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Activation of Rat Kupffer Cells to Tumoricidal Cells by the Immunomodulator Muramyl Tripeptide-Phosphatidylethanolamine Incorporated into the Novel Drug Carrier Lactosylated Low Density Lipoprotein

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SUMMARY

Lactosylated low density lipoprotein (lac-LDL) is a potential carrier for the site-specific delivery of lipophilic drugs to liver macrophages (Kupffer cells). In the present study we evaluated the application of lac-LDL as a carrier to target the immunomodulator muramyl tripeptide-phosphatidylethanolamine (MTP-PE) to rat Kupffer cells, to specifically activate these cells to tumor-killing cells. The drug carrier ^{125}I-labeled lac-LDL interacted with a galactose-specific recognition system on isolated rat Kupffer cells. The *in vitro* association of ^{125}I-lac-LDL at 37° was maximal after 20 min, whereas degradation of ^{125}I-lac-LDL was observed after a lag period of 10 min. Cultured rat Kupffer cells were activated after incubation with MTP-PE incorporated into lac-LDL. Lac-LDL-MTP-PE induced a 2-fold increase in the amount of newly synthesized proteins secreted by Kupffer cells. Lac-LDL-MTP-PE induced a concentration-dependent increase in the cytostatic and cytolytic activities of Kupffer cells towards tumor cells (B16F10 melanoma cells) *in vitro*. Treatment of rats with lac-LDL-MTP-PE also resulted in dose-dependent activation of Kupffer cells to tumoricidal cells, whereas the drug carrier alone had only a minor effect on this activity of Kupffer cells. The present data show that lac-LDL is an effective carrier for the delivery of the lipophilic immunomodulator MTP-PE to rat Kupffer cells. The specific activation of Kupffer cells to tumoricidal cells by lac-LDL-MTP-PE may be beneficial for the treatment of liver metastases.

Kupffer cells play an important role in protection against infections by the uptake of circulating bacteria (1). Kupffer cells are also able to capture and kill circulating tumor cells (1, 2). Kupffer cells may also be involved in several infectious diseases; it was shown that Kupffer cells within the livers of human immunodeficiency virus type 1-infected patients contain the human immunodeficiency virus type 1 virus (3).

Recently, a new potential carrier for the specific delivery of drugs to rat Kupffer cells was described. This drug carrier, lac-LDL, may be used to target drugs that increase the protective functions of Kupffer cells (4, 5). Lac-LDL is a highly lactosylated LDL particle that exposes about 400 lactose residues on the apo-B part of LDL. Lac-LDL is taken up *in vivo* by a galactose-specific receptor on the Kupffer cells (4, 5). The lipophilic part of the lac-LDL particle is highly suitable for the incorporation of lipophilic drugs. After incorporation, these drugs can be targeted effectively to Kupffer cells (4).

Several immunomodulating substances increase the aforementioned protective functions of Kupffer cells and other macrophages. Compounds derived from the bacterial cell wall, like LPS, MDP, and the lipophilic PE derivative of MDP, MTP-PE, increase the capacity of Kupffer cells to kill viruses, bacteria, and tumor cells (6–9). The increased tumoricidal activity of Kupffer cells after treatment with immunomodulators may be of great therapeutic interest for the prevention and/or destruction of liver metastases, which frequently occur after the resection of primary colorectal tumors (10). In this paper we describe the potential use of lac-LDL as a drug carrier to target the lipophilic immunomodulator MTP-PE to rat Kupffer cells *in vivo* and *in vitro*; the activation of Kupffer cells to tumoricidal cells by lac-LDL-MTP-PE is also described.

Experimental Procedures

Materials

Collagenase type I (from *Clostridium histolyticum*), antibiotics, and FCS were from Boehringer Mannheim (Mannheim, Germany). MEM

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ABBREVIATIONS: lac-LDL, lactosylated low density lipoprotein; LDL, low density lipoprotein; MTP, muramyl tripeptide; PE, phosphatidylethanolamine; MDP, muramyl dipeptide; MEM, minimal essential medium; DMEM, Dulbecco's modified Eagle's medium; apo-B, apolipoprotein B; FCS, fetal calf serum; BSA, bovine serum albumin; LPS, lipopolysaccharide; PBS, phosphate-buffered saline; TCA, trichloroacetic acid.

without L-methionine, RPMI 1640 medium, and DMEM were from GIBCO (Paisley, Scotland). BSA (fraction V) and LPS from *Escherichia coli* (026.B6) were from Sigma Chemical Co. (St. Louis, MO). Nycodenz was from Nycomed A/S (Oslo, Norway). Sephadex G-25 (coarse) was from Pharmacia (Uppsala, Sweden). L-[^{35}S]Methionine (specific activity, 800 Ci/mmol), [*methyl*-^{3}H]thymidine (specific activity, 83 Ci/mmol), ^{125}I (carrier free), Rainbow markers, and amplifier were from Amersham (Buckinghamshire, UK). Lactose (monohydrate) was from Merck (Darmstadt, Germany). MTP-PE was kindly provided by Ciba Geigy (Basel, Switzerland).

Methods

Kupffer cell isolation. For Kupffer cell isolation male rats (200–250 g) were anesthetized and Kupffer cells were isolated after collagenase (0.05%, w/v) perfusion of the liver at 37°. Kupffer cells were separated by differential centrifugation, density gradient centrifugation, and counterflow centrifugation as described in detail elsewhere (11), except for the first elutriation step, which was replaced by a centrifugation step (2 min, 75 × *g*). Kupffer cell fractions were >90% pure (contaminating cells were large endothelial liver cells), as judged by the presence of peroxidase activity in the cells, and viability was >95%.

For cytotoxicity studies Kupffer cells were cultured in 96-well tissue culture dishes, at a density of 0.25 × 10^6 cells/well, in RPMI 1640 medium supplemented with 20% FCS, 0.01% penicillin-streptomycin, and 2 mM L-glutamine. After an attachment period of 2 hr, medium was replaced by RPMI 1640 medium supplemented with 10% FCS, 0.01% penicillin-streptomycin, and 2 mM L-glutamine. In control experiments performed with pure liver endothelial cells, we never observed any cytolytic or cytostatic activity of these cells towards B16F10 melanoma cells.

Isolation, iodination, and lactosylation of LDL. LDL was isolated from human plasma (containing 1 mM EDTA), at density of $1.019 < d < 1.063$, by two repetitive centrifugation steps, as described (12, 13). The LDL preparation contained mostly apo-B (99.97%), and no degradation products of apo-B were observed by electrophoresis in sodium dodecyl sulfate-containing gels. With a high LDL concentration (5 mg of apolipoprotein/ml) in a radial immunodiffusion system (14), apolipoprotein E was noticeable at the detection limit and contributed at most 0.02–0.03% to the total apolipoprotein. Radioiodination of LDL was done according to a modification (15) of the iodine chloride method, as described (16). LDL was lactosylated by reductive amination in PBS at 37° for 3 days (under sterile conditions), as described before (4, 5). This resulted in the incorporation of 390 lactose residues/particle of LDL, determined as described before (4, 5).

Cell association and degradation of lipoproteins *in vitro*. To determine the binding and degradation of ^{125}I-lac-LDL by Kupffer cells, we incubated freshly isolated Kupffer cells with ^{125}I-labeled lac-LDL at 37° (17, 18). Incubations of Kupffer cells with ^{125}I-lac-LDL were performed in DMEM containing 1% (w/v) BSA and were carried out in Kartell plastic tubes, which were placed in a circulating laboratory shaker (Adolf Kühner AG, Switzerland) at 150 rpm. Viability was checked by measuring ATP content and trypan blue exclusion and remained above 95% throughout the incubations.

At the end of the incubations Kupffer cells were centrifuged (500 × *g*, 2 min) and the pellets were washed twice in 0.5 ml of buffer (50 mM Tris, 0.15 M NaCl, 2.5 mM $CaCl_2$, 0.2% BSA, pH 7.4) at 4° and once in the same buffer without BSA. Finally, Kupffer cells were suspended in the BSA-free buffer, the amount of ^{125}I was counted in a Packard γ-counter, and the amount of cell protein was determined according to the method of Lowry *et al.* (19). To 0.5 ml of the first supernatant, 0.2 ml of 35% TCA was added, followed by incubation for 15 min at 37°; the mixture was then centrifuged for 2 min at 10,000 × *g*. To 0.5 ml of the supernatants, 10 μl of 20% KI and 25 μl of 30% H_2O_2 were added. After 5 min at room temperature, 0.8 ml of $CHCl_3$ was added and the mixture was shaken for another 5 min. After centrifugation for 2 min at 10,000 × *g*, 0.4 ml of the aqueous phase (containing iodinated amino acids and small peptides) was counted.

Association was determined as the amount of ^{125}I-lac-LDL present in plus on the Kupffer cells (binding plus uptake) at a certain time point. The half-maximal association and the maximal specific association (B_{max}) were determined from displacement and binding curves according to a single-site displacement model, using a computerized nonlinear curve-fitting program (GraphPAD; ISI Software) (17).

Incorporation of MTP-PE into lac-LDL. MTP-PE was incorporated according to the method of Shaw *et al.* (20). Briefly, 1 mg of MTP-PE was dissolved in 1 ml of chloroform/methanol (7:3, v/v). This solution was evaporated to dryness under nitrogen, with continuous stirring with a Teflon-coated Miniflow, at room temperature in the dark. One milligram of lac-LDL [1 mg of protein/ml of PBS (10 mM sodium phosphate buffer, pH 7.4, containing 150 mM NaCl and 1 mM EDTA)] was added and stirring was continued for another 2 hr. To separate incorporated MTP-PE from free MTP-PE, the mixture was centrifuged for 10 min at 700 × *g* over a Sephadex G25 (coarse) column (saturated with PBS) and was then sterilized over a 0.45-μm Millipore filter saturated with BSA. The final yield of apo-B protein for the entire procedure starting with native LDL was about 60%. Approximately 40–130 molecules of MTP-PE could be incorporated into each LDL particle by this method (see Ref. 19).

Determination of Kupffer cell incorporation of L-[^{35}S]methionine into proteins. Kupffer cells cultured for 24 hr in 96-well culture dishes were washed for 30 min with RPMI 1640 medium without FCS. Thereafter, immunomodulators were added (volumes did not exceed 10% of total incubation volume of 200 μl). L-[^{35}S]Methionine (75 μCi/ml) was added in L-methionine-free MEM. At the end of the incubation supernatant was removed from the cells and the Kupffer cells were allowed to dissolve in 0.5 M NaOH. Both supernatant and cellular fractions were rapidly refrigerated and stored at −80°. L-[^{35}S]Methionine incorporation was determined by TCA precipitation. The samples (40 μl of the supernatant or cellular fraction) were added to 500 μl of water containing 0.1% (w/v) BSA and 0.1% (w/v) L-methionine. Proteins were precipitated by the addition of 250 μl of 35% (w/v) TCA, followed by centrifugation for 5 min at 7500 × *g*. TCA precipitates were redissolved in 500 μl of water containing 0.1% L-methionine plus one drop of 5.0 M NaOH and were precipitated again by the addition of 200 μl of 35% TCA solution. This procedure was repeated three times. All determinations were done in triplicate. Radioactivity was measured by scintillation counting in a Packard β-counter. The amount of radioactivity was correlated with the protein content of the Kupffer cells of corresponding incubations, as measured by the method of Lowry *et al.* (20), using BSA as the standard.

Tumor target cells and tumor cell labeling. B16F10 melanoma cells were grown in DMEM (supplemented with 10% FCS). Melanoma cells were diluted three times each week by short incubations with 0.05% (w/v) trypsin.

For the cytolytic assay, cells were allowed to grow in culture medium containing 1 μCi/ml [*methyl*-^{3}H]thymidine, which was added when cells were in the exponential growth phase. After 24 hr, cells were allowed to grow for another 4 hr in culture medium containing 0.2% thymidine, to deplete cytoplasmic pools of radiolabeled thymidine to reduce spontaneous release of [^{3}H]thymidine by the tumor cells. Cells were harvested after short incubations with 0.05% trypsin and were washed three times by centrifugation with DMEM. Total [*methyl*-^{3}H]thymidine incorporated into DNA was determined by dissolving 10,000 cells in 0.5 ml of Soluene (Amersham, Buckinghamshire, England), followed by scintillation counting.

Cytolytic assay. Kupffer cells cultured for 24 hr in 96-well plates were activated by the addition of immunomodulators. After 4 hr, 10,000 radiolabeled B16F10 melanoma cells/well were added to the Kupffer cells. Spontaneous release of label was determined in those wells containing tumor cells alone. After 48 hr of co-culture, radioactivity in the supernatant was determined by scintillation counting in a Packard β-counter. The number of tumor cells lysed was expressed as percentage

of cytolysis = $(A - B)/(C - B) \times 100\%$ or as percentage of specific cytolysis = $(A - D)/(C - D) \times 100\%$, where A is the radioactivity of ^3H in the supernatant of treated incubations, B is the radioactivity of ^3H in the supernatant of tumor cells (incubated without Kupffer cells), C is the total radioactivity (^3H) present in the added tumor cells, and D is the radioactivity (^3H) determined in the supernatant of tumor cells incubated with nonactivated Kupffer cells.

Cytostatic assay. Four hours after activation of Kupffer cells as described above, 10,000 unlabeled B16F10 melanoma cells/well were added. After 24 hr of co-culture, supernatants were removed and 200 μl of RPMI 1640 culture medium containing 1.0 μCi/ml [*methyl*-^3H] thymidine were added per well. After an additional co-culture of 24 hr, wells were washed three times with ice-cold RPMI 1640 medium. Finally cells were dissolved in 0.5 M NaOH and incorporated radioactivity was determined by scintillation counting. Growth inhibition was expressed as percentage of cytostasis = $[1 - (B/A)] \times 100\%$ or as percentage of specific cytostasis = $[1 - (B/C)] \times 100\%$, where A is the radioactivity in tumor cells grown without Kupffer cells, B is the radioactivity in tumor cells grown with treated Kupffer cells, and C is the radioactivity in tumor cells grown with untreated Kupffer cells.

***In vivo* activation of rat Kupffer cells.** Rats (anesthetized with ether) were given intravenous injections, via the vena penis, of lac-LDL (20, 25, 100, or 400 μg/kg of body weight) or the lac-LDL-MTP-PE complex (20, 25, 100, or 400 μg/kg of body weight), dissolved in 0.5 ml of PBS, or of 0.5 ml of saline solution. Four hours after injection, the livers of the treated rats were perfused and Kupffer cells were isolated as described before (11). Isolated Kupffer cells were plated at a density of 2.5×10^5 or 1.25×10^5 cells/well in 96-well plates. Cytolytic and cytostatic assays were started after adherence of the Kupffer cells (2 hr).

Results

Rat Kupffer cell association with and degradation of lac-LDL. The association of increasing concentrations of ^{125}I-lac-LDL with Kupffer cells is shown in Fig. 1. The association of ^{125}I-lac-LDL with Kupffer cells was specific and could be largely inhibited by 50 mM *N*-acetylgalactosamine. The same degree of inhibition of the association of ^{125}I-lac-LDL with rat Kupffer cells was observed in the presence of unlabeled lac-LDL (200 μg/ml). The association of ^{125}I-lac-LDL was saturated at concentrations between 4 and 10 μg/ml ^{125}I-lac-LDL. Maximal specific association was calculated to be 170 ng of lac-LDL/mg of Kupffer cell protein, whereas half-maximal association of lac-LDL with rat Kupffer cells was observed at a concentration of 6.3 μg/ml lac-LDL. The level of galactose-specific binding varied from isolation to isolation, and maximal association ranged from 150 to 450 ng of lac-LDL/mg of cell protein (five experiments), whereas half-maximal association of lac-LDL was rather constant and observed at concentrations ranging from 5.9 to 6.9 μg/ml lac-LDL (five experiments). In binding studies performed for 2 hr at 4°, Kupffer cells bound ^{125}I-lac-LDL with an apparent affinity of 1.3 ± 0.16 μg/ml lac-LDL, whereas the maximal specific binding of ^{125}I-lac-LDL to the Kupffer cells was 142 ± 42 ng of lac-LDL/mg of cell protein (corresponding to approximately 23,500 binding sites/Kupffer cell) (21).

Fig. 2 shows the time course of association with and degradation of ^{125}I-lac-LDL by rat Kupffer cells at 37°. The association of ^{125}I-lac-LDL reached equilibrium after 20 min of incubation. Degradation of ^{125}I-lac-LDL was observed after a lag phase of 10 min of incubation and continued for 120 min of incubation.

Effect of lac-LDL and lac-LDL-MTP-PE on protein

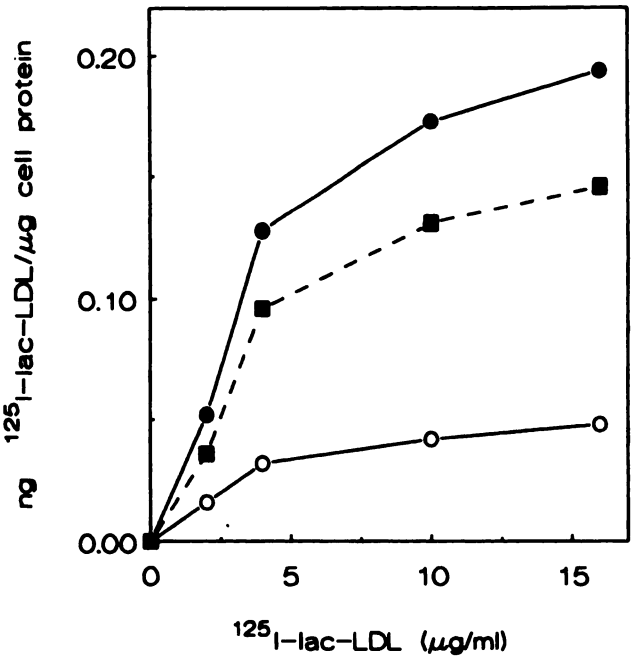

Fig. 1. Association of lac-LDL with Kupffer cells. Freshly isolated Kupffer cells were incubated with increasing concentrations of ^{125}I-lac-LDL at 37°. After 15 min Kupffer cells were washed and the amount of ^{125}I-lac-LDL/μg of cell protein was determined (●). Nonspecific association (○) was determined as the association of ^{125}I-lac-LDL with Kupffer cells in the presence of 50 mM *N*-acetylgalactosamine. Specific association of lac-LDL (■) was determined by subtracting nonspecific association from total association. Data are from one representative experiment of five.

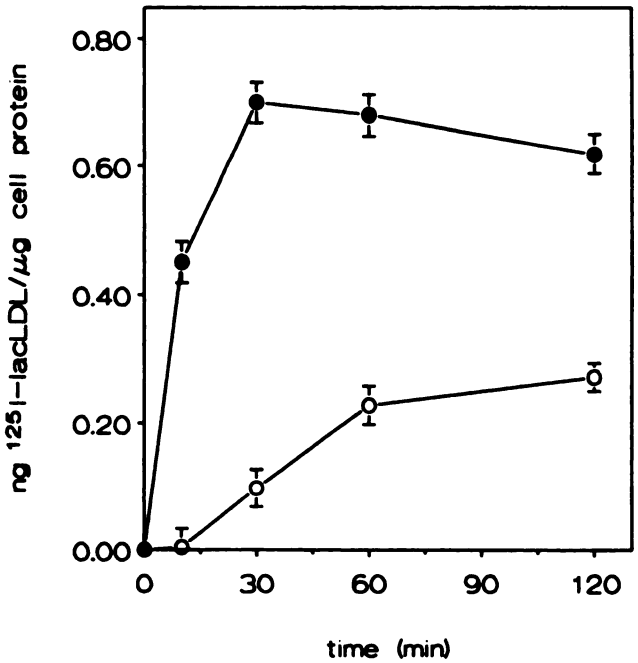

Fig. 2. Time course of Kupffer cell association with and degradation of lac-LDL. Kupffer cells were incubated at 37° in the presence of 5 μg of ^{125}I-lac-LDL. At the indicated times cells were washed and the amounts of degraded (○) and cell-associated (●) ^{125}I-lac-LDL were determined as described in Experimental Procedures.

synthesis by Kupffer cells. The effect of lac-LDL and lac-LDL-MTP-PE on the state of activation of Kupffer cells was studied by determining the rate of protein synthesis, which was previously shown to be increased after activation of macrophages. Table 1 shows the effects of the aforementioned compounds on the synthesis of secreted proteins by cultured Kupffer cells. Lac-LDL-MTP-PE had, in comparison with the drug carrier alone, a significant stimulatory effect on protein secretion by Kupffer cells. The carrier for MTP-PE on itself had also a small but significant stimulatory effect on the secretion of proteins by Kupffer cells, as did LPS derived from *E. coli*. However, no qualitative difference in newly synthesized proteins between control and either lac-LDL- or lac-LDL-MTP-PE-incubated Kupffer cells was observed, as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and autoradiography. Preliminary data on the production of tumor necrosis factor-α by Kupffer cells (in a L929 cell lysis assay) showed that, after activation with lac-LDL-MTP-PE or LPS, Kupffer cells produce significant amounts of tumor necrosis factor-α (data not shown).

Effect of lac-LDL-MTP-PE on the tumoricidal activity of Kupffer cells. The effect of MTP-PE incorporated into lac-LDL on the cytostatic activity of Kupffer cells is shown in Fig. 3. Control Kupffer cells already had a cytostatic activity of 50% towards B16F10 tumor cells. The cytostatic capacity of the Kupffer cells was clearly enhanced by activation with increasing amounts of lac-LDL-MTP-PE, up to 87% specific cytostatic activity after activation with 20 μg/ml lac-LDL-MTP-PE. The stimulatory effect of the drug carrier lac-LDL on the specific cytostatic activity of the Kupffer cells was maximally 10%.

The ability of Kupffer cells to kill tumor cells after *in vitro* activation was also tested (Fig. 3). Control Kupffer cells had only a marginal cytolytic effect on B16F10 tumor cells. The cytolytic activity of Kupffer cells was greatly enhanced after activation with lac-LDL-MTP-PE (20 μg/ml), to about 30% specific cytolysis. Uptake of the drug carrier lac-LDL by Kupffer cells had no effect on the specific cytolysis. Activation of Kupffer cells with free MTP-PE (4 μg/ml) did not significantly affect the cytolytic or the cytostatic activity of the Kupffer cells.

***In vivo* activation of Kupffer cells using lac-LDL-MTP-PE.** Experiments were carried out to test the use of the drug carrier lac-LDL-MTP-PE complex to activate rat Kupffer cells *in vivo*. Kupffer cells isolated 4 hr after the intravenous injection of lac-LDL, lac-LDL-MTP-PE, or saline into rats were tested for their cytostatic and cytolytic activities towards B16F10 cells (Fig. 4). Kupffer cells isolated from saline-treated animals had a cytostatic effect on the tumor cells of 50%, whereas these Kupffer cells had a moderate cytolytic effect (10%). Kupffer cells isolated from animals treated with lac-LDL-MTP-PE showed significantly higher cytostatic activity towards B16F10 tumor cells (90%). *In vivo* treatment with lac-LDL-MTP-PE also resulted in strong increases in the cytolytic activity of the Kupffer cells towards the tumor target cells. Treatment of the rats with the drug carrier lac-LDL alone did not change the cytostatic and cytolytic activities of the Kupffer cells significantly.

Finally, rats were treated with increasing amounts of lac-

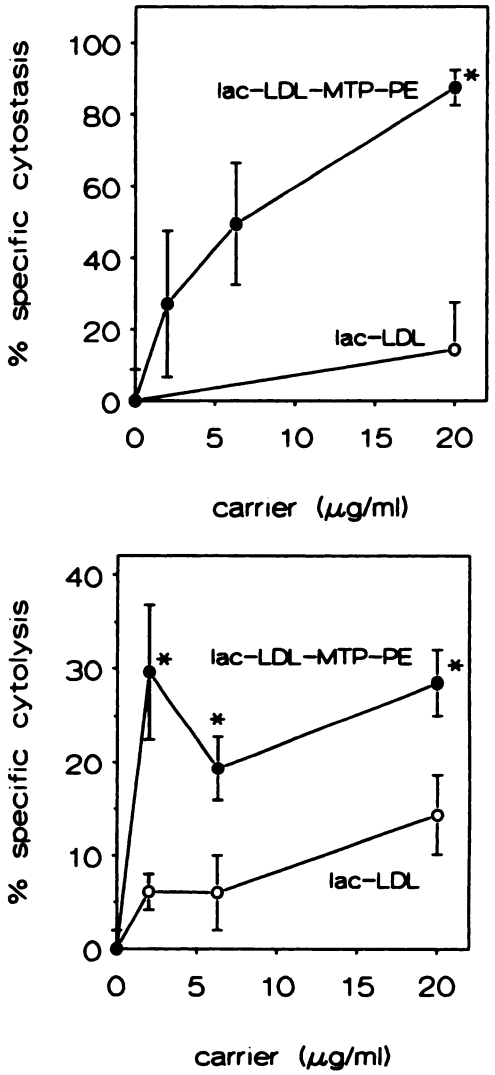

Fig. 3. Effect of lac-LDL-MTP-PE on Kupffer cell tumoricidal activity. The effects of increasing amounts of lac-LDL (○) and lac-LDL-MTP-PE (●) on the specific cytostatic (*top*) and specific cytolytic (*bottom*) activities of cultured Kupffer cells towards B16F10 tumor cells were determined. Assays were performed as described in Experimental Procedures. Data are mean ± standard deviation of three experiments. Control Kupffer cells had a cytostatic activity of 48 ± 9% and a cytolytic activity of 16 ± 2%. *, Significant difference ($p < 0.05$) between treatment with lac-LDL-MTP-PE and treatment with lac-LDL.

TABLE 1

Effect of lac-LDL, lac-LDL-MTP-PE, and LPS on the incorporation of L-[^{35}S]methionine into newly synthesized, secreted proteins

Kupffer cells were incubated for 24 hr with lac-LDL, lac-LDL-MTP-PE, or LPS in L-methionine-free DMEM, containing 75 μCi/ml L-[^{35}S]methionine, or with medium alone. Treatment and analysis of the samples were as described in Experimental Procedures. Data are expressed as percentage of control (mean ± standard error of three independent determinations). Control level of incorporation was 13,300 ± 500 dpm/μg of Kupffer cell protein.

Immunomodulator	Concentration	Secreted proteins
	μg/ml	% of control
Lac-LDL	6.3[a]	155 ± 22[b]
Lac-LDL-MTP-PE	6.3[a]	217 ± 15[c]
LPS	10[d]	141 ± 17[b]

[a] Concentration of the drug carrier.
[b] Statistically significant difference, compared with control (Student's *t* test), $p < 0.05$.
[c] Statistically significant difference, compared with lac-LDL (Student's *t* test); $p < 0.05$.
[d] Concentration of the immunomodulator.

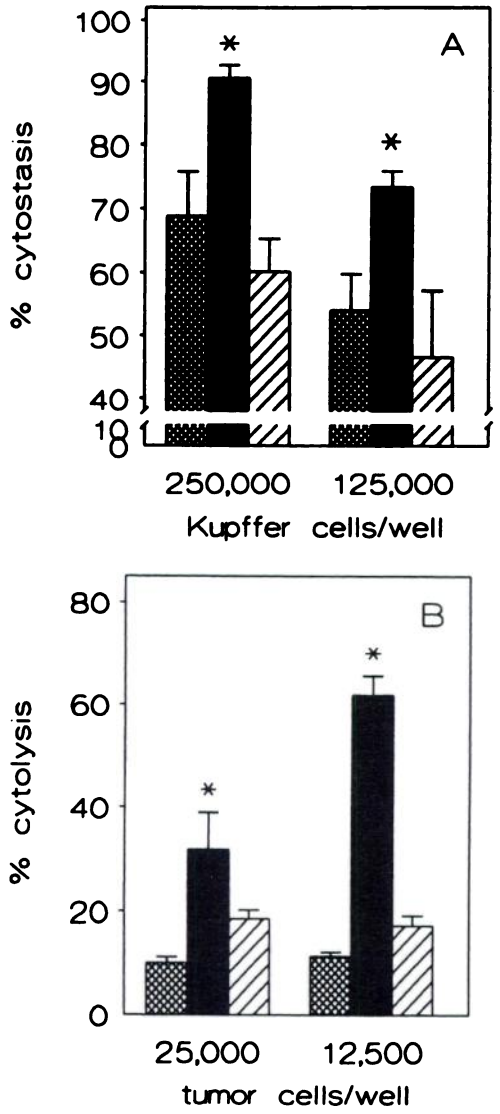

Fig. 4. *In vivo* activation of Kupffer cells with lac-LDL-MTP-PE. Four hours after the administration of lac-LDL (■), lac-LDL-MTP-PE (■) (both at 20 μg of carrier/kg of body weight), or saline (▨), Kupffer cells were isolated, plated, and tested for their cytostatic (A) and cytolytic (B) activities. For the cytostatic assay (A), 12,500 B16F10 cells were added to either 250,000 or 125,000 cultured Kupffer cells. For the cytolytic assay (B), either 25,000 or 12,500 [^{3}H]thymidine-labeled B16F10 tumor cells were added to 250,000 cultured Kupffer cells. Data are the mean ± standard deviation of eight experiments. *, Significant difference ($p < 0.05$) between lac-LDL-MTP-PE-treated Kupffer cells and lac-LDL- or saline-treated Kupffer cells.

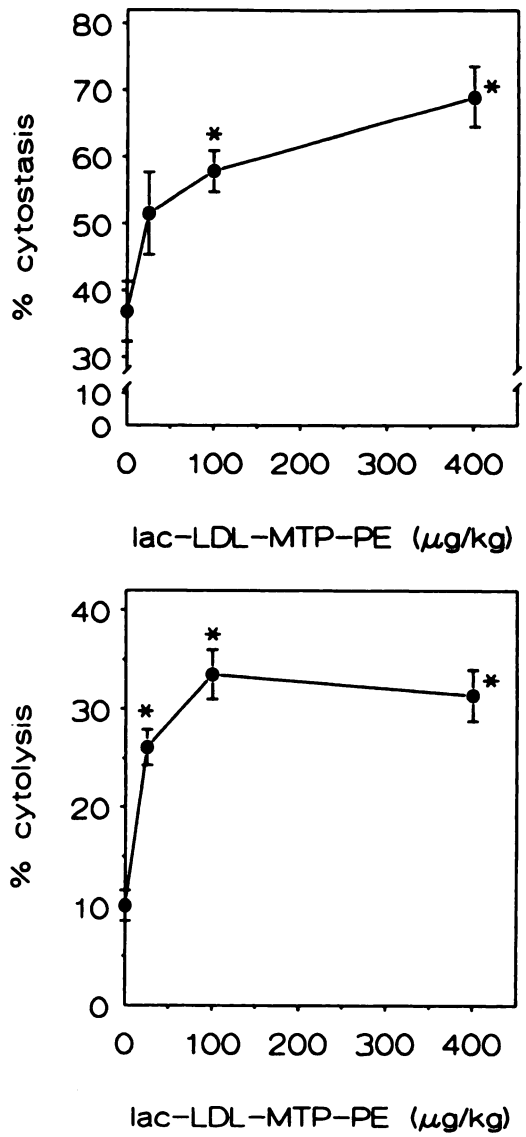

Fig. 5. Dose-dependent activation of Kupffer cells after *in vivo* administration of lac-LDL-MTP-PE. Rats were given intravenous injections of saline or 25, 100, or 400 μg of lac-LDL-MTP-PE/kg of body weight. Four hours after treatment of the rats, Kupffer cells were isolated and cultured at a density of 250,000 cells/well. For the cytostatic assay (*top*), 25,000 B16F10 tumor cells were added to the Kupffer cells. For the cytolytic assay (*bottom*), 12,500 radiolabeled B16F10 tumor cells were added to the Kupffer cells. Data are mean ± standard deviation of eight experiments. *, Significant difference ($p < 0.05$) between lac-LDL-MTP-PE-treated Kupffer cells and saline-treated Kupffer cells.

LDL-MTP-PE. We determined that the treatment of rats with increasing amounts of lac-LDL-MTP-PE resulted in increasing levels of both cytostatic and cytolytic activities of the Kupffer cells (Fig. 5). Maximal activation of the Kupffer cells was found at dosages of 400 μg of lac-LDL-MTP-PE/kg of body weight.

Discussion

In this paper we report that the immunomodulator MTP-PE can be targeted to rat liver macrophages (Kupffer cells) by the use of the drug carrier lac-LDL. The Kupffer cells are subsequently activated, both *in vivo* and *in vitro*, to tumoricidal cells. The immunomodulator MTP-PE is in fact a prodrug, which must be hydrolyzed into an active part (the MTP moiety) and the PE moiety (22). It is therefore important to note that the uptake of lac-LDL by Kupffer cells is mediated by a galactose-specific receptor. The receptor involved in the recognition of lactosylated particles, like lac-LDL, was recently identified (21) to be the well described fucose receptor (23, 24). The fucose receptor is uniquely localized in the body on Kupffer cells, which makes this receptor very attractive for the targeting of MTP-PE, via the drug carrier lac-LDL, to the Kupffer cells to specifically activate the Kupffer cells (23, 24). After binding to the fucose receptor, lac-LDL is endocytosed and degraded in the lysosomes, which is favorable for the hydrolysis of MTP-PE. It is anticipated that MTP, after hydrolysis in the lysosomes, subsequently activates the Kupffer cells. The incorpo-

ration of the prodrug MTP-PE into lac-LDL, which is internalized and degraded lysosomally after binding to the Kupffer cells, is relevant for the activation of Kupffer cells to tumoricidal cells.

MTP-PE has also been incorporated into the drug carrier acetylated LDL (20) and this particle, which is very comparable to lac-LDL with respect to its lipid moiety, could carry 40–130 molecules of MTP-PE. Acetylated LDL is, however, recognized by a scavenger receptor, which is present on many macrophages but is absent on the liver macrophages. In the liver acetylated LDL is predominantly taken up by the endothelial liver cells. To activate the Kupffer cells it was necessary to choose a different recognition marker (17, 25). The drug carrier lac-LDL is rapidly cleared from the plasma (plasma half-life, 3 min) after intravenous injection and is taken up very specifically in the liver. The specific uptake of lac-LDL was at least 20 times higher than the uptake in any other tissue in the body (4, 5). Within the liver, uptake of lac-LDL is very specifically performed by the Kupffer cells, which take up 6–7 times and 13 times more lac-LDL than do endothelial and parenchymal liver cells, respectively (4, 5). This extremely rapid plasma clearance of lac-LDL reduces the possible leakage of the prodrug MTP-PE from the drug carrier. With respect to this rapid clearance, lac-LDL may be a better carrier for MTP-PE than are liposomes containing galactose groups as recognition markers, because the plasma half-life of liposomes carrying trisgalactose-cholesterol or lactocylceramide is at least 5 times greater than that of lac-LDL, thereby increasing the risk of leakage of drugs (4, 26–28). Incorporation of MTP-PE into the drug carrier, in comparison with the use of the free drug (MDP or MTP-PE), is also beneficial, because the free drug is taken up by cells only through nonspecific pinocytosis (8, 29). The free drug also is not taken up very specifically by Kupffer cells after administration but most likely is taken up by the cell types lining the veins and arteries. It can be calculated that the dose of free drug needed to activate the Kupffer cells (2000 μg of MTP/kg of body weight) (8) is much larger than the amount of MTP-PE we needed, after incorporation in the drug carrier lac-LDL, to activate the Kupffer cells (20 μg of MTP-PE/kg of body weight).

In this study it was demonstrated that lac-LDL-MTP-PE can activate the Kupffer cells with respect to increased protein. Another immunomodulator, LPS, also stimulated protein secretion by Kupffer cells, but to a lesser extent. The drug carrier lac-LDL itself had an unexpected stimulatory effect on protein secretion, but the effect was significantly increased after the incorporation of MTP-PE in lac-LDL. More importantly, lac-LDL-MTP-PE increased specifically the cytostatic and cytolytic activities of Kupffer cells towards tumor cells. We did not observe any effect of the drug carrier on the cytostatic and cytolytic activities; control and lac-LDL-treated Kupffer cells had comparable cytostatic activities and cytolytic effects towards B16F10 melanoma cells. Both the cytostatic activity and the cytolytic activity were greatly enhanced by the uptake of lac-LDL-MTP-PE by Kupffer cells, both *in vivo* and *in vitro*.

In summary, we demonstrate in this study that the novel drug carrier lac-LDL can be used to deliver drugs effectively to Kupffer cells. The lipophilic immunomodulator MTP-PE was targeted to rat Kupffer cells via a galactose-specific uptake mechanism, by the incorporation of this drug into the lipophilic part of the drug carrier lac-LDL. The targeting of MTP-PE, via a receptor-mediated pathway, to rat liver macrophages activated these cells to tumoricidal cells both *in vivo* and *in vitro*. The activation of Kupffer cells to tumoricidal cells may be useful in the immunotherapeutic treatment of liver metastasis (10), which frequently occur after the surgical treatment of primary colorectal tumors. As shown before (30), activation of Kupffer cells will be most effective for the treatment of liver metastases when activation is induced directly after the primary (colorectal) tumor is detected.

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