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POLYMYXIN B COMPLEXES WITH AND CATIONIZES LOW DENSITY LIPOPROTEINS

THE CAUSE OF POLYMYXIN B-INDUCED ENHANCEMENT OF ENDOCYTOTIC CATABOLISM OF LOW DENSITY LIPOPROTEINS

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Abstract—We previously reported that polymyxin B (PMB) enhances cellular catabolism of low density lipoproteins (LDLs) through a non-LDL receptor-mediated endocytotic pathway. These data were obtained mainly by using Hep G2 cells, a well differentiated human hepatoma cell line. In the current study, we explore the mechanisms of PMB-mediated endocytotic catabolism of LDL. We found that PMB enhanced LDL catabolism also in homozygous familial hypercholesterolemia fibroblasts, thereby establishing that PMB-mediated cellular catabolism of LDL does not involve LDL receptors. By using [^{14}C]sucrose, and ligands for the asialoglycoprotein (ASGP) receptors, possibilities were excluded that PMB enhances cellular endocytosis of LDL, by inducing a general increase of cellular pinocytic activity or by causing endocytosis of LDL via the ASGP receptors in Hep G2 cells. We further show, by using polymyxin B coupled Sepharose 4B (PMB–Sepharose 4B) beads, that PMB binds to LDL to form a complex. This binding was tight, and changes in pH and salt concentrations had no significant effect on the binding, but unlabelled LDL competed with ^{125}I-LDL to bind to PMB–Sepharose 4B. Urea and endotoxins decreased this binding, suggesting that PMB binds to LDL at least partially through hydrophobic interactions. Agarose gel electrophoresis of PMB–LDL indicates that PMB cationizes LDL. In conclusion, PMB binds to LDL to form a PMB–LDL complex presumably through interactions between lipid groups. This endows LDL with positive charges, which enhances LDL binding to negatively charged cell membranes, and such bound LDL is rapidly internalized through absorptive endocytosis.

Recently, we have been studying the influence of endotoxin on cellular endocytotic catabolism of low density lipoproteins (LDLs†), mostly in Hep G2 cells. This cell-line is of human origin and is well differentiated [1]. It has been widely used and is well suited to studying lipoprotein catabolism as it has functional LDL receptors [2]. We found that endotoxin inhibits cellular catabolism of LDL [3, 4]. To try to define which part(s) of the endotoxin molecule play(s) a role in the inhibitory effect of endotoxin on cellular endocytosis of LDL, we used polymyxin B (PMB). Besides its antimicrobial activity, PMB has antiendotoxin properties as it binds to the lipid A part of endotoxin [5]. We then found that PMB dramatically enhances LDL catabolism in Hep G2 cells and extrahepatic cells (human skin fibroblasts, bovine endothelial cells and bovine smooth muscle cells) through an endocytotic pathway [6]. PMB-mediated LDL catabolism appears to be due to an effect of PMB on LDL rather than on the cells *per se*, as pretreatment of cells with PMB does not increase the subsequent cellular uptake and degradation of LDL [6]. PMB-mediated endocytosis of LDL has characteristics of an adsorptive endocytotic process, i.e. sensitivity to treatment by enzymes such as phospholipase C and pronase, Ca^{2+} dependency, microtubules involved in the endocytotic process and degradation in lysosomes. However, LDL receptors seem not to be involved in this process, as a monoclonal anti-LDL receptor antibody has no effect on PMB-mediated endocytotic catabolism of LDL [6]. In the present study, we further explore the mechanisms of this process. The results show that PMB forms a complex with LDL and cationizes LDL. This then causes an enhancement of endocytotic catabolism of LDL.

MATERIALS AND METHODS

Materials. PMB sulfate, *N*-acetylgalactosamine, *N*-acetylglucosamine, fetuin, asialofetuin and polymyxin B coupled Sepharose 4B (PMB–Sepharose 4B, which contains 1 mg PMB per 40 mg Sepharose in a volume of 1 mL) used in this study were obtained from the Sigma Chemical Co. (St Louis, MO, U.S.A.); Sepharose 4B, Sepharose CL 4B and polyacrylamide gradient gels PAA 2/16 (4.9 × 82 × 82 mm) were obtained from Pharmacia (Uppsala, Sweden); [^{14}C]sucrose (sp. act. 604 mCi/mmol sucrose) from Amersham (Bucks, U.K.); Spectra/Por Standard Cellulose Dialysis Tubing (M_r cut off: 12,000–14,000) from Spectrum® (Houston, TX, U.S.A.).

Cell cultures. The established hepatoblastoma cell

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† Abbreviations: LDLs, low density lipoproteins; PMB, polymyxin B; PMB–Sepharose 4B, polymyxin B coupled Sepharose 4B; FCS, foetal calf serum; HSA, human serum albumin; PBS, phosphate-buffered saline; BSA, bovine serum albumin; ASGP, asialoglycoprotein.

line Hep G2 was obtained from the American Tissue Type Culture Collection (Rockville, MD, U.S.A.). The cells were grown at 37° in 80 cm^2 flasks (NUNCLON, Roskilde, Denmark), in 25 mL RPMI 1640 with glutamine (Life Technologies Ltd, Paisley, U.K.) containing 10% heat inactivated (56° for 30 min) foetal calf serum (FCS, from Flow Laboratories, Irvine, U.K.). The cells were grown in humidified air with 5% CO_2 and subcultured approximately once every week with a split ratio of 1:6.

Human skin fibroblasts were from skin punch biopsies which were obtained from the medial aspect of left upper arm of healthy adults and a patient with homozygous familial hypercholesterolemia. The latter cells were from a 7-year-old girl with hypercholesterolemia (over 18 mmol/L) and tendon xanthomas. Her cultured fibroblasts showed no LDL receptor activity. Cell culture was established as described previously [7] and the cells were maintained in minimum essential medium with 25 mM Hepes, supplemented with 10% FCS, 4 mM glutamine, 1% non-essential amino acids, 75 U/mL penicillin and 100 μg/mL streptomycin at 37° in an atmosphere of air. Fibroblasts were, in the present experiments, used from passages five to seven.

To initiate the experiments, cells were trypsinized with 0.05% trypsin–0.02% EDTA (Life Technologies Ltd) and plated into 35-mm plastic Petri dishes (NUNCLON, Roskilde, Denmark) and grown in 2 mL medium containing 10% FCS. The culture medium was changed every 2–3 days. The experiments started when cells became subconfluent, i.e. covering around two-thirds of the bottom of the dish.

LDL isolation and labelling. LDL was isolated from plasma from normo-cholesterolemic subjects by sequential preparative ultracentrifugation [8] as described earlier [3, 4, 6]. A narrow density range (1.034–1.054 kg/L) was used in the experiments to minimize contamination of LDL with apoliprotein E.

LDL was iodinated with ^{125}I by the iodine monochloride method [9] as modified for lipoproteins [10]. Unbound ^{125}I was removed by chromatography on a Sephadex G-25 column followed by extensive dialysis against 0.15 M NaCl with 1 mM EDTA, pH 7.4. The specific activity of LDL was between 150 and 400 cpm/ng LDL protein.

LDL degradation assay. When cells became subconfluent, the growth medium was changed to 1 mL medium containing 0.5% human serum albumin (HSA, fraction V, from Sigma). ^{125}I-LDL (2 μg LDL/mL medium) was then added to the dishes for 4 hr of incubation, with exceptions indicated, at 37°. After the incubation, the medium was removed to measure degradation products, i.e. the trichloroacetic acid-soluble non-iodine ^{125}I radioactivity [11]. The cells were washed three times with 1 mL cold phosphate-buffered saline (PBS) and then scraped in 1 mL 0.5 M NaOH for measurement of cell protein. The results are expressed as ng LDL degraded/mg cell protein, or as a percentage of the control.

Assay for pinocytic uptake of [^{14}C]*sucrose.* When cells became subconfluent, the growth medium was changed to 1 mL medium containing 0.5% HSA. [^{14}C]Sucrose (5 × 10^5 dpm/mL medium, the final activity in the medium was 0.5 μCi/mL) was then added to the dishes for 4 hr of incubation in a CO_2 incubator. After incubation, the medium was removed and the cells were washed three times with 1 mL cold PBS. The cells were dissolved in 1 mL of 0.5 M NaOH and then mixed with 10 mL of Luma Gel (from Lumac, The Netherlands). The mixture was left for 24 hr at the ambient temperature of the beta-counter (13°) to avoid chemiluminescence. The radioactivity was then measured in a beta-counter (Packard 300 CD Liquid Scintillation Spectrometer). The radioactivity count is a measure of cellular pinocytic uptake and related to cell protein. The volumes of fluid pinocytosed by the cells were calculated and expressed as nL/mg cell protein [12].

In vitro *binding of* ^{125}I*-LDL to PMB–Sepharose 4B.* ^{125}I-LDL were mixed with PMB–Sepharose 4B or Sepharose 4B in 250 μL phosphate buffer (0.01 M, pH 7.4) and kept at room temperature usually for 60 min with exceptions indicated. The beads were then washed with 2 mL phosphate buffer, usually, once. The bound radioactivity was measured in a gamma-counter. The results are expressed as LDL bound i.e. as a percentage of the added amount or as a percentage of the control.

Gradient gel slab electrophoresis. LDL and the mixture of LDL/PMB (25 μg LDL with various amounts of PMB in a volume of 45 μL) was mixed (3:1, v/v) with a solution of 40% sucrose/0.1% bromophenol blue (14.4 μL) and then applied on to a polyacrylamide gradient gel PAA 2/16 (fractionation range being about 100,000–5,000,000) for electrophoresis on a Pharmacia GE-4 apparatus in electrophoresis buffer (10.9 g Tris, 4.95 g boric acid, 0.93 g Na_2EDTA per litre adjusted to pH 8.73). Before applying the samples, the gradient gel was pre-equilibrated for 20 min at 70 V. After applying the samples on to the gel (2.9 μg LDL in a volume of 7 μL for each sample), pre-electrophoresis of the samples was made for 20 min at 70 V. Then further electrophoresis was carried out for 16 hr at 150 V. The gels were stained with 0.1% Coomassie blue R250 in 25% methanol/10% acetic acid for 24 hr at room temperature and then destained with 25% methanol/10% acetic acid.

Column chromatography. Gel filtration of the mixture of PMB/^{125}I-LDL was done on a column of Sepharose CL 4B. Chromatography was carried out with 0.5 mL sample aliquots containing 3 μg ^{125}I-LDL diluted with 30 μg unlabelled LDL with or without 5500 μg PMB. The column (35 × 1 cm) was equilibrated and eluted with 0.04 M phosphate buffer (pH 7.4) at room temperature and at a flow rate of 12 mL/hr. Fractions of 425 μL were collected using LKB 2112 Redirac fraction collector.

Agarose gel electrophoresis. The agarose gel electrophoresis was carried out in barbital buffer at pH 8.6 according to Nobel [13] using Sudan Black B to stain. In some instances, we instead used 0.025 M phosphate buffer (pH 5.84) as electrophoresis buffer. The agarose solution was prepared by dissolving 700 mg of Agarose A (with medium electroendosmosis, from Pharmacia, Uppsala,

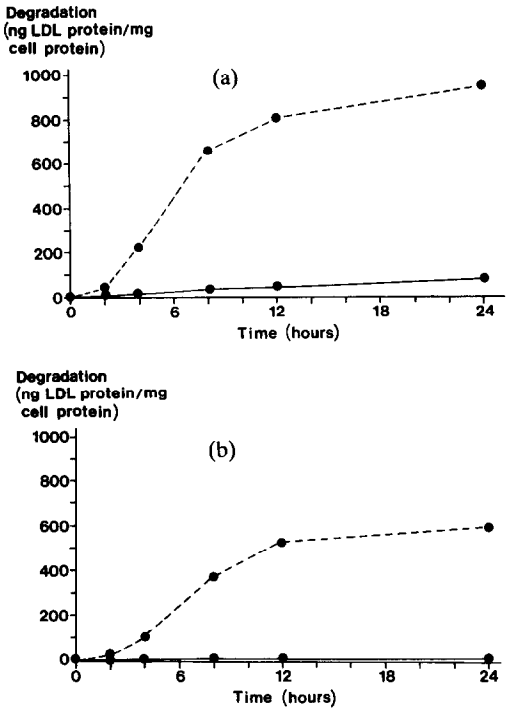

Fig. 1. PMB enhances catabolism of LDL in both normal (a) and familial homozygous hypercholesterolemia fibroblasts (b). ^{125}I-LDL (2 μg LDL/mL) with or without PMB (100 μg/mL) were added to the dishes and incubated for various time intervals to measure LDL degradation as described in Materials and Methods. (●—●) and (●- - -●) show LDL degradation in the absence or presence of PMB, respectively. Values are means of two dishes. Data from one of two similar experiments.

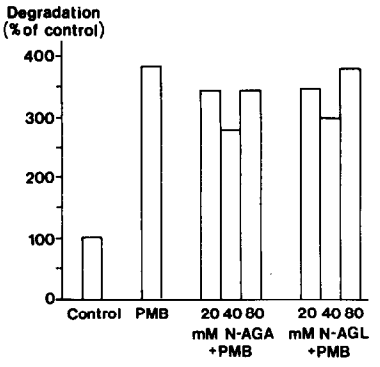

Fig. 2. The effect of *N*-acetylgalactosamine on PMB-enhanced degradation of LDL in Hep G2 cells. ^{125}I-LDL (2 μg LDL/mL) and PMB (100 μg/mL) together with 20, 40 or 80 mM of *N*-acetylgalactosamine (N-AGA) or *N*-acetylglucosamine (N-AGL) were added to the dishes and 4-hr degradation of LDL was measured as described in Materials and Methods. Values are means of two dishes.

Sweden) by boiling in 100 mL electrophoresis buffer (0.075 M barbital buffer with pH 8.6 or 0.025 M phosphate buffer with pH 5.84). After the agarose had dissolved completely, the solution was allowed to cool to about 55°. Then 2 mL of 10% bovine serum albumin (BSA, fraction V, from BDH Ltd, Poole, U.K.) was added. After that 25 mL of the mixture was gently coated on to a Gel Bond® film (204 × 109 × 0.2 mm, from FMCBioProduct, Rockland, ME, U.S.A.). A slit-forming device with teeth 8 × 0.6 mm was immediately placed in the hot agarose. After applying the samples (4.4 μg LDL in a volume of 8 μL for each sample), electrophoresis was run in barbital buffer (pH 8.6) for about 2.5 hr at 185 V and 90 mA, or in phosphate buffer (pH 5.84) for 24 hr at 60 V and 70 mA. After electrophoresis, the films were immediately transferred to a solution of 5% acetic acid/75% ethanol for 1 hr fixation and then rinsed several times with distilled water. After drying with an electric fan, the films were coloured with Sudan Black B solution (200 mg Sudan Black B dissolved in 100 mL of 60% ethanol) for 1–2 hr and then destained by rinsing several times with 50% ethanol.

Protein determination. The protein content of the cells and of LDL was determined with the method of Lowry *et al.* [14] using HSA as a standard.

Statistical analysis. Experimental results were analysed for their statistical significance by Student's *t*-test. A P value less than 0.05 was considered significant.

RESULTS

PMB-enhanced endocytotic catabolism of LDL is not dependent on functional LDL receptors

In order to confirm that PMB-mediated endocytosis of LDL is not dependent on LDL receptors, homozygous familial hypercholesterolemia fibroblasts were used, as these cells lack functional LDL receptors [15]. The results presented in Fig. 1 show that the homozygous fibroblasts catabolized little, if any, ^{125}I-LDL during a 24-hr incubation. However, PMB (100 μg PBM/mL) dramatically enhanced ^{125}I-LDL catabolism in the homozygous fibroblasts (583 ng/mg cell protein/24 hr, about 38 times increase of LDL degradation) as well as in the normal fibroblasts (952 ng/mg cell protein/24 hr, about 11 times increase of LDL degradation). These data do confirm that PMB-mediated endocytotic catabolism of LDL is not dependent on functional LDL receptors.

PMB does not mediate endocytotic catabolism of LDL via the asialoglycoprotein (ASGP) receptors

In order to exclude the possibility that PMB changes the configuration of LDL so that LDL exposes galactose residues and is recognized by the ASGP receptors on Hep G2 cells, the following experiments were done. The data shown in Fig. 2 indicate that *N*-acetylgalactosamine (a ligand for the ASGP receptors [16]) and *N*-acetylglucosamine (used as control) seemed to have no effect on PMB-mediated endocytotic catabolism of LDL. Although the presence of very high concentrations of

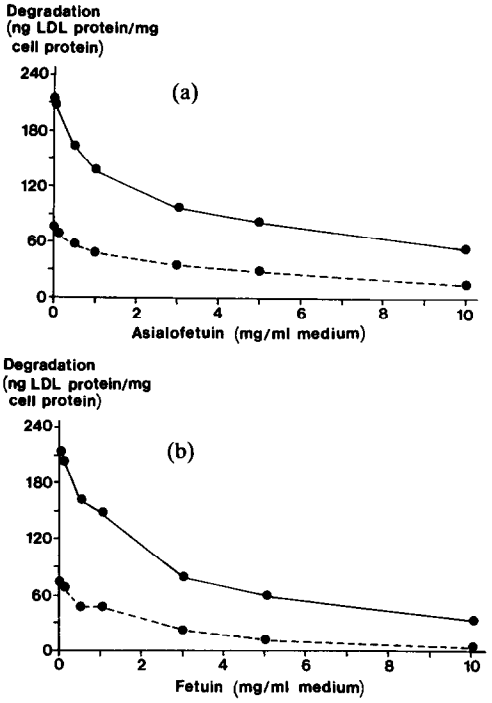

Fig. 3. The effect of asialofetuin and fetuin on PMB-enhanced degradation of LDL in Hep G2 cells. ^{125}I-LDL (2 μg LDL/mL) with or without PMB (100 μg/mL) were added to the dishes for a 4-hr degradation measurement in the presence of increasing amounts of asialofetuin (a) or fetuin (b) as described in Materials and Methods. (●--●) and (●—●) show LDL degradation in the absence or presence of PMB, respectively. Values are means of two dishes. Data from one of two similar experiments.

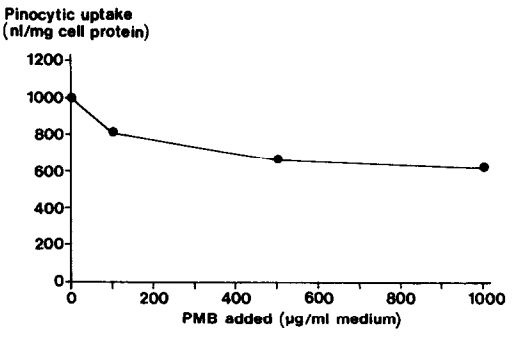

Fig. 4. The effect of PMB on the pinocytic uptake of [^{14}C]-sucrose in Hep G2 cells. [^{14}C]Sucrose (5×10^5 dpm/mL medium) with increasing amounts of PMB were added to the dishes for a 4-hr pinocytic uptake assay as described in Materials and Methods. Values are means of three dishes. Data from one of two similar experiments.

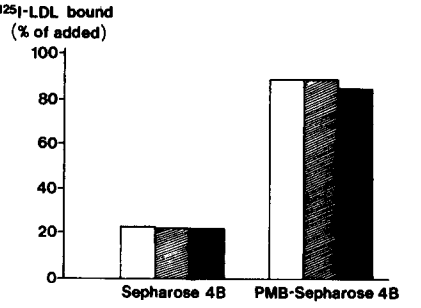

Fig. 5. Binding of LDL to PMB–Sepharose 4B and Sepharose 4B. ^{125}I-LDL (2 μg) was mixed with 0.1 mL of PMB–Sepharose 4B or Sepharose 4B, and the binding of LDL was then measured as described in Materials and Methods. Opened, slashed and closed bars show the binding after the first, the second and the third wash, respectively. Values are means of two determinations. Data from one of two similar experiments.

asialofetuin, a ligand for the ASGP receptors [17], caused a dose-dependent inhibition on LDL degradation and on PMB-mediated LDL degradation (Fig. 3a) in Hep G2 cells, fetuin (used as control) also had a similar effect (Fig. 3b). These results thus suggest the PMB-enhanced LDL catabolism does not occur through the ASPG receptors.

PMB does not cause a general increase in cellular pinocytic activity

[^{14}C]Sucrose was employed, as it meets the criteria for a tracer of fluid phase endocytosis [18], in order to exclude the possibility that PMB enhances cellular LDL catabolism by inducing a general increase of cellular pinocytic activity. The results show that PMB does not increase the uptake of [^{14}C]sucrose in Hep G2 cells (Fig. 4). Thus, PMB does not cause a general increase in cellular pinocytic activity.

PMB complexes with and cationizes LDL

PMB–Sepharose 4B was employed to investigate whether PMB binds to LDL to form a complex. The results in Fig. 5 show that when 2 μg ^{125}I-LDL was mixed with 0.1 mL of PMB–Sepharose 4B and maintained for 60 min, about 90% of the added ^{125}I-LDL bound to PMB–Sepharose 4B, whereas only about 20% of the added ^{125}I-LDL was retained on Sepharose 4B beads ($P < 0.001$). The amount of the bound ^{125}I-LDL after the first wash was nearly the same as after the third wash (Fig. 5). So, in all following experiments the beads were washed only once. When a fixed amount of ^{125}I-LDL was mixed with increasing amounts of PMB–Sepharose 4B and with increasing amounts of Sepharose 4B, the results further indicated that PMB–Sepharose 4B had a higher capacity to bind LDL than Sepharose 4B (Fig. 6). Binding of LDL to the PMB–Sepharose 4B was a rapid process as immediately after ^{125}I-LDL was mixed with the PMB–Sepharose 4B, half of the added ^{125}I-LDL was bound, and within 40 min, more than 80% of the added ^{125}I-LDL was bound. ^{125}I-LDL binding to PMB–Sepharose 4B was tested at different temperatures, i.e. 4°, 24° and 37°. The amount of ^{125}I-LDL bound was considerably lower at 4°, only 77% of the binding which occurred at 37° ($P < 0.05$). However, binding at room temperature

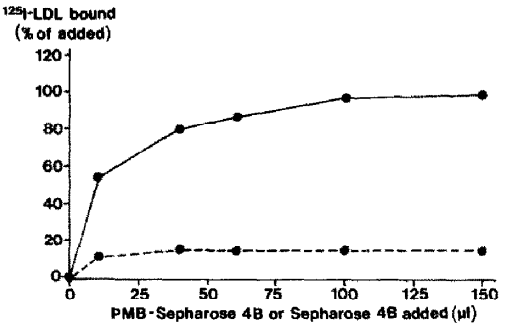

Fig. 6. Dose–response of the binding of LDL to PMB–Sepharose 4B and Sepharose 4B. A fixed amount of ^{125}I-LDL (2 µg) was mixed with increasing amounts of PMB–Sepharose 4B or Sepharose 4B to measure the binding of LDL as described in Materials and Methods. (●—●) and (●– –●) show the binding of ^{125}I-LDL to PMB–Sepharose 4B and Sepharose 4B, respectively. Values are means of two determinations.

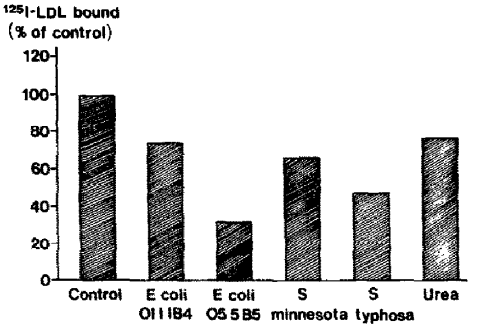

Fig. 8. Effect of endotoxins and urea on the binding of LDL to PMB–Sepharose 4B. ^{125}I-LDL (0.2 µg) was mixed with 0.01 mL of PMB–Sepharose 4B in the presence of 100 µg of different endotoxins or in the presence of 6 M urea, and the binding of LDL to PMB–Sepharose 4B was then measured as described in Materials and Methods. Values are means of two determinations. Data from one of two similar experiments.

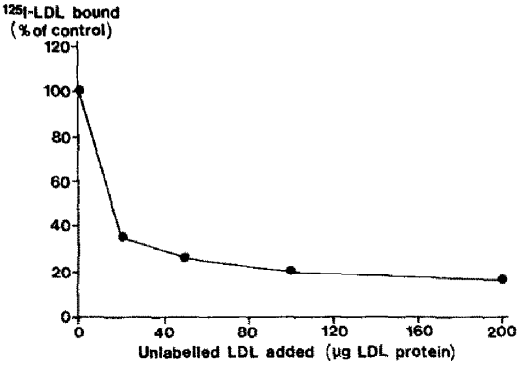

Fig. 7. Unlabelled LDL competes with ^{125}I-LDL to bind to PMB–Sepharose 4B. ^{125}I-LDL (0.2 µg) was mixed with 0.01 mL of PMB-Sepharose 4B in the presence of increasing amounts of unlabelled LDL, and the binding of ^{125}I-LDL to PMB–Sepharose 4B was then measured as described in Materials and Methods. Values are means of two determinations.

was the same as at 37°. Changes in the pH, tested from 6.1 to 8.3, and salt concentrations (NaCl), tested from 0 to 500 mM, appeared to have no significant effect on binding of ^{125}I-LDL to PMB–Sepharose 4B (data not shown). Unlabelled LDL competed with ^{125}I-LDL to bind to PMB–Sepharose 4B (Fig. 7). The competitive effect of unlabelled LDL on ^{125}I-LDL binding to PMB–Sepharose 4B levelled off when 50–100 µg of unlabelled LDL were added. Urea (6 M) which lessens hydrophobic bonds, decreased binding of ^{125}I-LDL to PMB–Sepharose 4B by 23% ($P < 0.005$) (Fig. 8). Endotoxin may interfere with binding of LDL to PMB–Sepharose 4B as PMB tightly binds to the lipid A part of endotoxin [5]. Thus, binding of ^{125}I-LDL to PMB–Sepharose 4B was measured in the presence of endotoxins. The results show that binding of ^{125}I-LDL to PMB–Sepharose 4B was reduced by 26–69% in the presence of 100 µg of endotoxins from *Escherichia coli* O111 B4 ($P < 0.005$), *E. coli* O55 B5 ($P < 0.001$), *Salmonella minnesota* ($P < 0.001$) and *Salmonella typhosa* ($P < 0.001$) (Fig. 8).

The size of PMB–LDL complex was estimated by gradient gel electrophoresis on polyacrylamide gradient gel PAA 2/16 and gel chromatography on Sepharose CL 4B. LDL was mixed with increasing amounts of PMB and then maintained at room temperature for more than 60 min. The mixtures were then applied on to the gradient gel for electrophoresis. The results show that there is one band in the native LDL preparation (migration of about 18 mm, Fig. 9). However, with increasing amounts of PMB, the native LDL band gradually got fainter, whereas new bands with sizes larger than native LDL (migration distance less than 18 mm) appeared (Fig. 9, lanes C, D, E and F). When the ratio of PMB/LDL was more than 40:1 (µg/µg), no LDL entered the gel (Fig. 9, lanes G, H, I and J), whereas protein aggregates could be seen in the sample slots, suggesting that complexes with a size larger than 5,000,000 were formed. We then chose a mixture of PMB/LDL (the ratio was 166:1, µg/µg, in which all LDL was in aggregates with sizes larger than 5,000,000 as judged by gradient gel electrophoresis) for gel filtration on Sepharose CL 4B. Gel chromatography of the mixture of PMB/LDL showed, however, that only a very small radioactive peak appeared before the main radioactive peak corresponding to ^{125}I-LDL (Fig. 10). This suggests that only a small portion of LDL was in a complex with a size larger than native LDL. Therefore, we presume that the size of the PMB–LDL complexes themselves are not significantly larger than the size of native LDL. The formed aggregates that are significantly larger than native LDL are PMB–LDL complexes that aggregate

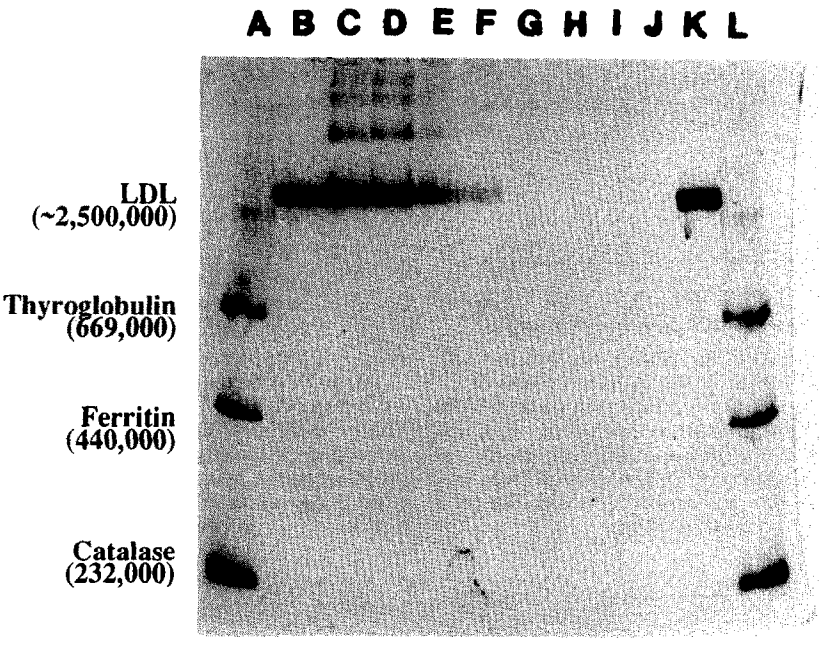

Fig. 9. Gradient gel electrophoresis of LDL and LDL/PMB mixture. LDL (25 μg) was mixed with increasing amounts of PMB in a volume of 45 μL. The mixture was then maintained at room temperature for more than 60 min. To the mixture 14.4 μL of 40% sucrose/0.1% bromophenol blue was added. Then the samples were applied on to the polyacrylamide gradient gels PAA 2/16 for electrophoresis as described in Materials and Methods. Each applied sample contained 2.9 μg LDL with 0 (lanes B and K, controls), 7.0 (lane C), 14.5 (lane D), 29 (lane E), 58 (lane F), 116 (lane G), 232 (lane H), 464 (lane I) and 928 (lane J) μg PMB. Lanes A and L show reference proteins. Data from one of two similar experiments.

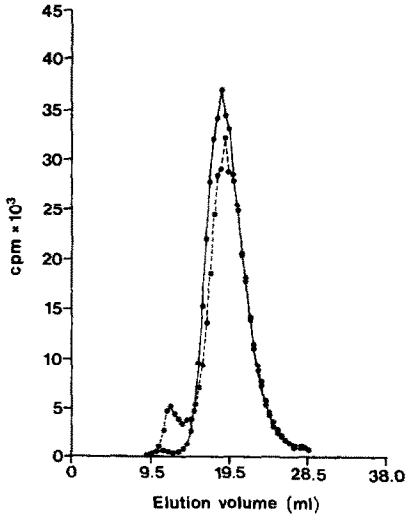

Fig. 10. Gel chromatography of PMB/LDL mixture. ^{125}I-LDL (3 μg) diluted with 30 μg unlabelled LDL was mixed with 5500 μg PMB in a volume of 0.5 mL 0.04 M phosphate buffer (pH 7.4), and the mixture was maintained at room temperature for more than 60 min. The mixture was chromatographed on a Sepharose CL 4B as described in Materials and Methods. Column fractions were assayed for the presence of LDL by measurement of gamma-radioactivity. (●—●) shows LDL and (●- -●) shows PMB/LDL mixture.

together perhaps due to the influence of the electrophoresis conditions.

LDL was mixed with increasing amounts of PMB and then maintained at room temperature for more than 60 min. The mixture was then applied on to an agarose gel plate and electrophoresed at pH 8.6 in barbital buffer. The results showed that PMB significantly delayed LDL electrophoretic mobility in a dose-dependent manner (Figs 11 and 12). The PMB–LDL complex showed a marked reduction of mobility compared to LDL towards the anode. The PMB/LDL mixture was also dialysed against 0.15 M NaCl with 1 mM EDTA (pH 7.4) for 24 hr at 4° using a dialysis bag with a relative molecular mass cut-off of 12,000–14,000 to dialyse away free PMB. The PMB–LDL complex was then applied on to an agarose gel plate and electrophoresed under identical conditions as above. Similar results were obtained. Although the electrophoretic mobility in agarose gel is mainly dominated by the electric charge, we can not completely exclude the possibility that change of LDL size may be responsible for the retarded LDL electrophoretic mobility towards the anode at pH 8.6 in barbital buffer, as the PMB–LDL complexes may aggregate together as described above. The mixture of PMB/LDL was, however, also electrophoresed in 0.025 M phosphate buffer at pH 5.84 in which LDL moves slowly from anode to cathode. The results then showed that PMB accelerated LDL electrophoretic mobility towards the cathode in a dose-dependent manner (Fig. 12),

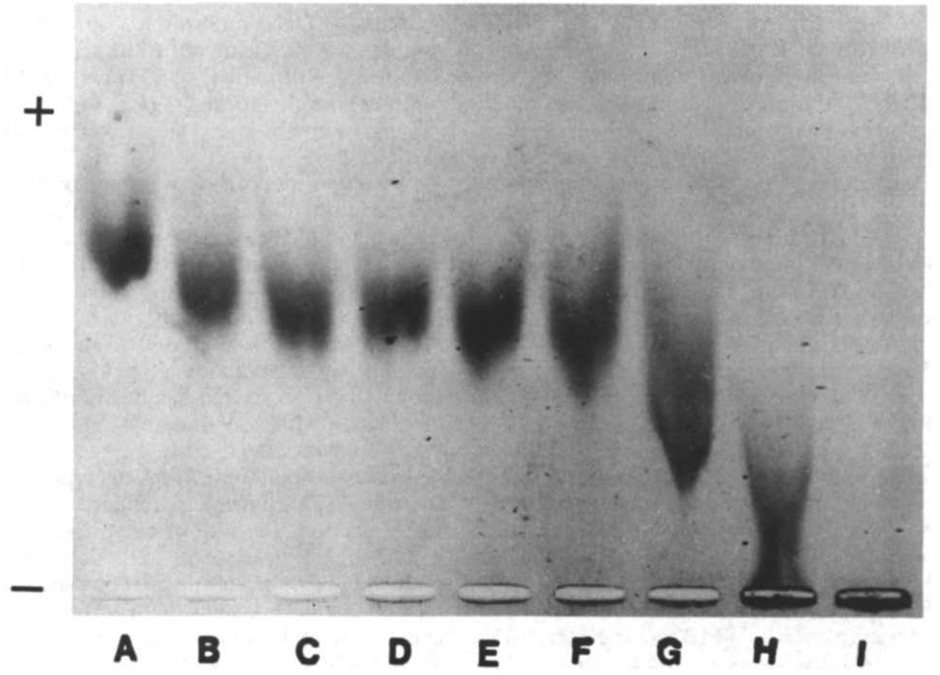

Fig. 11. Agarose electrophoresis of PMB/LDL in barbital buffer with pH 8.6. LDL was mixed with increasing amounts of PMB and then maintained at room temperature for more than 60 min. The mixture was then applied on to an agarose gel plate and electrophoresed at pH 8.6 in barbital buffer as described in Materials and Methods. Each applied sample contained 4.4 μg LDL with 0 (lanes A, control), 12.25 (lane B), 24.5 (lane C), 49 (lane D), 98 (lane E), 196 (lane F), 392 (lane G), 784 (lane H) or 1568 (lane I) μg PMB.

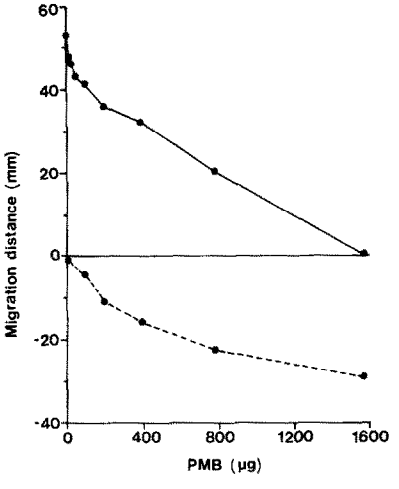

Fig. 12. Effect of PMB on LDL migration on agarose electrophoresis. LDL was mixed with increasing amounts of PMB and then maintained at room temperature for more than 60 min. The mixture was then applied on to an agarose gel plate (4.4 μg LDL with increasing amounts of PMB) and electrophoresed in barbital buffer (pH 8.6) or in phosphate buffer (pH 5.84) as described in Materials and Methods. Migration distance was measured from the application point to the middle of the front. In the barbital buffer with pH 8.6 (top part of the figure), LDL moved from cathode to anode (●—●, positive migration), whereas in the phosphate buffer with pH 5.84 (bottom part of the figure), LDL moved from anode to cathode (●- - -●, negative migration). Values are means of two independent determinations.

suggesting that PMB endows LDL with positive charges. The conclusions of these experiments are that PMB complexes with LDL and makes it more cationic.

DISCUSSION

PMB is a peptide antibiotic with antiendotoxin properties with a relative molecular mass of about 1000. PMB contains both lipophilic and hydrophillic groups within the molecule, and thus has amphipathic properties. Its main mode of action is to interact with lipids and penetrate into and disrupt the structure of cell membranes, and to bind to the lipid A part of the endotoxin molecule. Systemic administration of PMB has been hampered by its nephrotoxicity and neurotoxicity. We have earlier shown that PMB enhances cellular catabolism of LDL by a process presumably not involving LDL receptors, as a monoclonal anti-LDL receptor antibody had no effect on PMB-mediated endocytotic catabolism of LDL [6]. In the present study by using fibroblasts from a patient with familial homozygous hypercholesterolemia, we further confirm that LDL receptors are not involved in the PMB-mediated endocytotic catabolism of LDL.

Apolipoprotein B, the main protein of LDL has been shown to be highly glycosylated, and may function in the asialo form as ligands for galactose-recognizing lectins. Windler *et al.* [9] showed that apolipoprotein B100, B48 and E of human LDL and rat chylomicron remnants contain terminal galactose residues. These studies suggest that LDL may have

a potential structure for binding to the ASGP receptors. Windler *et al.* [19] further suggested that the ASGP receptors may contribute to the endocytosis of LDL and of chylomicron remnants. It is possible that PMB might change the molecular configuration of LDL to allow LDL to expose potential structures for binding to the ASGP receptors. We have now, however, excluded the possibility that PMB-mediated enhancement of LDL catabolism occurs through binding of the PMB–LDL complex to the ASGP receptors. This is based on experiments where *N*-acetylgalactosamine, a ligand for the ASGP receptors [16], had no effect on PMB-mediated endocytotic catabolism of LDL. Although the presence of very high concentrations of asialofetuin, a ligand for the ASGP receptors [17], blocked PMB-mediated degradation of ^{125}I-LDL in Hep G2 cells, fetuin at the same concentration also caused a similar inhibition. This suggests that the inhibitory effect of asialofetuin on PMB-enhanced degradation of ^{125}I-LDL in Hep G2 cells is not due to a specific interference with the ASGP receptors. Furthermore, the ASGP receptors exclusively exist on hepatic cells. However, PMB also enhances LDL catabolism in extrahepatic cells [6].

[^{14}C]Sucrose is generally accepted to meet criteria as a tracer of fluid phase endocytosis, because it is impermeable to the cell membrane, and it does not significantly bind to the cell surface [18]. We excluded the possibility that PMB caused a general increase of cellular pinocytic activity as PMB does not increase uptake of [^{14}C]sucrose.

In our previous study [6], we prepared a mixture of PMB and ^{125}I-LDL, and the mixture was then dialysed, to dialyse away free PMB. This preparation still retained the ability to enhance ^{125}I-LDL catabolism by the cells. We then hypothesized that PMB complexes with LDL and that PMB–LDL complex enters into the cells by an endocytotic pathway after binding to cell membranes.

The present study strengthens this hypothesis by using PMB–Sepharose 4B beads. PMB–Sepharose 4B was originally designed for removal of endotoxins from solutions [20–23]. This is based on the fact that PMB tightly binds to the lipid A part of endotoxin [5]. We found that PMB–Sepharose 4B had a much stronger capacity to bind ^{125}I-LDL than did Sepharose 4B. This is solely attributed to PMB as the difference between PMB–Sepharose 4B and Sepharose 4B is that the former is coupled with PMB. The binding of ^{125}I-LDL to PMB was tight as ^{125}I-LDL can not simply be washed away. Binding of PMB to LDL presumably occurs through lipid group interactions as urea and endotoxin decrease this binding and as changes in pH and salt concentrations have little effect on this binding, although we cannot exclude entirely the possibility that ionic interactions may also be involved in the interactions between PMB and LDL. In this assay system, decrease in binding of ^{125}I-LDL to PMB–Sepharose 4B bead by endotoxins may not be solely due to endotoxin binding to PMB, as endotoxins also bind to lipoproteins including LDL to form endotoxin–lipoprotein complexes [24–27]. Thus, endotoxin may interfere with binding of ^{125}I-LDL to PMB–Sepharose 4B bead by formation of endotoxin–PMB and/or endotoxin–LDL complexes. The size of PMB–LDL complex itself appears not to be significantly larger than the size of native LDL. However, PMB–LDL complexes tend to aggregate together to form aggregates with sizes larger than native LDL particles.

Formation of PMB–LDL complexes will lead to cationization of LDL because of the polycationic character of PMB. Agarose electrophoresis of PMB–LDL obviously indicates that PMB endows LDL with positive charges, because PMB not only delayed LDL electrophoretic mobility towards the anode at pH 8.6, but also accelerated LDL electrophoretic mobility to the cathode at pH 5.84. It has been shown that ferritin that has been rendered cationic by reaction with *N,N*-dimethyl-1,3-propanediamine binds to widespread sites on negatively charged plasma membranes of various cell types [28, 29], and that when LDL have been rendered cationic by the same reaction, the rate of catabolism of LDL was increased by more than 100-fold in homozygous familial hypercholesterolemia fibroblasts [30]. Thus, it is reasonable to assume that PMB mediates cellular catabolism of LDL through a similar mechanism. Presumably the positively charged LDL endowed by PMB interacts with negative charges on the cellular plasma membrane, which causes an enhanced endocytotic catabolism of LDL by the cells.

As shown in this study, PMB is a pharmacological agent, which very firmly interacts with lipoproteins. It is presumably carried on lipoproteins in blood. What is noteworthy is that when bound to lipoproteins, it actually changes the catabolic pathway of the carrier, the LDL particles. This raises the possibility that LDL may enter the cell largely via an endocytotic pathway, other than the LDL receptor pathway, after forming complexes with other pharmacological agents. Also, other experiments have shown that if the galactose-moieties (which are specifically recognized by hepatic ASGP receptors) are introduced into specific Fab fragments of anti-apolipoprotein B antibodies, such Fab fragments caused a specific change of LDL endocytotic metabolism from the LDL receptor-mediated pathway to the ASGP receptor-mediated pathway [31, 32]. This line of research might be fruitful for treatment of patients with homozygous familial hypercholesterolemia as their tissue cells lack functional receptors for LDL.

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