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Lipopolysaccharide-Mediated Protection against *Klebsiella pneumoniae*-Induced Lobar Pneumonia: Intranasal *vs*. Intramuscular Route of Immunization

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ABSTRACT. Immunoprotective potential of delivered lipopolysaccharide (LPS) preparation from *Klebsiella pneumoniae* was determined in a murine model of lobar pneumonia. Protection was assessed with three doses of LPS (25, 50 and 100 μg; without any adjuvant) administered intranasally or intramuscularly. After evaluation of lung tissue (bacterial load and histopathology), no significant protection was observed at 25 μg with either application. A significant decrease in lung bacterial load coupled with fall in severity of lung lesions was observed with 50 μg (again both applications). At 100 μg dose, with intramuscular route, a further decrease in the lung bacterial load was shown compared to the 50 μg dose. In contrast, 100 μg LPS, when given intranasally, resulted in a higher bacterial colonization of the lung tissue and higher lung pathology; thus we recommend intramuscular instead of the intranasal route for developing protection against *K. pneumoniae*-mediated pneumonia with intact LPS-based vaccines.

The magnitude of *Klebsiella pneumoniae*-induced pneumonia especially in a hospital setting requires the attention of researchers to develop effective preventive strategy (Podschun and Ullman 1998). In *K. pneumoniae*, the outermost cell-surface component which is a capsular polysaccharide (CPS), has been explored as vaccine candidate with good success. CPS based vaccines have been reported to be successful even in human trials (Cryz *et al.* 1986). However, this candidate vaccine has not been used for further studies because of an inherent disadvantage, as the immunity provided is only type specific. Also, since at least 77K types of *K. pneumoniae* are known to be causing disease in humans (Ørskov and Ørskov 1984), attention has shifted to the LPS molecule (O-antigens), which is also a surface antigen. Besides, only 9 known serotypes based on this antigen are recognized for the genus *Klebsiella* (Held *et al.* 2000). Of these, O1 is the most common type associated with clinical situations (Trautmann *et al.* 1996). The presence of CPS does not interfere with the penetration of antibodies directed towards the LPS molecule (Meno and Amano 1990); therefore, this molecule has been a subject of study in different laboratories including ours (Chhibber *et al.* 2003).

Past vaccination efforts have employed the intramuscular or subcutaneous route for immunization (Rani *et al.* 1990; Chathley *et al.* 1996), following which the response at the mucosal sites is less effective. The new generation vaccines are specially being aimed at stimulation of local antibody response in addition to systemic immunity. It is for this reason that the antigen has to be administered *via* the route of entry of the pathogen. In the case of *K. pneumoniae*-induced lung infections there is paucity of information in relation to the intranasal route of immunization with the LPS molecule. Our study was undertaken to investigate the immunoprotective potential of intranasally delivered LPS molecule in a murine lobar pneumonia model, and to compare it with the intramuscular route of administration of the vaccine.

MATERIALS AND METHODS

Bacterial strain. Klebsiella pneumoniae strain B5055 (K^+O^+) procured from Dr. M. Trautman (*University of Ulm*, Germany) was used and maintained on nutrient agar slants at 4 °C.

Animals. BALB/c strains of mice procured from the *Central Animal House, Panjab University* (Chandigarh, India) with animals weighing 20–30 g, were used; they were fed on standard antibiotic-free

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synthetic feed. The study protocol was approved by the *Institutional Ethical Committee* for animal experimentation.

Induction of pneumonia by the intranasal route. For intranasal instillation of the bacterial inoculum, the method of Held *et al*. (1998) was employed. Fifty μL of bacterial inoculum was instilled into the nasal opening while holding the mice upright.

Purification of LPS. LPS was extracted by the conventional phenol extraction method of Westphal and Jann (1965) as modified by Morrison and Leive (1975); it was purified by sequential ultracentrifugation (Johnson and Perry 1976). The final material was analyzed for proteins, DNA (Burton 1956) and RNA (Munro and Fleck 1966); it was also tested for pyrogenicity and toxicity according to Rani *et al.* (1990).

Protection studies. A group of 6 BALB/c mice in sets of 3 were immunized with 3 concentrations (25, 50, 100 μg/mL) of intact LPS administered *via* the intramuscular and the intranasal route. Mice were then challenged 2 weeks post-immunization with *K. pneumoniae* B5055, sacrificed 3 d post challenge and the lung tissues were examined for bacterial counts and histopathological lesions. For bacteriological load assessment lung tissue was sectioned into halves. One-half of each lung was placed in a sterile tube and weighed. Tissue was homogenized in sterile PBS and serial dilutions of the homogenized mass in PBS were plated on MacConkey agar plates.

For histopathological evaluation lung tissue preserved in aqueous formaldehyde was dehydrated in ascending series of ethanol (70–100 %). The tissue was embedded in paraffin wax, sectioned and stained with hematoxylin–eosin (*Hi-Media*, India). Grading of the severity of pathological lesions was also done for evaluation; a section of each lung was assessed on a semi-quantitative scale of 0–8. A total score indicative of the overall severity of lesions was determined by adding the individual score.

RESULTS AND DISCUSSION

Presence of macromolecules in purified LPS preparation. LPS contained 42 % polysaccharide, 2.0 % protein, 20.1 % lipid and negligible amounts of DNA and RNA. No significant difference was observed in the lung bacterial counts and the severity scores of the lung tissue in mice immunized with 25 μg LPS either *via* intranasal or intramuscular route. A significant ($p < 0.05$) decrease in the lung bacterial load of 2.2 and 2.5 log range was observed for mice immunized with 50 μg LPS *via* both routes, respectively ($p < 0.05$). This decrease in the bacterial load was in correlation with the histopathological studies which revealed less severe changes in the tissue of mice immunized with this dose. Lungs of mice immunized with 100 μg intramuscularly showed a further decrease in the bacterial counts ($p < 0.01$). However, when the same dose was given intranasally, an increase in the lung bacterial load and severity of lung lesions was observed (Table I).

Table I. Lung bacterial counts (log CFU/g) and severity scores (scale 0–8) of mice challenged 2 weeks after intranasal or intramuscular immunization with LPS (25–100 μg)

LPS	Control	Intranasal route	Intramuscular route
Bacterial counts			
25	8.4 ± 0.45	7.8 ± 0.46	8.1 ± 0.45
50	8.4 ± 0.52	5.9 ± 0.50	6.2 ± 0.46
100	8.4 ± 0.32	8.2 ± 0.57	5.6 ± 0.45
Severity scores			
25	5.7 ± 0.45	5.5 ± 0.45	5.8 ± 0.45
50	5.7 ± 0.70	4.3 ± 1.25	4.5 ± 0.10
100	5.7 ± 0.40	6.0 ± 0.54	3.3 ± 0.81

Efforts have been directed during the last two decades to understand the pathogenetic mechanisms operative at mucosal surfaces. The thrust is to develop good local immune response for effective prevention, and for this it is being advocated that the route of immunization should be similar to the one adopted by the pathogen for causing infection. In a report on immunological basis of asthma, it has been suggested that the effects of endotoxins are dose-dependent (Braun-Fahrlander *et al*. 2002); whether exposure to endotoxin is protective or harmful, depends on multiple factors. In our study, out of the three doses (25, 50, 100 μg) of LPS administered intranasally for conferring protection, only the 50-μg dose exhibited acceptable protec-

tion. The lower doses, 25 and also 50 μg when given without bacterial challenge, caused tissue damage, which resolved by 14 d post immunization (Fig. 1). However, with the 100 μg dose the pathology in the lung persisted beyond 2 weeks (Fig. 2). Our results show that the LPS antigen of *K. pneumoniae*, when administered intranasally, although has an immunoprotective potential at 50-μg dose level, is associated with undesirable damage to the lung tissue. This effect could be observed even with the smallest dose tested (25 μg) and was far more pronounced with the 100 μg dose. Exposure of the host to other respiratory pathogens, with

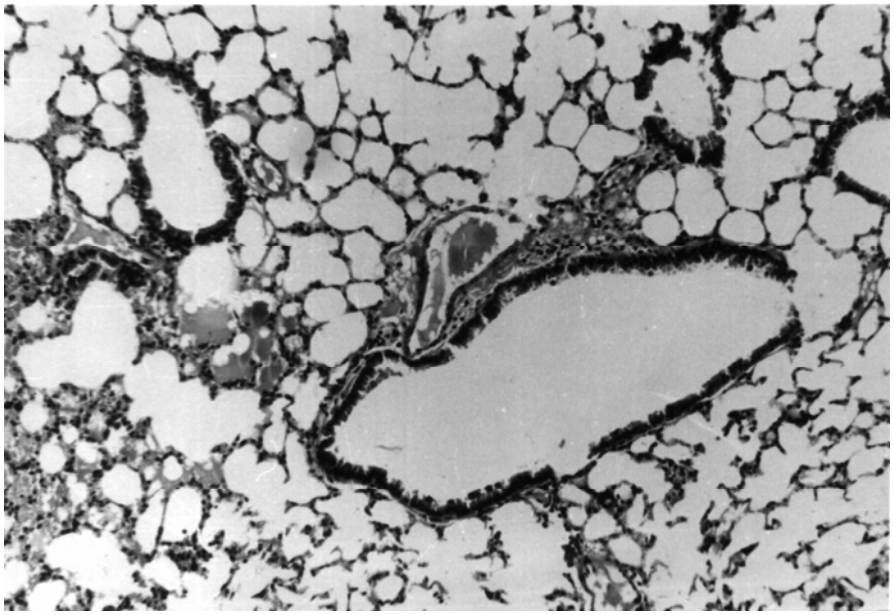

Fig. 1. Photomicrograph showing a bronchiole lined by intact epithelium while there is minimal peribronchiolar infiltrate; the alveoli in the peribronchiolar area show some edema fluid (hematoxylin–eosin, ×100).

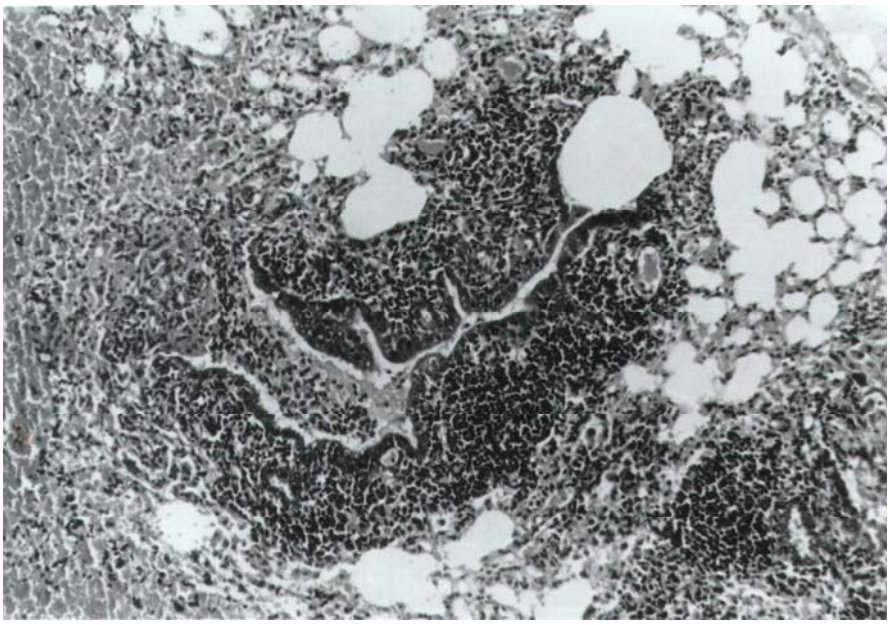

Fig. 2. Photomicrograph showing peribronchial and intra-alveolar exudation while there is focal destruction of alveolar septal walls; the lining of the bronchiole is also disrupted and the lumen contains exudates (hematoxylin–eosin, ×100).

this type of underlying tissue damage especially in the hospital setting is therefore likely to cause complications in the patients. Direct instillation of LPS to the lungs has earlier been shown to exert an effect on macro-

phages resulting in an inflammatory cascade, defined by production of proinflammatory cytokines (Ulich *et al.* 1990). This is accompanied by a huge influx of polymorphonuclear leukocytes into the airway spaces which possibly contributes to acute injury (Hirano 1996).

An alternate route tested for administration of the LPS molecule was the instramuscular one. There was significant protection noticeable with the higher dose, *i.e*. 100 μg, administered to mice. This corroborates the earlier observations of our laboratory in which the LPS antigen was found to be protective at the 100 μg dose level, given intramuscularly in a rat lobar pneumonia model (Rani *et al.* 1990). The same dose of LPS antigen was also reported to be protective in mice against homologous experimental infection of urinary tract, when immunized *via* intramuscular and/or intraurethral route (Chathley *et al.* 1996). A complete LPS-based vaccine strategy, although it appears attractive (cf. Mittal *et al*. 2002; Toky *et al*. 2003), still requires an alternative approach especially for local immunization.

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