density of $2 \times 10^4$ cells per well and were incubated in mycoplasma-free and serum-free medium instead of fetal calf serum. The growth rates of the cells were not influenced by the addition of penicillin or streptomycin and serum-free medium in comparison with the growth rates of the controls.

**MTT assay.** The conversion of MTT to MTT-formazan product by means of the mitochondrial electron transport chain is used as an indicator of cell toxicity (11). A decrease in the amount of MTT converted indicates increased cell toxicity. MTT (10 μl of a 5-mg/ml solution per well) was incubated with drug-treated or untreated cells for 60 min at 37°C. Following incubation, the optical density (OD) at a wavelength of 570 nm ($OD_{570}$) was determined for each well by a microELISA plate reader. Percent cytotoxicity was calculated by the following formula

$$\frac{OD_{570\ \text{treated cells}}/\text{number of cells per well}}{OD_{570\ \text{untreated cells}}/\text{number of cells per well}} \times 100$$

**Trypan blue exclusion assay.** The trypan blue exclusion assay has been well characterized from previous work (10). Briefly, 20 μl of 0.04% trypan blue solution was incubated with cells for 10 min. Following incubation, cells were washed twice with phosphate-buffered saline (PBS; 1 ml), and the $OD_{595}$ was determined. Cells that prevented the uptake of the trypan blue solution, and that thus had low OD values, were resistant to AmpB toxicity. The cell membranes of cells that did not prevent the uptake of trypan solution were considered to have encountered toxic effects.

**$^{125}$I-labelling of serum lipoproteins.** HDLs and LDLs were labelled with $^{125}$I by the iodine monochloride method (15). An aliquot of lipoprotein (100 μg, on the basis of the protein content) was added to 10 μl of 0.1 M Tris-HCl (pH 8.0) after the lipoprotein was cooled in an ice water bath. A 0.5 mCi aliquot of $^{125}$I in PBS was added; this was followed by the addition of 10 μl of chloramine-T solution (100 pg/ml in PBS). To terminate the reaction, 10 μl of a saturated solution of tyrosine in water was added; this was followed by the addition of PBS (2.5 ml) containing 0.1% bovine serum albumin and 10 mM $NaN_3$. The mixture was then applied to a disposable PD-Sephadex column (Pharmacia Inc., Piscataway, N.J.) equilibrated with PBS. The column was then eluted with 5.0 ml of PBS, and the effluent was collected in 1-ml-volume fractions. These fractions were analyzed by using a scintillation counter (Beckman Instruments, Houston, Tex.). The appropriate fractions were precipitated with trichloroacetic acid, and 10-μl aliquots were removed. The $^{125}$I in both the supernatant and the precipitated pellet was counted to determine the percentage of $^{125}$I incorporated into the lipoprotein.

**Dose-response and time course effects of AmpB and L-AmpB.** AmpB and L-AmpB (0.5 to 50 μg of AmpB per ml) were incubated in serum-free medium containing $2.0 \times 10^4$ LLC PK1 cells per well. Cells were incubated with either no drug (controls), AmpB, or L-AmpB for 18 h at 37°C; cell toxicity was then determined. In a second set of experiments, AmpB and L-AmpB at 20 μg of AmpB per ml were incubated with LLC PK1 cells for different times (3 to 72 h). L-AmpB and AmpB at 20 μg of AmpB per ml were chosen because that is where the maximal percent cytotoxicity was observed (see Fig. 1A). Percent cytotoxicity was determined by an MTT assay and was confirmed by the trypan blue exclusion assay as described above.

**Influence of serum lipoprotein-associated AmpB and L-AmpB on in vitro antifungal activity.** *C. albicans* 336, which was obtained from an infected patient, was diluted in normal saline to 85% transmittance at a wavelength of 540 nm, which was measured by using a spectrophotometer (Spectrometer 20; Milton Roy Co., St. Petersburg, Fla.). The *C. albicans* cell suspension (100 μl) was then applied onto a 5% glucose-agarose agar plate containing yeast nitrogen broth (100 μl). After 24 h of incubation at 37°C, colonies of *C. albicans* were transferred to agar plates containing AmpB (0.1 to 2.0 μg/ml), L-AmpB (0.1 to 2.0 μg/ml), HDLs (0.5 mg of protein per ml), LDLs (0.5 mg of protein per ml), mixtures of AmpB (0.1 to 2.0 μg/ml) with HDLs or LDLs, and mixtures of L-AmpB with HDLs or LDLs; these plates were incubated for 18 h at 37°C. The MIC of each treatment was determined by comparing the number of CFU of each plate after the total incubation time (42 h) with that of untreated control plates. The same procedure was repeated with *Aspergillus niger*.

**Influence of serum lipoprotein-associated AmpB and L-AmpB on drug toxicity to LLC PK1 renal cells.** LLC PK1 cells were incubated for 18 h at 37°C in serum-free media containing various treatments: AmpB (20 μg/ml), L-AmpB (20 μg/ml), HDLs (0.5 mg of protein per ml), LDLs (0.5 mg of protein per ml), mixtures of AmpB (20 μg/ml) with HDLs or LDLs, and mixtures of L-AmpB (20 μg/ml) with HDLs or LDLs. Percent cytotoxicity was determined by an MTT assay and was confirmed by the trypan blue exclusion assay as described above.

**Equilibrium binding of $^{125}$I-LDL and $^{125}$I-apo A-I to LLC PK1 renal cells.** LLC PK1 cells ($3.2 \times 10^5$ cells per well) were grown for 4 days in mycoplasma-free culture medium containing 10% fetal calf serum. Twenty-four hours before the experiment, the medium was replaced with culture medium containing 10% delipidized serum. After 24 h, the culture medium containing delipidized serum was replaced with serum-free medium containing $^{125}$I-LDL or $^{125}$I-apoprotein A-I ($^{125}$I-apo A-I; (1 to 100 ng/ml). Apo A-I is one of the major protein components of HDLs. Since radiolabeled HDLs are unstable and the apoprotein portion is responsible for the interaction with lipoprotein receptors, apo A-I was used to determine the presence of HDL receptors. Nonspecific binding was assessed by using a 300-fold excess of LDLs or apo A-I in the presence of its radiolabelled counterpart. Plates were incubated for 2 h at 4°C, after which the medium was removed and a 100-μl aliquot was counted to determine free $^{125}$I-LDLs or $^{125}$I-apo A-I. The cells were removed from the plate with 10% disodium EDTA (10 min at 37°C) and centrifuged at $15,000 \times g$ for 10 min. An aliquot of cell supernatant (100 μl) was removed and counted. The cell pellet was also counted to determine total cell-bound $^{125}$I-LDL or $^{125}$I-apo A-I. $K_d$ values, representing the binding affinity of $^{125}$I-LDLs or $^{125}$I-apo A-I to LLC PK1 cells and the number of binding sites per cell, were determined by Scatchard analysis by using nonweighted linear regression (17).

**Influence of reduced LDL receptor expression on apo A-I- and LDL-associated AmpB toxicity to LLC PK1 renal cells.** LLC PK1 cells were treated with a 0.075% trypsin solution for 2 min to remove high-affinity LDL receptors. Then the cells were incubated for 18 h at 37°C in serum-free medium containing the following various treatments: AmpB (20 μg/ml), L-AmpB (20 μg/ml), HDLs (0.5 mg/dl), LDLs (0.5 mg/ml), mixtures of AmpB (20 μg/ml) with HDLs or LDLs, and mixtures of L-AmpB (20 μg/ml) with HDLs or LDLs. Percent

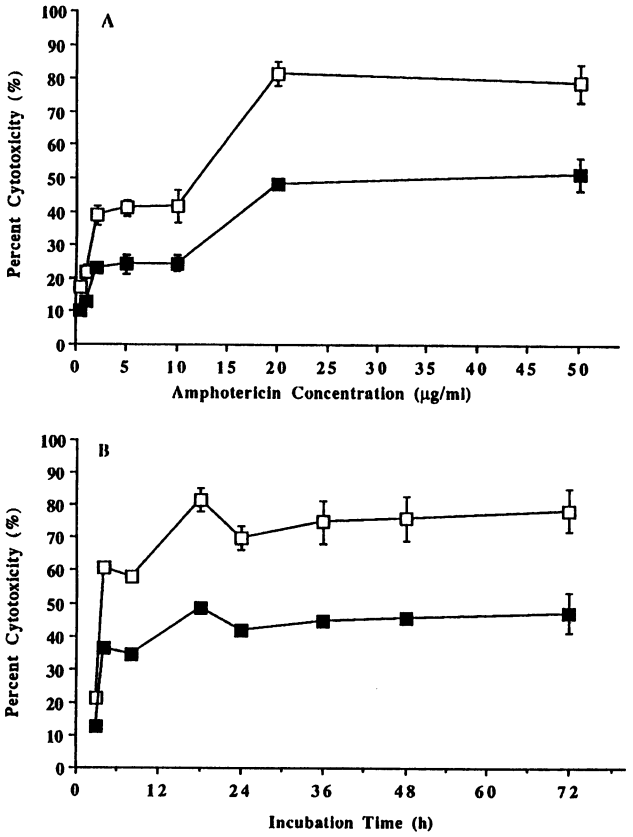


FIG. 1. Dose-response and time course effects of AmpB and L-AmpB. Percent cytotoxicity of LLC PK1 cells in serum-free medium after 18 h of incubation at 37°C with various concentrations of AmpB-deoxycholate (Fungizone) (□) and L-AmpB (■) (A) and after different incubation times at 37°C with AmpB-deoxycholate (□) and L-AmpB (■) at 20 μg of AmpB per ml (B) ($n$ = 6; values are means ± standard deviations).

cytotoxicity was determined by an MTT assay and was confirmed by the trypan blue exclusion assay as described above.

**Statistical analysis.** Differences in cell toxicity in the presence of different treatment groups were determined by a two-way analysis of variance (PCANOVA; Human Systems Dynamics). Critical differences were assessed by Neuman-Keuls posthoc tests. Differences were considered significant if $P$ was <0.05. All data are expressed as means ± standard deviations and are corrected for the number of samples by Student's t test.

## RESULTS

**Dose-response and time course curves of AmpB and L-AmpB.** The toxicities of various concentrations of AmpB to LLC PK1 cells increased in a dose-dependent fashion (from 17.3% ± 1.0% to 81.3% ± 3.6%). No further increase in toxicity was observed at AmpB concentrations greater than 20 μg/ml (Fig. 1A). The maximal toxicity observed with L-AmpB was significantly lower than that observed with AmpB (48.3% ± 1.5% versus 81.3% ± 3.6% cytotoxicity; $P < 0.05$) (Fig. 1A). In the time course studies, when AmpB and L-AmpB (at 20 μg of AmpB per ml) were incubated with LLC PK1 cells for different times (3 to 72 h), peak cell toxicity was achieved after 18 h of incubation for both AmpB and L-AmpB (Fig. 1B).

**Influence of serum lipoprotein-associated AmpB and L-AmpB on in vitro antifungal activity.** For *C. albicans*, the MICs of AmpB and L-AmpB after 18 h of incubation in the presence or absence of HDLs and LDLs were not significantly different (Fig. 2). Similar results were obtained for *A. niger* (data not shown). HDLs alone, LDLs alone, deoxycholate alone, and empty DMPC-DMPG liposomes did not inhibit fungal growth (data not shown).

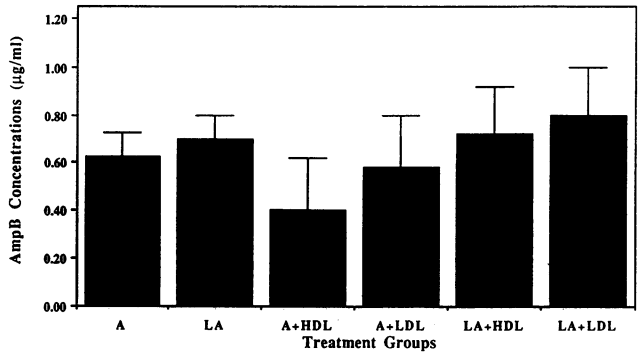


FIG. 2. Influence of serum lipoprotein-associated AmpB and L-AmpB on in vitro antifungal activity of *C. albicans*. The MICs of the following treatments are given: AmpB (A), L-AmpB (LA), HDLs (0.5 mg of protein per ml), LDLs (0.5 mg of protein per ml), mixtures of AmpB with HDLs (A+HDL) or LDLs (A+LDL), and mixtures containing L-AmpB with HDLs (LA+HDL) or LDLs (LA+LDL) ($n$ = 6; values are means ± standard deviations). *, $P < 0.05$ versus AmpB treatment alone.

**Influence of serum lipoprotein-associated AmpB and L-AmpB on drug toxicity to LLC PK1 renal cells.** L-AmpB was significantly less toxic than AmpB-deoxycholate at 20 μg of AmpB per ml (81.3% ± 3.6% versus 48.3% ± 1.5% cytotoxicity) (Fig. 3A). HDL-associated AmpB, HDL-associated L-AmpB, and LDL-associated L-AmpB were less toxic than AmpB to LLC PK1 cells (53.0% ± 2.5%, 25.5% ± 2.2%, and 52.2% ± 2.5% versus 81.3% ± 3.6% cytotoxicity), while AmpB and LDL-associated AmpB were equally toxic.

**Determination of LDL and apo A-I receptors on LLC PK1 cell culture.** LLC PK1 renal cells expressed high-affinity ($K_d$ = 0.054 ng/ml; 96,000 sites per cell) and low-affinity ($K_d$ = 222.22 ng/ml; 77 sites per cell) LDL receptors, but only a low-affinity ($K_d$ = 71.43 ng/ml; 2 sites per cell) apo A-I receptor (Table 1). When the cells were treated with 10% trypsin for 10 min, only the high-affinity LDL receptors were not detected. Incubation of cells with trypsin did not alter the expression of the low-affinity LDL receptors or the apo A-I receptor (Table 1).

**Influence of reduced LDL receptor expression on HDL- and LDL-associated AmpB toxicity to LLC PK1 renal cells.** Following incubation with trypsin, HDL-associated AmB and LDL-associated AmpB were found to be less toxic than AmpB to the cells (46.6% ± 10.9% and 16.8% ± 15.98% versus 74.7% ± 7.7% cytotoxicity) (Fig. 3B). HDL-associated L-AmB and LDL-associated L-AmpB were also less toxic than AmpB to the cells (20.4% ± 6.2% and 13.5% ± 8.6% versus 74.7% ± 7.7% cytotoxicity).

## DISCUSSION

Our results demonstrate that HDL-associated AmpB and HDL-associated L-AmpB are less toxic than AmpB or LDL-associated AmpB to LLC PK1 renal cells. The reduced level of toxicity of HDL-associated AmpB may be explained by the low level of expression of HDL receptors in LLC PK1 cells. The sustained toxicity observed with AmpB alone in trypsinized

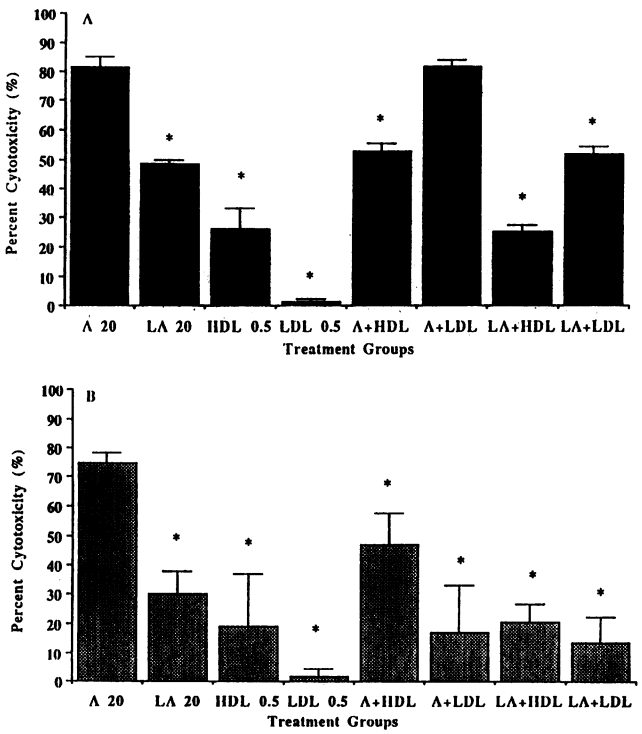


FIG. 3. (A) Influence of serum lipoprotein-associated AmpB and L-AmpB on drug toxicity to LLC PK1 renal cells as percent cytotoxicity of LLC PK1 cells in serum-free medium at 37°C containing the following treatments: AmpB (20 μg/ml) (A 20), L-AmpB (20 μg/ml) (LA 20), HDLs (0.5 mg of protein per ml; HDL 0.5), LDLs (0.5 mg of protein per ml; LDL 0.5), mixtures of AmpB (20 μg/ml) with HDLs (A+HDL) or LDLs (A+LDL), and mixtures of L-AmpB (20 μg/ml) with HDLs (LA+HDL) or LDLs (LA+LDL) ($n = 3$; values are means ± standard deviations). * $P < 0.05$ versus treatment with AmpB at 20 μg/ml. (B) Influence of reduced LDL receptor expression on HDL- and LDL-associated AmpB toxicity of LLC PK1 renal cells as percent cytotoxicity of LLC PK1 cells in serum-free medium at 37°C after a trypsin wash containing the same treatments as described for panel A ($n = 3$; values are means ± standard deviations). *, $P < 0.05$ versus treatment with AmpB at 20 μg/ml.

cells may be related to a direct effect on the membrane. However, when AmpB is associated with LDLs, the toxicity is maintained, suggesting that both direct membrane- and non-membrane-related toxicities may occur.

Lipoprotein-associated or free AmpB and L-AmpB were equally toxic to fungal cells, suggesting that the presence of lipoproteins does not alter the antifungal activities of AmpB and L-AmpB. Such effects may be related to the liberation by fungal lipases of monomeric AmpB from AmpB and L-AmpB when AmpB is either associated or not associated with lipoproteins (6, 16). The low concentrations of unbound and water-soluble monomeric AmpB present in L-AmpB (2, 9, 10) may be sufficient for fungal toxicity but not adequate for forming AmpB aggregates that are toxic to mammalian cells (2, 10). AmpB-induced toxic effects on mammalian cells are reduced when AmpB is complexed with lipid compares with that when it is in the monomeric form (6, 16).

TABLE 1. Equilibrium binding of $^{125}$I-LDL and $^{125}$I-apo A-I (HDL) to LLC PK1 renal cells before and after treatment with trypsin in serum-free media by Scatchard analysis

| Lipoprotein fraction | No. of surface-binding sites/cell | $K_d$ (ng/ml) |
|---|---|---|
| $^{125}$I-apo A-I | | |
| Before trypsin wash | 2 | 71.43 |
| After trypsin wash | 17 | 70.49 |
| $^{125}$I-LDL (low affinity) | | |
| Before trypsin wash | 77 | 222.22 |
| After trypsin wash | 69 | 198.23 |
| $^{125}$I-LDL (high affinity) | | |
| Before trypsin wash | 96,000 | 0.0538 |

We have previously shown that L-AmpB predominantly associates with HDLs (18) and that the amount of AmpB associated with HDLs increases when AmpB is incorporated into negatively charged liposomes. These results further suggest that modification of the distribution of drugs to specific lipoproteins within the bloodstream may determine the drug's biological fate and pharmacological activity within the body. In the case of AmpB, the relative distribution of AmpB among serum lipoproteins seems to be a major factor influencing the enhanced therapeutic index of L-AmpB.

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