The Transferrin Receptor at the Blood-Brain Barrier - exploring the possibilities for brain drug delivery

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Chapter 7

Interaction of liposomes with lipopolysaccharide: influence of time, serum and liposome composition

Submitted for publication

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Abstract

The central nervous system is protected by the blood-brain barrier (BBB). Under inflammatory conditions (such as bacterial meningitis) brain homeostasis is changed as a result of a disrupted BBB. This may be caused in part by lipopolysaccharide (LPS), that has many effects, among which the formation of free radicals. These free radicals cause a disruption of the tight junctions between brain capillary endothelial cells (BCEC). We have investigated whether liposomes are able to scavenge LPS by measuring the TransEndothelial electrical resistance as a measure of BBB tightness. After 6 hours of pre-incubation of LPS and liposomes (egg phosphatidylcholine (EPC-35) and cholesterol, with 0, 5 or 10 % polyethylene glycol (PEG-PE)), the opening of the BBB *in vitro* was delayed and less pronounced compared to LPS that was not pre-incubated. However, since the functional read-out of the effect of LPS in the *in vitro* BBB model was highly variable, it was not possible to quantify this liposomal scavenging effect by a functional assay. Therefore, we have chosen to investigate this physico-chemically. We found that liposomes, consisting of EPC-35 alone (least stable) or of EPC-35 and cholesterol with 5 or 10 % PEG-PE (more stable) are able to scavenge fluorescently labelled LPS in a time dependent manner. The surface density of PEG or the absence or presence of serum (10 %) did not influence the ability of liposomes to scavenge LPS.

We conclude that liposomes used for the targeting of drugs under inflammatory disease conditions are able to scavenge LPS in a time dependent manner.

Introduction

The central nervous system is protected by the blood-brain barrier (BBB). This barrier maintains homeostasis in the brain, by minimising paracellular and transcellular transport across the endothelial cells (1). The main barrier is formed by brain capillary endothelial cells (BCEC), which are stimulated by astrocytic endfeet (2). Between BCEC, so called tight junctions, are formed. Lipopolysaccharide (LPS) can induce an opening of the tight junctions and thereby decrease BBB functionality. LPS induces transcription of acute phase proteins and activates protein kinases (3). Furthermore, Gaillard *et al* (4) have shown that LPS induces the formation of free radicals, which in turn cause an opening of the tight junctions. By pre-incubation of BCEC with high concentrations of the radical scavenger N-acetyl-L-cysteine (NAC), opening of the BBB by LPS *in vitro* was prevented (4). However, NAC is very hydrophilic and, therefore, poorly penetrates the cellular membrane. Incorporation of NAC into the aqueous compartment of liposomes can potentially increase the intracellular delivery of NAC. We have therefore prepared Tf-tagged liposomes, consisting of EPC-35 and cholesterol, encapsulating NAC. After overnight pre-incubation of the *in vitro* BBB these liposomes were able to counteract LPS induced BBB opening (unpublished results). However, liposomes that did not contain NAC also showed protection of the BBB against LPS after overnight pre-incubation. From literature it is known that LPS, when incorporated into liposomes, is still endocytosed by macrophages, but has a reduced potency to induce tumour cytotoxicity and tumor necrosis factor (TNF) secretion (5). In addition, it has been shown that lipoproteins in serum have the ability to scavenge LPS (6).

We have investigated whether liposomes can scavenge LPS by pre-incubation of liposomes and LPS before addition to the *in vitro* BBB model. After at least 6 hours of pre-incubation with liposomes, the effect of LPS on the *in vitro* BBB model was reduced. However, as was observed earlier by Gaillard *et al* (4), the LPS effect on the *in vitro* BBB model was found to be variable. In addition, the effect of the combination of liposomes and LPS on the *in vitro* BBB model was variable, which made it difficult to quantify these effects by a functional read-out. Therefore, we have chosen to extend our investigations by a physico-chemical approach.

For these experiments we have incubated fluorescently labelled LPS (LPS-Alexa) with liposomes for 2, 6 or 16 hours and separated the liposomes and LPS micelles on a

Sepharose CL4B column. We have performed incubations in the absence or presence of serum (10 %) and with different polyethylene glycol-2000 (PEG) densities on the surface of the liposomes. This method enabled us to directly measure the capacity of liposomes to scavenge LPS and to relate this to the results we have found in our *in vitro* BBB model.

Experimental

Liposomes

Liposomes of egg phosphatidylcholine (EPC-35) only or EPC-35 and cholesterol (2:1 molar ratio) were prepared by hydration of a lipid film in HEPES buffered saline (HBS), pH 7.0, at a phospholipid concentration of 18 µM. Liposomes of EPC-35 and cholesterol were stabilised with 0, 5 or 10 % PEG-PE After hydration of the lipid film, liposomes were extruded 8 times through 200 and 100 nm polycarbonate filters with a hand extruder (Alabaster, AL, USA) to obtain a homogeneous dispersion of liposomes. The final phospholipid content was determined according to Rouser (7). Liposomal size was determined by dynamic light scattering with a Malvern 4700 system (Malvern Ltd. Malvern, UK). The polydispersity index (p.i.) was used as an indication for the size distribution. The p.i. can range from 0 (monodisperse) to 1 (polydisperse).

LPS effect in the in vitro BBB model

The preparation of the *in vitro* BBB model was described previously (8). To characterise the effect of LPS on the *in vitro* BBB model the TransEndothelial electrical resistance (TEER) was measured, as described by Gaillard et al (4).

LPS was pre-incubated at 37 °C for 2, 6 or 16 hours with liposomes (1: 750 weight ratio) in PBS (0.11 mM KH_2PO_4, 0.56 mM Na_2HPO_4 and 150 mM NaCl) without serum. Subsequently, the LPS-liposome mixture was added to the apical side of the *in vitro* BBB model, obtaining a final concentration of 100 ng/ml LPS. The cells in the *in vitro* BBB model were cultured in the presence of 10 % serum and this medium was not changed before addition of LPS-liposome mixtures. TEER was measured each hour and was corrected for background and surface area of a transwell filter. TEER is expressed as a percentage of control (PBS).

Association of fluorescent LPS to liposomes

Fluorescently labelled LPS (LPS-Alexa, 400 ng) was incubated with 400 nmol of liposomes (1:750 weight ratio) in 200 μl PBS for 2, 6 or 16 hour. Incubation was performed in the absence or presence of 10 % serum, at 37 °C. After incubation, 150 μl of the liposome-LPS mixture was separated on a Sepharose CL4B column. Elution was based on gravity, and fractions of approximately 0.5 ml were collected every 2 minutes. Between each separation the column was rinsed with PBS for at least 30 minutes; overnight the column was rinsed with 20 % ethanol.

Fractions were analysed for LPS-Alexa by fluorescence analysis (Fluostar Optima, BMG Labtech, Offenburg, Germany). The LPS-liposome incubation mixture that was not separated was diluted to a standard curve of 0 – 0.4 ng/ml LPS-Alexa. Excitation was set at 480 nm, emission at 530 nm and each well was scanned with 20 flashes in the matrix mode. The emission wavelength of LPS-Alexa was 519 nm according to Molecular probes. However, we obtained more reproducible results with a 530 nm emission wavelength filter. The 530 nm filter had a smaller range (12 nm), while the filter for the 520 nm wavelength had a broad range (30 nm). We obtained a full absorption- and emission spectrum for LPS-Alexa, which showed an excitation maximum at 495 nm and an emission maximum at 520 nm. The gain was 3173 ± 43 for each plate that was measured. The gain is set to the well containing the highest concentration fluorescent label (in our case LPS-Alexa) and is used to automatically recalculate the measured value to the fluorescent intensity. Each curve was corrected for its own PBS background, which was 1967 ± 838.

The AUC of each fluorescence peak was determined with the trapezium rule, in which the average LPS concentration in two following samples was multiplied by the time interval between those samples. Initially in this research black fluorescent 96-wells plates from 2 suppliers were compared. No differences were found in the results.

To determine which fractions contained the liposomes, an enzymatic phospholipid assay was performed on fraction numbers 5 – 15, 17, 20, 22 and 24.

After each incubation and separation, the LPS-Alexa micelles were present in the same fractions, independent of time of incubation and liposomes present during the incubation. Furthermore, for LPS alone, for LPS + EPC-35 (2 h, in the presence of serum) and for LPS + liposomes with 5 % PEG-PE (16 h, in the absence of serum) we have repeated the incubation and separation to verify our results. The variation between the two occasions of each sample was less than 5 %.

Materials

LPS-Alexa (serotype 055:B5) was obtained from Molecular Probes (Leiden, the Netherlands). EPC-35 was purchased from Lipoid GmbH (Ludwigshafen, Germany) and PEG_{2000}-DSPE from Avanti Polar Lipids Inc. (Alabaster, AL, USA). LPS (serotype 055:B5) and cholesterol were obtained from Sigma (Zwijndrecht, the Netherlands) and Sepharose CL4B was obtained from Amersham Pharmacia Biotech (Uppsala, Sweden). Fetal calf serum from BioWhittaker Europe (Verviers, Belgium), while other cell culture media were obtained from BioWhittaker Europe (Verviers, Belgium). Transwell polycarbonate filters (surface area 0.33 cm^2, pore size 0.4 μm) and black fluorescent 96-wells plates

were obtained from Corning Costar (Cambridge, MA, USA). Black fluoresecent 96-wells plates were also obtained from Greiner Bio-one GmbH (Frickenhausen, Germany). Phospholipid B reagent was obtained from Wako chemicals GmbH (Neuss, Germany).

Results

The liposomes that were prepared contained 12.5 – 15.4 mM phospholipid, while liposomes with 5 and 10 % PEG-PE also contained cholesterol. Table I summarises the liposome characteristics. The liposomal size ranged from 124 to 135 nm in diameter and the polydispersity index ranged from 0.07 to 0.12. The size increased to approximately 140 nm in 2 weeks, but did not change thereafter. The polydispersity index increased slightly to 0.14, indicating that liposomes, even after 2 weeks, had a narrow particle size distribution.

LPS was pre-incubated for 2, 6 or 16 hours with liposomes. The functional read-out (i.e. measuring the TEER of the *in vitro* BBB model) was used to estimate the effect of the interaction between liposomes and LPS. Figure 1 shows some typical graphs of the effect of LPS on TEER. The effect of LPS on TEER is variable: in figure 1B, for example, TEER is decreased after addition of LPS, but shows a recovery at 3 hours, while in figure 1A, LPS decreases TEER without a subsequent recovery of TEER. The effect of 6 hours pre-incubation with liposomes was also variable between experiments. Figure 1A shows a delayed and less steep decrease in TEER, while figure 1B shows a less intense TEER decrease at 2 hours, but no difference between LPS and LPS with liposomes at later time-points. Figure 1C shows that LPS which is 6 hours pre-incubated without liposomes at 37 °C does not show a difference compared to LPS that is added directly to the *in vitro BBB* model.

Table I: Characteristics of liposomes, with regards to their phospholipid concentration, size and polydispersity index (p.i., a p.i. < 0.2 indicates as rather narrow particle size distribution). The final column indicates for which experiment the liposomes are used.

liposomes	PEG-PE	phospholipid (mM)	size (nm)	p.i.	experiment
EPC-35 : cholesterol	5 %	18.1	154	0.07	BBB model
EPC-35 : cholesterol	5 %	10.5	156	0.07	fig. 1A
EPC-35 : cholesterol	-	6.9	160	0.05	BBB model
EPC-35 : cholesterol	5 %	10.3	158	0.06	fig. 1B
EPC-35 : cholesterol	10 %	7.5	163	0.08	
EPC-35	-	15.4	124	0.12	LPS-Alexa
EPC-35 : cholesterol	5 %	12.5	135	0.07	fig. 2
EPC-35 : cholesterol	10 %	14.2	125	0.08	

Subsequently, a physico-chemical approach was applied to quantify the interaction of liposomes and LPS. Liposomes were incubated with LPS-Alexa at 37 °C for 2, 6 or 16 hours. Figure 2 shows the elution profile of LPS-Alexa and EPC-35 liposomes after separation of the liposome-LPS mixture on a Sepharose CL4B column. When LPS-Alexa alone was applied on the Sepharose CL4B column a fluorescence peak between 22 and 50 minutes was visible, while after pre-incubation with liposomes also a fluorescence peak between 16 and 22 minutes was visible. For the calculation of the LPS concentration a standard curve of 0 - 0.4 ng/ml LPS-Alexa was used. The presence of liposomes did not affect fluorescence measurement as is shown from the standard curves in the absence or presence of EPC-35 (figure 3). The elution profiles of the other LPS-liposome mixtures were similar (data not shown).

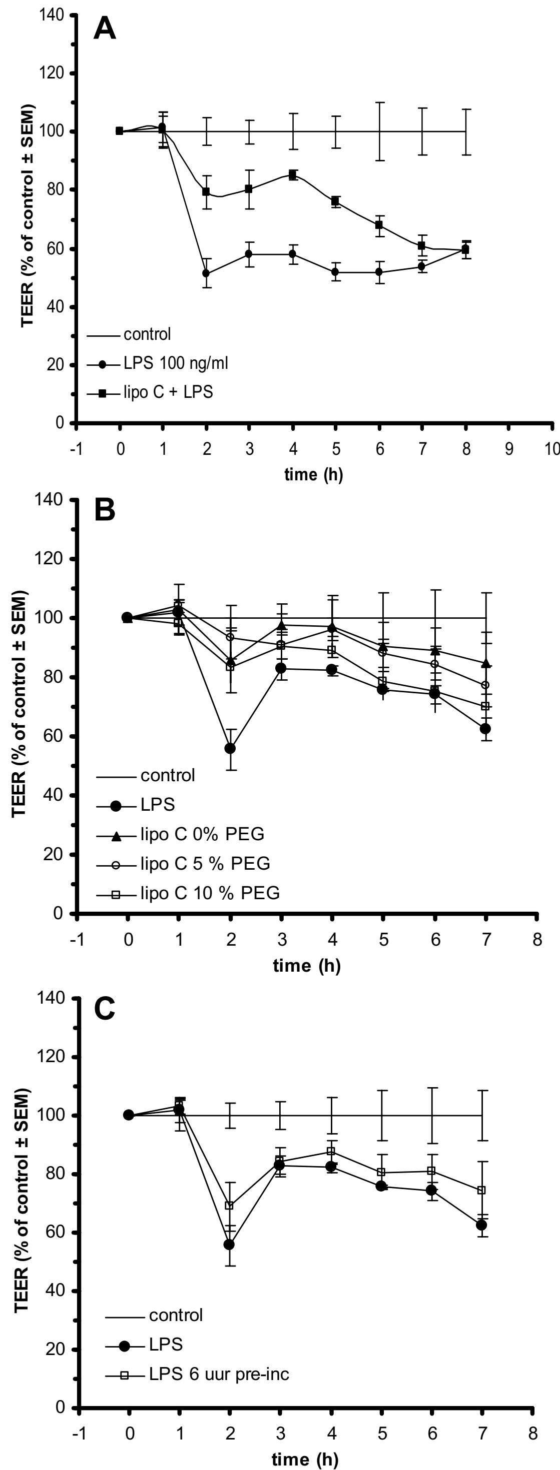

Figure 1: LPS and liposomes were pre-incubated for 6 hours after which TEER was measured up to 8 hours after addition to the *in vitro* BBB model. Figure A shows a delayed and less intense decrease in TEER after pre-incubation of LPS with liposomes containing 5% PEG-PE (lipo C) and transferrin (lipo T). Figure B only shows a less intense decrease at 2 hours after pre-incubations with liposomes containing 0, 5 or 10 % PEG-PE. Figure C shows that 6 hours of pre-incubation at 37 °C without liposomes does not affect the LPS effect itself. The graph in figure B and C are from the same experiment and have therefore the same LPS and control curve.

Phospholipid determination showed that the liposomes eluted in peak 1 (between 16 and 22 min), while no phospholipid was detected in peak 2 (between 22 and 50 min). This indicates that the fluorescence in peak 1 is liposome associated LPS. The AUC of both peaks was determined and the LPS that was associated with the liposomes was calculated as the percentage AUC_{peak1} of the total AUC of both peaks (table II). The association of LPS with liposomes was shown to be time dependent (table II). The absence or presence of 10 % serum did not change the association of liposomes and LPS. When LPS was incubated overnight at 37 °C in the presence of serum only a small peak was detected between 16 and 22 min (figure 2C). The AUC of this peak was only 2 % of the total AUC.

Discussion

Bacterial meningitis and sepsis can affect the BBB (9). This is mainly caused by LPS from gram negative bacteria. One of the effects of LPS is the formation of free radicals, which in turn cause an opening of the tight junctions between BCEC (4). From literature it is known that the effect of LPS on tumour cytotoxicity and TNF secretion diminishes when it is incorporated into liposomes (5). In these investigations both free LPS and liposomal LPS were endocytosed by macrophages, but liposomal LPS showed a diminished effect. We have investigated whether liposomes can scavenge LPS, thereby decreasing the effect on the BBB *in vitro*. For this we have used liposomes of EPC-35 and cholesterol with 0, 5 or 10 % PEG-PE. Initially, LPS was incubated with liposomes (1:750 weight ratio) in PBS without serum at 37 °C for 2, 6 or 16 hours. After incubation, the mixture was added to the *in vitro* BBB model (containing 10 % serum), developed by Gaillard (8) and the TEER was measured every hour up to 8 hours as a measure of BBB functionality (figure 1). The *in vitro* BBB model is a well characterised and established model for BBB transport (8). However, the effect of LPS on this model shows variability in the initial effect on TEER, as well as on the recovery phase. This was also observed by Gaillard *et al* (4). The high variability in LPS effect made it difficult to quantify the effect of the liposomes on LPS. Figure 1A shows a clearly delayed and less intense effect of LPS after pre-incubation with liposomes, while figure 1B only shows an effect of liposomes on LPS at 2 hour after addition of the LPS-liposome mixture to the *in vitro* BBB model. Pre-incubation of LPS at 37 °C without liposomes did

not affect the LPS effect in the *in vitro* BBB model (figure 1C). Therefore, the changed effect of LPS on the *in vitro* BBB model was due to the pre-incubation with liposomes. However, due to the high variability of the results obtained with this model, we have chosen for a purely physico-chemical approach to assess the interaction between LPS and liposomes that was hypothesised to be the basis of the "neutralising" effect by liposomes. For this fluorescently labelled LPS was used. Separation of liposomes from LPS was performed on a Sepharose CL4B column. This method was used previously to separate liposomes from PEG-PE micelles (10). LPS can form aggregates or micelles as well. For LPS from E.coli 055:B5 a crititical aggregation concentration of 38 μg/ml is reported (11). We have used a final concentration of 2 μg/ml LPS from the same serotype, at which no micelles or aggregates are formed. At this concentration a clear separation between liposomes and LPS on the Sepharose CL4B column was observed (figure 2).

Table II: The AUC of the liposomal peak and the AUC of the LPS peak were calculated using the trapezium rule. The fluorescently labelled LPS associated with liposomes is represented as a percentage of the total AUC. Each incubation and separation was performed only once.

	without serum			with serum (10%)		
	2 hours	6 hours	16 hours	2 hours	6 hours	16 hours
EPC-35	10	19	23	15	21	21
5 % PEG-PE	7	n.d.*	20	12	15	12
10 % PEG-PE	10	16	21	10	22	32

* not determined

For this research we have used liposomes of EPC-35, which are not very stable in the presence of serum. In addition, the liposomal bilayer was stabilised by cholesterol (EPC-35: cholesterol is 2:1) and stealth properties were induced in the liposomes by the addition of PEG-PE. In general, liposomes with 5 % PEG-PE are used for *in vivo* drug delivery to prevent liposomes from elimination from the bloodstream. In our previous work we also used liposomes with 5 % PEG-PE for drug targeting *in vitro* (10). Liposomal size was approximately 130 nm in diameter and the polydispersion index was approximately 0.1 which indicates a narrow size distribution.

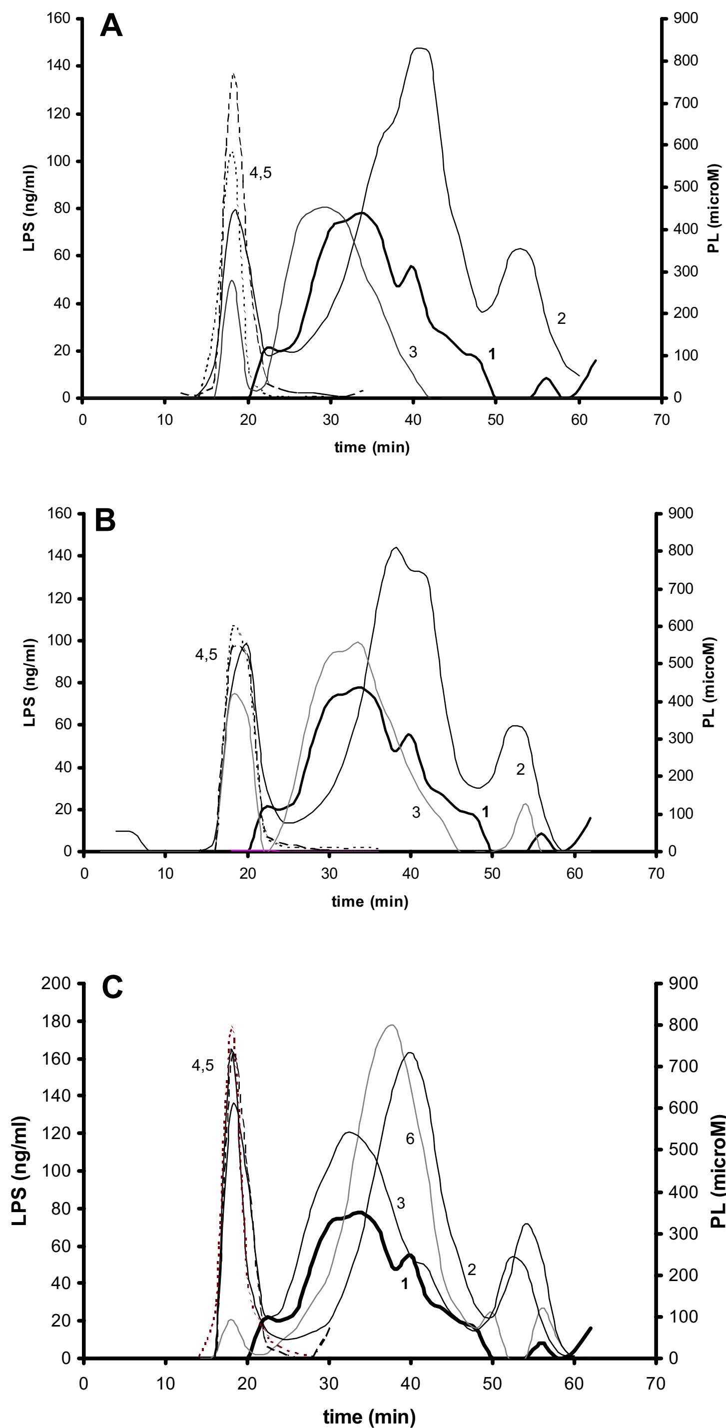

Figure 2: Liposomes of EPC-35 were pre-incubated with fluorescently labelled LPS in the absence or presence of 10 % serum for 2 hours (A), 6 hours (B), or 16 hours (C) before separation on a Sepharose CL4B column. Solid lines is the LPS concentration in ng/ml (left y-axis), dotted lines is the phospholipid (PL) concentration in μM (right y-axis). In each graph lines represent an elution profile of (1) LPS, which is not pre-incubated, (2) LPS + EPC-35 with 10 % serum, (3) LPS + EPC-35 without serum, (4) PL curve of LPS + EPC-35 with 10 % serum, (5) PL curve of LPS + EPC-35 without serum. Figure C also represents an elution profile of LPS pre-incubated with serum (6).

LPS-Alexa was incubated with liposomes (1:750 weight ratio) at 37 °C for 2, 6 or 16 hours. The liposomal peak was separated from the LPS peak by one or two fractions (figure 2), although in some samples overlap of the peaks was seen. The size distribution of the LPS was probably broad, since a broad fluorescent peak was visible. It was not possible to determine size and p.i. of the fluorescent LPS, since we could not prepare a high enough concentration of LPS-Alexa. For LPS of the same serotype, but without a fluorescent label we obtained a diameter of 40 nm and a p.i. of 0.4 at a LPS concentration of 1 mg/ml, as measured by dynamic light scattering. The relatively high p.i. indicates a broad particle size distribution. Sonication of the LPS solution had no effect on the size distribution as determined by fluorescence analysis after separation on a Sepharose-CL4B column (data not shown). The presence of the liposomes did not change the fluorescent signal as is shown from the standard curves diluted from the incubation mixtures with or without liposomes (figure 3).

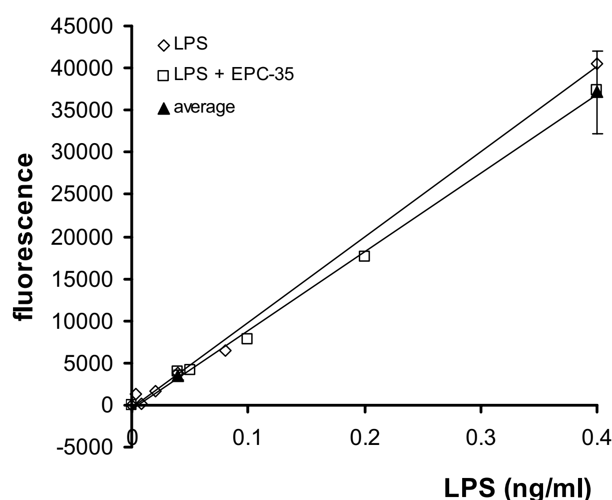

Figure 3: Standard curve of LPS-Alexa and LPS-Alexa in the presence of liposomes. At 0.04 and 0.4 ng/ml LPS-Alexa the averages (± s.d.) of all samples are represented. The slopes of the standard curves are $86*10^3 \pm 8*10^3$ (mean ± s.d.), the interception with the y-axis was 0.

With an enzymatic phospholipid assay it was determined which peak contained the liposomal fraction. LPS also contains a lipid tail which could be measured by a phospholipid determination, but the concentration was too low to be detected under the chosen conditions. Therefore, the phospholipid determination was representative for the liposomal fractions. Calculating the AUC with the trapezium rule revealed that for

liposomes consisting of EPC-35 the association of LPS was time dependent, but not dependent on the presence of 10 % serum (table II). We assumed that serum might have an inhibitory effect, but, in contrast, it had a slightly increasing effect on the association of LPS by liposomes. Wurfel and Wright (12) have reported that LPS-binding protein enhances the interaction of LPS with the phospholipid content of lipoproteins. This may account for the higher association of LPS by liposomes in the presence of serum, since LPS-binding protein is present in serum. Incubation in the presence of serum caused a shift to the right for the peak containing the LPS micelles (figure 2). This may be explained by the fact that lipoproteins present in serum have a diameter ranging from 5 - 12 nm for HDL to 30 – 80 nm for VLDL and are able to scavenge LPS (13). Therefore, a re-distribution in LPS particles or aggregates might have caused the shift in the peak containing the LPS micelles. It was shown that LPS that was pre-incubated in the presence of 10 % serum for 16 hours gave a small peak between 16 and 22 min. However, the AUC of this peak was only 2 % of the total AUC, indicating that serum does not interfere with the liposome-LPS interaction.

The composition of the liposomes, as well as the surface density of PEG-PE did not interfere with the ability of liposomes to interact with LPS (table II). In the *in vitro* BBB model we have found that after 6 hours pre-incubation of liposomes with LPS, the decrease in TEER was delayed and less pronounced, compared to LPS which was not pre-incubated. Gaillard *et al* (4) have shown that the effect on TEER was dependent on the LPS concentration. With the fluorescently labelled LPS we found that after 6 hours of incubation with liposomes, 15 - 20 % of the LPS was associated by liposomes (table II). Apparently, a decrease of 15 – 20 % in the “free” concentration LPS is already enough to alter the effect on the BBB *in vitro* (figure 1). We have not determined whether LPS was inserted into the bilayer or attached on the outer surface of the liposomes. However, since the liposomes with 5 and 10 % PEG-PE are sterically stabilised, it is unlikely that LPS is able to enter the liposomal bilayer. Although the position of the LPS on or in the liposomal bilayer is different, the decrease in LPS effect after pre-incubation with liposomes was comparable with results from Dijkstra *et al* (5). They have found that active incorporation of LPS into the liposomal bilayer did not affect the endocytosis of LPS, but reduced the effect of LPS on inflammatory mediators, such as tumour necrosis factor and interleukin-1.

For our work towards the delivery of liposomes encapsulating the free-radical scavenger NAC, the effect of liposomes on LPS is important. To discriminate between the effect of the drug and the effect of the liposomes on LPS-induced opening of the BBB *in vitro*, we have applied liposomes apically (directly in the BCEC compartment). In addition, LPS was added to the basolateral side of the transwell filter. By doing so, LPS and liposomes are in different compartments, thereby revealing the drug effect. However, the effects of LPS and NAC on the tightness of the BBB *in vitro* was too variable to draw solid conclusions about the effect of NAC.

In conclusion, in this short communication we have shown that liposomes are able to scavenge LPS in a time-dependent manner. The density of PEG-PE on the surface of the liposomes, as well as the absence or presence of serum had no effect on the ability of liposomes to scavenge LPS. These results are interesting for research that is performed with liposomes under inflammatory disease conditions, since the direct effect of liposomes on LPS may interfere with the interpretation of the effect of the drug that is incorporated into the liposomes.

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