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Original Article

Protective Role of Liposome Incorporated Lipopolysaccharide Antigen of *Klebsiella pneumoniae* in a Rat Model of Lobar Pneumonia

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SUMMARY: In a lobar pneumonia model of *Klebsiella pneumoniae*, the immunoprotective role of free lipoploysaccharide (LPS) and liposome-incorporated LPS was studied. An alteration in the biological activity of the LPS molecule, in terms of its pyrogenicity and lethal toxicity, was observed on incorporation in the liposome. Compared at equal doses, liposome-incorporated LPS was found to be non-pyrogenic and 10 times less toxic than free LPS. Liposome-incorporated LPS was more effective in providing protection against *K. pneumoniae* induced lobar pneumonia in rats. The immunological mechanism underlying protection revealed involvement of both nonspecific and specific immune response. Alveolar macrophage activation was observed after 4 and 14 days of treatment with the free and liposome-entrapped forms of LPS, respectively. Specific immunity in terms of plaque-forming cells was seen with both forms of LPS. Delayed type hypersensitivity reaction was observed only with liposome-incorporated LPS. It is concluded that a non-toxic and immunogenic form of *K. pneumoniae* LPS can be obtained by incorporation of the native preparation into liposomes.

INTRODUCTION

Nosocomial pneumonia, because of its high morbidity and mortality rates, is considered to be one of the most serious hospital-acquired infections (1). Among Gram-negative bacilli, *Klebsiella pneumoniae* alone accounts for 25-43% of nosocomial pneumonias (2). What makes it all the more important is that the pneumonia caused by this organism has a rapidly progressive clinical course that is often complicated by multilobular involvement and lung abscesses, leaving little time to institute effective antimicrobial treatment (3). This problem is further complicated by the emergence of multidrug-resistant *K. pneumoniae* isolates (4). As a result, development of new immunoprophylactic and immunotherapeutic agents against *Klebsiella* infections has been suggested. Vaccine development against *Klebsiella* infections has concentrated mainly on the capsular polysaccharide (CPS) (5,6). However, the protective capacity of CPS has been shown to be type-specific in various models (7). Recent observations that *K. pneumoniae* CPS does not present any permeability barrier to immunoglobulins have opened the possibility of exploring the vaccine potential of lipopolysaccharide (LPS) antigen (8). Its toxicity, however, remains a limiting factor of its potential as a potent candidate vaccine. Therefore, in this study, attempts were made to reduce its toxicity and improve its immunogenecity by incorporating it into phospholipid multilayered vesicles (liposomes) without altering its native structure.

MATERIALS AND METHODS

Bacterial strains: *K. pneumoniae* NCTC 5055 (K_2^+ O_1^+) procured from Dr. P. Williams (Microbiology Research Groups, Aston Triangle, Birmingham, UK) was used in this study. The strain was maintained on nutrient agar slants at 4°C.

Extraction of LPS: LPS from *K. pneumoniae* NCTC 5055 was extracted by the conventional hot phenol extraction procedure of Westphal and Jann (9) as modified by Morrison and Leive (10). LPS was further purified by sequential ultracentrifugation as described by Johnson and Perry (11). The final material was analysed for DNA, RNA, and protein content by standard methods (12-14).

Preparation of liposomes and incorporation of LPS antigen: The method of Dijkstra et al. (15) was followed. In brief, the phospholipid vesicles were prepared from a mixture of egg phosphatidylcholine, phosphatidylserine, and cholesterol in chloroform (Molar ratio 4:1:4). The bulk of organic solvent was removed by rotary evaporation. The dried lipid mixture was resuspended by vortexing in 1 ml Tris-buffer (pH 8.0). To prepare multilamellar vesicles (liposomes), the lipid suspension was vigorously vortexed for 1 min, and this treatment was repeated five times over the next hour. To incorporate LPS into liposomes, the phospholipids were resuspended by vortexing in pyrogen-free water containing LPS. The suspension was heated to 45°C for 5 min, sonicated for 1 min, and finally vortexed. This treatment was repeated two times.

Biological studies: Various antigens were tested for lethal toxicity and pyrogenicity (16).

Lethal toxicity assay: A group of four LACA strain females mice weighing 20-25 g were used for assaying the lethal toxicity of free and of liposome-incorporated LPS (normal and D-galactosamine sensitized mice). The mice were sensitized by injecting 18 mg of D-galactosamine intraperitoneally followed within 30 min. by intravenous (i.v.) injection of the preparation to be tested. Free LPS, liposomal LPS, and free LPS along with control liposomes were tested for lethal toxicity at various doses. Animals were observed up to 72 h, and mortality rates were noted.

Pyrogenicity assay: For each sample, a group of three New Zealand white rabbits (body weight 1.5 ± 0.2 kg) was used. The following doses of various preparations were injected

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i.v. through the marginal ear vein in each group (n = 3) of rabbits: (i) 50 μg and 100 μg of free LPS, (ii) 50 μg and 100 μg liposomal LPS, and (iii) 50 μg and 100 μg of free LPS along with control liposomes. The rectal temperature was recorded before and at 1, 2, and 3 h after injection. A preparation was considered to be pyrogenic if the sum of increase of temperature was >1.15°C for the relevant three rabbits.

Protection studies:

Treatment: Female adult Wistar rats, weighing 100-125 g and 60-90 days old, were used. Each group was comprised of 12 rats. An equal number of rats were taken as control. The following preparations were injected intramuscularly (i.m.) in each animal of the respective groups: (i) 100 μg of free LPS, (ii) 100 μg of liposome incorporated LPS, and (iii) 100 μg of free LPS coinjected with control liposomes. Four rats each in group were challenged with *K. pneumoniae* NCTC 5055 at 4 h, 4 days, and 14 days after treatment, respectively, to induce lobar pneumonia. All experiments were carried out in accordance with institutional policies and guidelines for animal use.

Development of pneumonia: Pneumonia was induced as described by Rani et al. (16). In brief, rats were anaesthetized by injecting sodium pentobarbitone (30 mg/kg body weight) (Sigma Fine Chemicals, St. Louis, Mo., USA) intraperitoneally, and fixed on a wax board in the supine position. The skin over the neck region was shaved and cleaned with methylated spirit. An incision (1 cm) was made just above the sternum to expose the trachea for the insertion of a catheter (1 mm diameter). A fixed dose of *K. pneumoniae* (1×10^7 cfu) was injected directly into the lungs through the trachea. The incision was sutured with an unabsorbable ethicon 3/0 thread, and the antibiotic ointment nitrofurazone was applied to the sutured cut. The animals were given a regular diet (Hindustan Levers Ltd., Bombay, India) and water ad libitum.

Quantitiation of bacteria: The rats were sacrificed 4 days post-challenge, and the lungs were excised and homogenized under aseptic conditions. Serial 100-fold dilutions were spread-plated on MacConkey's agar plates. The plates were incubated at 37°C for 24 h, after which viable counts were taken and the results expressed as cfu/g of lung tissue.

Phagocytic assay: Phagocytosis was performed according to the method of Allen et al. (17). The rats were divided into three groups of four rats each. An equal number of animals served as controls. The following preparations were injected i.m. in each animal of the respective groups: (i) 100 μg of free LPS, (ii) 100 μg of liposome-incorporated LPS, and (iii) 100 μg of free LPS along with control liposomes.

Two rats from each group were sacrificed at 4 h, 4 days, and 14 days after the day of injection. Bronchoalveolar macrophages from the lungs of these rats were collected by the method of Sugar et al. (18), and later used for the phagocytic assay. To study the uptake of bacteria by macrophages, mixtures of 0.5 ml of alveolar macrophages (10^7 cells/ml), 0.4 ml of diluted mouse serum, and 0.1 ml of bacterial suspension (2×10^8 cfu/ml) were prepared and incubated at 37°C in 5% CO_2 atmosphere. Samples (20 μl) were taken after 30 min and 60 min and suspended in cold RPMI-1640 (2 ml). Macrophages were pelleted by centrifugation. The number of viable bacteria in the supernatant was determined by plating appropriate serial dilutions in triplicate on MacConkey agar plates. Results were expressed as the percentage of bacteria taken up by macrophages at each sampling interval (30 min, 60 min).

Plaque-forming cell (PFC) assay: Direct PFC assay was performed to assess the number of antibody-forming cells by the slide version technique of localized hemolysis in gel according to the method of Hiernaux et al. (19). In brief, sheep red blood cells (RBCs) were sensitized by incubating 10% sheep RBC suspension with LPS (1 mg/ml, boiled for 2 h) for 30 min. Spleen cell suspension from LPS-treated rats was obtained by gently teasing the spleen in cold Eagle's essential medium (HiMedia, Bombay, India). The count of spleen cells was adjusted to 2×10^6 cells/ml for PFC assay. To tubes containing 0.5 ml of melted 0.7% agarose and 0.2 ml of sensitized RBC, 50 μl of spleen cell suspension was added. The contents were mixed properly and spread evenly over the surface of a glass slide previously coated with a thin film of 0.1% agarose. Upon gelation, the slides were incubated for 2 h at 37°C, and subsequently in the presence of guinea pig complement (1:20 dilution) for 1 h. The slides were drained free of complement and examined under low power magnification for the presence of LPS-specific PFC. The mixture of immune spleen cells and the unsensitized sheep RBC were processed similarly and served as controls.

Delayed type hypersensitivity (DTH) response: DTH response was checked according to the method of Desiderio and Campbell (20). Groups of four mice were each treated with one of the following test preparations: (i) heat-killed *K. pneumoniae* NCTC 5055 (5×10^8 cells in 0.1 ml), (ii) formalin-killed *K. pneumoniae* NCTC 5055 (5×10^8 cells in 0.1 ml), (iii) liposomal LPS (10 μg), (iv) free LPS (10 μg of LPS in 0.1 ml), and (v) control liposomes. All these preparations were administered i.v. Animals in each group were treated with 2 doses of the above-mentioned preparation at an interval of 1 week.

Mice were examined for DTH response to liposomal LPS and free LPS, respectively, as test inocula. At 2 weeks after the second dose, each mouse received 0.025 ml of liposomal LPS and free LPS intradermally in the left hind footpad, and the same volume of PBS in the right hind foot pad. Footpad thickness was measured 48 h later with vernier calipers accurate to 0.01 mm.

Statistical Analysis: Data was analyzed statistically using Student's t test.

RESULTS

Purified LPS showed almost negligible amounts of DNA (0.1%, wt/wt), RNA (1.05%, wt/wt), and protein (2%, wt/wt), thereby confirming the purity of the preparation. Both free LPS and free-LPS respectively co-injected with control liposomes were found to be pyrogenic at doses of 50 μg and 100 μg. However, liposome-incorporated LPS was not pyrogenic even at a dose of 100 μg and was 10 times less toxic than free LPS (Table 1, 2).

The respective roles of free and liposome-incorporated LPS were compared in *K. pneumoniae* NCTC 5055-induced lobar pneumonia in rats. The rats were treated with free LPS (100 μg), liposomal LPS (100 μg), or free LPS (100 μg) along with control liposomes, and challenged on the 14th day post-treatment with a fixed dose (1×10^7 cfu) of *K. pneumoniae* cells then sacrificed 4 days post-challenge. A decrease in bacterial count of nearly 5.0 and 6.5 logcycles in free- and liposomal LPS-treated rats, respectively, was observed as compared to the controls (Fig. 1). Similarly, a decrease of 4.5 and 5.0 log cycles in lung bacterial count was observed in

Table 1. Lethal toxicity of free and liposome-incorporated LPS in normal and D-galactosamine sensitized mice

Preparation	Dose μg/mouse	Number of mice killed	
		Normal	Sensitized
Free LPS	100	0	ND
	50	0	ND
	10	0	4
	1	0	4
	10^{-2}	0	4
	10^{-3}	0	0
Liposome-incorporated LPS	100	0	ND
	50	0	ND
	10	0	4
	1	0	4
	10^{-2}	0	0
	10^{-3}	0	0

ND: Not done.

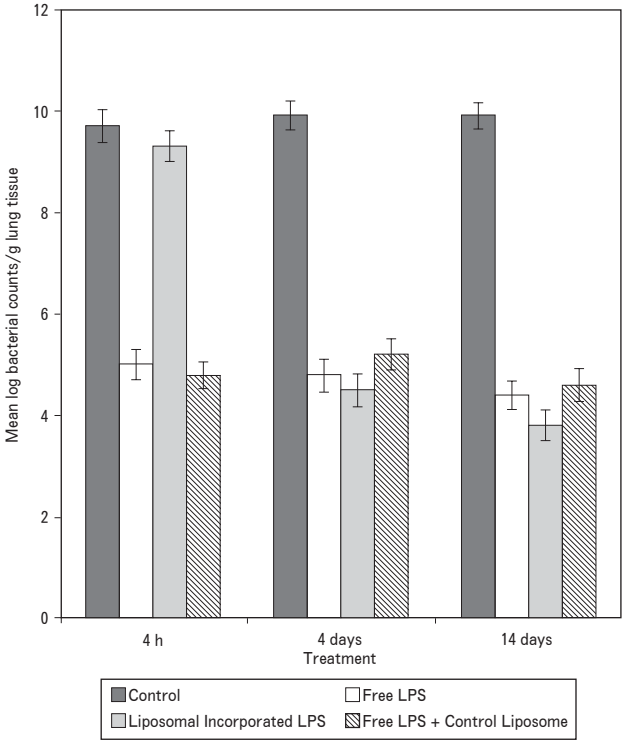

Fig. 1. Bacterial count per g of lung tissue in rats treated with 100 μg of control free LPS, liposomal LPS, or free LPS plus control liposomes, and challenged at 4 h, 4 days, and 14 days after treatment with *Klebsiella pneumoniae* NCTC 5055. Each value represents the mean of counts from four rats.

rats treated for 4 days with 100 μg of free LPS and liposomal LPS, respectively (Fig. 1). However, treatment with liposomal LPS for 4 h did not appear to afford any protection, as no decrease in bacterial count was observed, whereas free LPS treatment resulted in a decrease of nearly 4.5 logcycles (Fig. 1). The decrease in bacterial counts in the lungs of rats treated with free LPS along with control liposomes was nearly the same as that of free LPS-treated rats in the respective groups (Fig. 1).

The underlying immunological mechanism providing protection against *K. pneumoniae* NCTC 5055-induced lobar pneumonia was studied in terms of non-specific and specific immunity. The phagocytic function of alveolar macrophages from free and from liposomal LPS treated animals at 4 h, 4 days, and 14 days after treatment was compared with that of controls (Table 2a - 2c). It was observed that the percentage survival of bacteria with respect to controls was much less in both groups on exposure to activated alveolar macrophages collected at 4 days and 14 days after treatment, but not to those collected at 4 h after treatment (Table 2a, 2b). Notably, these figures show no marked differences between the phagocytic functions of alveolar macrophages obtained from animals treated with free LPS and those treated with liposomal LPS preparations at 4 days after treatment, but show an approximately 10% reduction in the overall phagocytic function in both groups at 14 days after treatment. In the control experiment in which free LPS was co-injected with control liposomes, the native effect of free LPS was not affected by liposomes. Both free LPS and liposome-incorporated LPS were found to induce the formation of LPS-specific PFCs. The number of PFC observed in liposomal LPS-treated animals was 490 ± 73 per 10^6 spleen cells, whereas those observed in free LPS and free LPS plus control liposome-treated rats at 14 days after treatment were 200 ± 67 and 184 ± 70, respectively (Table 3). This count of PFC was found to be significant at $P < 0.01$, thereby indicating that incorporation of LPS into phospholipid vesicles produces a better humoral immune response. DTH to liposomal LPS and to free LPS was checked in mice treated with various preparations made from *K. pneumoniae* NCTC 5055. Mice treated with various preparations at 2 doses, 1 week apart, injected intradermally with liposomal LPS, showed DTH at 48 h

Table 2. Pyrogenic response to free and liposome-incorporated LPS of intravenous injection in rabbits

Preparation	Dose body weight μg/kg	Sum of increase of temperature in three rabbits (°C) Time (h)			Pyrogenicity[1)]
		1	2	3	
Free LPS	50	2.1	1.80	1.5	+
	100	2.8	3.0	2.9	+
Liposomal LPS	50	0.1	0.1	0.6	–
	100	0.4	0.46	0.2	–
Free LPS + control liposomes	50	1.0	1.64	1.90	+
	100	1.18	2.4	2.6	+

[1)]: A preparation was considered to be pyrogenic if the sum of increase of temperature was >1.15°C for a group of three rabbits.

Table 2a. Survival values of *Klebsiella pneumoniae* NCTC 5055 in the presence of activated and normal alveolar macrophages at 4 h after treatment with 100 μg of free LPS, liposomal LPS, and free LPS plus control liposomes

Preparation	Bacterial CFU ± S.D.		
	0 min	30 min	60 min
Control	$(8.10 \pm 0.54) \times 10^6$	$(7.68 \pm 0.59) \times 10^6$	$(7.60 \pm 0.62) \times 10^6$
Free LPS	$(7.50 \pm 0.68) \times 10^6$	$(4.60 \pm 0.61) \times 10^6$	$(3.81 \pm 0.65) \times 10^6$
Liposomal incorporated LPS	$(8.30 \pm 0.53) \times 10^6$	$(7.66 \pm 0.70) \times 10^6$	$(7.51 \pm 0.65) \times 10^6$
Free LPS + control liposome	$(8.25 \pm 0.70) \times 10^6$	$(5.20 \pm 0.59) \times 10^6$	$(4.50 \pm 0.65) \times 10^6$

Table 2b. Survival values of *Klebsiella pneumoniae* NCTC 5055 in the presence of activated and normal alveolar macrophages at 4 days after treatment with 100 μg of free LPS, liposomal LPS, and free LPS plus control liposomes

Preparation	Bacterial CFU ± S.D.		
	0 min	30 min	60 min
Control	$(8.20 \pm 0.61) \times 10^6$	$(7.82 \pm 0.73) \times 10^6$	$(7.79 \pm 0.70) \times 10^6$
Free LPS	$(8.10 \pm 0.58) \times 10^6$	$(2.70 \pm 0.59) \times 10^6$	$(1.38 \pm 0.50) \times 10^6$
Liposomal incorporated LPS	$(7.92 \pm 0.66) \times 10^6$	$(2.98 \pm 0.64) \times 10^6$	$(2.70 \pm 0.60) \times 10^6$
Free LPS + control liposome	$(8.70 \pm 0.68) \times 10^6$	$(2.70 \pm 0.55) \times 10^6$	$(1.70 \pm 0.50) \times 10^6$

Table 2c. Survival values of *Klebsiella pneumoniae* NCTC 5055 in the presence of activated and normal alveolar macrophages at 14 days after treatment with 100 μg of free LPS, liposomal LPS, and free LPS plus control liposomes

Preparation	Bacterial CFU ± S.D.		
	0 min	30 min	60 min
Control	$(8.83 \pm 0.51) \times 10^6$	$(8.34 \pm 0.55) \times 10^6$	$(8.20 \pm 0.53) \times 10^6$
Free LPS	$(8.30 \pm 0.82) \times 10^6$	$(3.00 \pm 0.61) \times 10^6$	$(3.94 \pm 0.60) \times 10^6$
Liposomal incorporated LPS	$(7.90 \pm 0.55) \times 10^6$	$(3.00 \pm 0.61) \times 10^6$	$(3.94 \pm 0.60) \times 10^6$
Free LPS + control liposome	$(8.20 \pm 0.71) \times 10^6$	$(3.50 \pm 0.52) \times 10^6$	$(3.00 \pm 0.73) \times 10^6$

Table 3. Counts of LPS-specific PFCs in the spleens of animals treated for 14 days with free and liposomal LPS

Preparation	Dose (μg)	LPS-specific PFCs per		Significance
		10^6 cells	Spleen	
Control	Normal saline	20 ± 7	172 ± 60	
Free LPS	100	200 ± 67	1200 ± 405	$P < 0.01$
Liposomal LPS	100	490 ± 73	2940 ± 438	$P < 0.01$
Free LPS + control liposomes	100	184 ± 70	1104 ± 420	$P < 0.01$

PFC: Plaque-forming cell.

after injection. Liposomal LPS along with heat-killed and formalin-killed *Klebsiella* stimulated the induction of DTH. A significant increase in the footpad thickness was seen at 48 h in these groups (Table 4). However, no significant increase in the same was seen when free LPS was injected intradermally (Table 4).

DISCUSSION

The development of vaccines based on LPS obtained from *K. pneumoniae* and other Gram-negative bacteria is now attracting much attention. The main approach to solving the problems associated with these attempts consists of elimination of lipid A and conjugation of the carbohydrate component of LPS with a protein carrier (21). However, lipid A has, in addition to its toxic action, an adjuvant effect. For this reason, retaining and simultaneously reducing the toxicity of lipid A are of special interest for the development of new LPS-based vaccines.

In the present study, liposososmal LPS was found to be non-pyrogenic and 10 times less toxic than free LPS at a given dose, indicating that the procedure used to incorporate LPS into liposomes does not result in its inactivation. Dijkstra et al. (22) suggested that stable insertion of LPS into liposomal membrane accounts for its reduced toxicity, which thereby prevents a direct interaction of lipid A with appropriate plasma membrane components, which is necessary to efficiently trigger biological responses. As a result, such liposome-incorporated LPS fails to activate macrophages. Contrary to these findings, liposomal LPS in this study was found to activate alveolar macrophages at 4 days and 14 days after treatment. It is possible that such response might be due to the relatively longer interaction of the LPS molecule with the alveolar macrophages. Dijkstra et al. (22) studied the interaction between liposomal LPS and the macrophage membrane only up to 20 h after treatment. In the present study, liposomal LPS, in contrast to free LPS, had not activated alveolar macrophages at 4 h after treatment. It appears that longer contact between liposomal LPS and macrophages, as suggested by Desiderio and Campbell (20), resulted in endocytosis of the liposomal LPS complex, followed by disruption of the liposomal bilayer within the phagolysosomes, which exposed the free LPS molecule to direct interaction with macrophages.

The results of the protection studies showed that immunization with free as well as that with liposomal LPS provided protection at 4 days and 14 days after treatment. Our results

Table 4. DTH reaction to free LPS and liposome-incorporated LPS injected intradermally in mice previously injected with various preparations

Group No.	Preparation injected	Mean footpad thickness (mm) + S.D.		Significance
		Preinoculation	48h	
Free LPS				
	Heat killed *Klebsiella pneumoniae*	1.85 ± 0.161	1.867 ± 0.167	N.S.
	Formalin killed *Klebsiella pneumoniae*	1.773 ± 0.148	1.786 ± 0.143	N.S.
	Liposomes incorporated LPS	1.634 ± 0.223	1.647 ± 0.218	N.S.
	Free LPS	1.788 ± 0.117	1.797 ± 0.125	N.S.
	Control liposomes	1.703 ± 0.157	1.712 ± 0.149	N.S.
Liposomal LPS				
	Heat killed *Klebsiella pneumoniae*	2.70 ± 0.108	4.333 ± 0.321	$P < 0.01$
	Formalin killed *Klebsiella pneumoniae*	1.933 ± 0.207	3.80 ± 0.637	$P < 0.01$
	Liposomes entrapped LPS	1.750 ± 0.125	3.475 ± 0.180	$P < 0.01$
	Free LPS	1.782 ± 0.151	1.795 ± 0.180	N.S.
	Control liposomes	1.845 ± 0.151	1.863 ± 0.158	N.S.

DTH: Delayed type hypersensitivity, N.S.: Not significant.

are consistent with those of Bennett-Guerrero et al. (23), who recently showed that active immunization of mice with liposomal complete core LPS provides protection against lethal challenge with *Escherichia coli* O81 LPS. Free LPS provided protection as early as 4 h after treatment, whereas liposome-entrapped LPS was effective from the 4th day onwards. This early phase non-specific resistance can be explained as probably due to the activation of phagocytic cells, especially the macrophages. Further, at a longer time interval, entrapment of LPS into liposomes also resulted in activating the specific immune response. This was measured by the number of direct PFCs. The number of LPS-specific PFC was significantly higher in animals treated with liposomal LPS, than in those treated with free LPS. Humoral immune response to liposomal antigen can be described in terms of the system's ability to function as an antigen depot (24,25). This, along with the fact that nearly the entire antigenic mass is concentrated in the macrophage population of the reticulo-endothelial system, might explain the longer retention time of the LPS, which would lead to prolonged antigenic stimulation in terms of specific and non-specific mechanisms of protection.

In the present study, liposomal LPS as well as killed bacteria induced a positive DTH reaction. Free LPS was not able to induce a DTH response, and lacked the DTH-inducing activity not only at the sensitization levels but also at the elicitation level. The increased internalization of hydrophobic lipid antigens by macrophages ultimately improves antigen presentation to cells (26). These macrophages function as antigen-presenting cells (APC) which take up antigen, catabolize them, and express the antigenic determinants in an energy requiring process before being presented to antigen specific T-cells (27,28). It is also possible that these LPS-activated macrophages contribute towards positive regulating effects on the induction of specific immune response. Thus, the results of the present study elucidate that incorporation of *K. pneumoniae* LPS into liposomes not only makes it possible to achieve a considerable immune response to lipid A-based immunogens by circumventing toxicity associated with lipid A, but also to convert polysaccharide antigens into thymus-dependent antigens (29). However, whether antibodies induced by liposomal LPS have bactericidal action and cross react with other *K. pneumoniae* immunotypes warrants further investigation.

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