Salicylate-Enhanced Exposure of *Klebsiella pneumoniae* Subcapsular Components

**Introduction**

Bacterial diseases caused by encapsulated bacteria are both common and serious infections in the world human population [1]. This is especially the case for pneumonia, where they are implicated in the vast majority of cases [2]. Gram-negative bacteria account for more than half of the pneumonias that occur in hospitalized patients, or patients in extended-care facilities [2]. An important characteristic shared by these pneumonia-causing bacteria is their ability to produce capsular polysaccharide (CPS). The most notable gram-negative CPS producer is *Klebsiella pneumoniae*. Virulent strains of this organism are invariably surrounded by an extensive layer of CPS, which shields bacteria from host defenses, and is an important virulence factor [3]. Typically, the larger the capsule produced, the more pathogenic the strain [4–6].

Increasing resistance of *K. pneumoniae* to antibiotics and high mortality rates [7–10] have impelled the need for alternative preventive and therapeutic measures. One alternative is a vaccine against the type-specific CPS antigens (K antigens) [11–13]. Although serotype-specific immunotherapy for *K. pneumoniae* is a logical approach, it may not be clinically or economically suitable because at least 70 serologically distinct K antigens have been identified within this species [13]. Therefore, vaccines against O-saccharide side chains of LPS (O antigens) are being considered, because *K. pneumoniae* expresses relatively few O antigens in comparison to K antigens. Among clinical isolates, the O1 antigen is the most common O antigen encountered [6]. One problem with targeting the O1 antigen for immunotherapy is that it is not surface exposed in certain capsule types of *K. pneumoniae* [14].

Therapeutic agents that reduce CPS production may provide an effective adjunct to existing therapy against encapsulated pathogenic bacteria. In our hands, salicylate and/or bismuth salts have been used to reduce CPS production not only in several serotypes of *K. pneumoniae* [15, 16], but also in other medically important bacteria [17]. Previously, we have shown that *K. pneumoniae* grown in the presence of salicylate were more readily killed by human polymorphonuclear leukocytes (PMNL) in the presence of pooled normal human serum [18] and phagocytosed by PMNL in the presence of K-antigen specific antisera [19]. In this study we examined the effects of salicylate on exposing subcapsular antigens and components important for phagocytosis, complement activation, and the immunoreactivity of monoclonal antibodies. In addition, the effect of salicylate on the survival of mice infected with *K. pneumoniae* was studied.

**Materials and Methods**

*Bacteria and media:* *K. pneumoniae* strains KP1LC (O1:K1), 52145 (O1:K2), KT759 (O1:K10), KT762 (O1:K16), C3 (O1:K66), KT791 (O1:K), and KT707 (O:K66) used in this study were grown in Luria–Bertani medium at 37°C, 150-200 rpm. The cultures were harvested when optical density at 600 nm was 0.7. To test the effect of salicylate on exposure of subcapsular antigens and components, the bacteria were grown in medium containing salicylate at a concentration of 250 µg/ml.

**Results**

**Salicylate enhances exposure of subcapsular antigens and components.**

Salicylate, which inhibits CPS production, was used to expose subcapsular antigens and components that may play an important role in host defense. Salicylate treatment greatly increased phagocytosis of five O1 serotypes by human polymorphonuclear leukocytes with normal rabbit serum and rabbit antisera against purified O1 lipopolysaccharide (O1LPS) as opsonins (p<0.01 or <0.05). Similar results were obtained with rabbit antiserum against a non-encapsulated isogenic strain. To further determine how salicylate increases susceptibility to phagocytosis, the binding of monoclonal antibodies against O1LPS or the LPS core and the binding of complement component C3b were measured by ELISA. The data indicate that salicylate reduced the barrier of CPS in serotypes O1:K1, O1:K10, and O1:K16 and unmasked subcapsular antigenic components in serotypes O1:K2 and O1:K66 so that bound opsonins could react with receptors on phagocytes. Serum bacterial assays supported this conclusion. Therefore, decapsulating agents such as salicylate accentuate phagocytosis of *K. pneumoniae* by making subcapsular antigens and components accessible to immune and nonimmune host defences and vaccination with subcapsular antigens may exhibit optimal protection against lethal infection when combined with salicylate therapy.
study have been described [20–22]. For experiments on the inhibition of CPS production and phagocytosis, bacteria were grown at 37°C for 16–18 h in a defined medium that promoted capsule production [23]. For all other experiments bacteria were grown in Luria broth [24]. Sodium salicylate (Sigma Chemical Co., St. Louis, MO) was added as indicated to culture media from concentrated stock solutions prepared within 7 days prior to use. *Extraction and quantitation of capsular polysaccharide:* Methods for CPS determination have been described [15,25]. Cultures grown at 37°C for 18 h in defined medium containing varying concentrations of sodium salicylate were mixed with Zwittergent® 3–14 detergent (Calbiochem, LaJolla, CA) in citric acid and mildly heated to solubilize extracellular polysaccharides. The CPS content of ethanol-precipitated extracts was assayed by measuring uronic acid [26], since glucuronic acid is a unique component of Klebsiella CPS. Nutrient agar plates were used for enumerating viable bacteria from broth cultures. Measurements of CPS were expressed as nanograms of glucuronic acid per 10⁶ colony-forming units (ng UA/10⁶ CFU).

**Antisera:** A non-encapsulated variant of strain 52145, 52145-NCV, [18] was used to prepare a lipopolysaccharide antigen, designated O1LPS. The O1LPS antigen was separated from CPS by Sepharose 6B gel filtration chromatography and further purified by DEAE ion-exchange chromatography [25, 26]. New Zealand white rabbits were injected subcutaneously with mixtures of O1LPS and Freund’s adjuvant for immunization followed by booster injections with O1LPS and incomplete Freund’s adjuvant at 2-week intervals. Similarly, rabbits were immunized with formaldehyde-killed 52145-NCV. Rabbits were monitored for antibody production by testing serum agglutination of 52145-O1LPS or anti-NCV serum. Mixtures were incubated 30 rain at 37°C with rabbit anti-C3b (Calbiochem) diluted 1:100 or PBS for negative controls. Bound antibody was detected with a protein A-alkaline phosphatase conjugate as described above. Results were expressed as the mean A₄₀₅ of triplicate samples of at least two experiments.

**C3b binding to bacteria:** The enzyme immunoassay to detect complement component C3b binding to K. pneumoniae has been described [31]. Briefly, bacteria were preincubated for 5 to 20 min with 90% NHS at 37°C, washed with PBS, and then incubated for 45 min at 37°C with rabbit anti-C3b (Calbiochem) diluted 1:100 or PBS for negative controls. Bound antibody was detected with a protein A-alkaline phosphatase conjugate as described above. Results were expressed as the mean A₄₀₅ of triplicate samples of at least two experiments.

**Inhibition of serum bactericidal activity:** The serum sensitive K. pneumoniae strain KT707 [21] was used to determine if salicylate-treated bacteria could prevent the bactericidal activity of NHS as described previously [31]. Bacteria (10⁶ CFU) grown in the absence or presence of salicylate were incubated with NHS for 1 h at 37°C and then the bacteria were removed from the serum by centrifugation and filtration. The treated serum (0.9 ml) was then added to 0.1 ml K. pneumoniae strain KT707 (5 x 10⁵ CFU) and incubated at 37°C for 3 h. Samples were taken at time zero and hourly to determine CFU/ml. Results were expressed as percent survival after 3 h of incubation. The survival of strain KT707 with untreated NHS was <0.1%.

**Animal studies:** Swiss-Webster male mice weighing 20–25 g (Taconic, Valhalla, NY) were injected intraperitoneally (IP) with variable amounts of rabbit anti-NCV serum and simultaneously with 100 µl of salicylate (200 mg/kg) in propylene glycol or propylene glycol alone. Mice were subsequently challenged IP with encapsulated K. pneumoniae strain 52145. Survival data were recorded for 5 days.

**Statistics:** The PI and %P data were converted to natural logs for analysis of variance. The data of other experiments were analyzed using Student’s t test and the coefficient of correlation.

**Results**

**CPS Inhibition**

CPS production from five different K. pneumoniae serotypes was inhibited in a concentration-dependent manner by salicylate (Figure 1). Serotype K1 was most refractory to salicylate treatment, while serotype K2 was most susceptible. At 0.25 mM salicylate, 40% of O1:K2 and 10% of O1:K1 CPS production was inhibited. At 1 mM salicylate,