Evaluation of Importance of Toll-like Receptor 4 in Acute Streptococcus pneumoniae Sinusitis in Mice

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Objectives: To investigate the effect of RC-527, a synthetic toll-like receptor 4 (TLR4) agonist, on stimulating the immune response before acute Streptococcus pneumoniae sinusitis in a mouse model, and to determine the importance of TLR4 in modulating the response to S pneumoniae. Toll-like receptor 4 agonists have been shown to induce protective innate immune responses when administered before some bacterial or viral challenges in mice.

Design: We intranasally inoculated BALB/c, TLR4 complex–deficient C3H/HeJ, and wild-type C3H/HeOuJ mice with S pneumoniae 24 hours after treatment with 10 or 1 μg of RC-527 or vehicle. Bacterial counts from nasal lavage culture and the cell markers GR1, CD11b, CD3, CD4, and CD8 in sinus tissue were quantified at postinoculation days 2, 5, and 14.

Main Outcome Measure: Immune response induced by RC-527.

Results: Treatment with RC-527 induced an immune response through TLR4, as demonstrated by recruitment of phagocytes in uninfected wild-type C3H/HeOuJ mice, but not in TLR4 complex–deficient C3H/HeJ mice. However, the enhancement of the immune response induced by the TLR4 agonist showed a limited effect on bacterial clearance.

Conclusions: Our studies in mice suggest that stimulation of TLR4 plays a minor role in the overall response to S pneumoniae infection of the upper airway, and stimulating this receptor before infection does not significantly enhance the immune response of immunocompetent mice to clear S pneumoniae infection.

adaptive immunity mediated through TLR signaling.\textsuperscript{11,12} We thought that stimulation of the TLR4 before infection would speed the resolution of acute bacterial rhinosinusitis.

Prophylactic administration of purified LPS was found to induce protection from subsequent bacterial or viral challenge in various animal models.\textsuperscript{13-15} Monophosphoryl lipid A, derived from the LPS of Salmonella minnesota R595, reduces toxicity and pyrogenicity compared with the parent LPS. Monophosphoryl lipid A activity is mediated via binding to the TLR4 complex, and data in vivo demonstrate that mice pretreated with monophosphoryl lipid A are nonspecifically protected from bacterial and viral challenge not thought to involve TLR4.\textsuperscript{16-21} More recently, synthetic lipid A mimetics that are chemically unique, acylated monosaccharides called aminoolkyl glucosaminide 4-phosphates (AGPs) were developed. The general structure of AGPs consists of a monosaccharide unit with an acylated amino-olalkyl aglycon spacer arm. For the protective AGPs, the secondary acyl chain is the most critical determinant of activity when combined with a primary acyl chain standardized at 14 carbons. The TLR4 agonist RC-527, with three 10-carbon secondary acyl chains and 2 negatively charged residues on its backbone, was chosen because of its maximal activity.\textsuperscript{22,23}

Our objectives were to evaluate whether treatment with RC-527 before exposure to \textit{S pneumoniae} speeds the resolution of infection, and to determine the importance of TLR4 in response to \textit{S pneumoniae} infection. Although gram-negative bacteria such as pneumococci usually interact through TLR2, Mally et al\textsuperscript{24} demonstrated that TLR4 mediates an innate immune response to \textit{S pneumoniae} through its interaction with 1 of the major virulence factors of the organism, the cholesterol-dependent cytolysin pneumolysin. In addition, TLR4 stimulation drives the immune response toward a helper T cell 1 (T\textsubscript{h1}) response. Thus, we chose to study BALB/c mice, which favor a T\textsubscript{h2} response, anticipating that a shift in their immune tendency would favor rapid clearance of \textit{S pneumoniae}, as occurs in C57Bl/6 mice, which favor a T\textsubscript{h1} response. A positive response in the prophylactic paradigm would have caused us to pursue studies on the effect of this drug after the initiation of infection.

**METHODS**

**MICE**

We obtained pathogen-free BALB/c, TLR4 complex-deficient C3H/HeJ, and wild-type (wt) C3H/HeOuJ mice aged 6 to 8 weeks (Jackson Laboratory, Bar Harbor, Me). The animals were kept in the Carlson Biocontainment Suite Facility at the University of Chicago, Chicago, Ill, 1 week before the beginning of experiments. All protocols were approved by the Animal Care and Use Committee of the University of Chicago.

**RC-527 ADMINISTRATION**

The RC-527 was provided by Corixa Corp (Hamilton, Mont). The stock 1-mg/mL solution was diluted with vehicle to concentrations of 10 and 1 µg in 50 µL. Mice were anesthetized by means of intraperitoneal administration of a preparation containing ketamine hydrochloride (50 mg/kg) and xylazine hydrochloride (5 mg/kg). We placed 50 µL of RC-527 intranasally (25 µL per nostril) in mice 24 hours before infection. In the first experiment, we used the TLR4 complex-deficient C3H/HeJ and wt C3H/HeOuJ strains of mice. Within each strain, the mice were stratified into infected and noninfected groups, with each subgroup receiving the different concentrations (10 and 1 µg in 50 µL) of RC-527 or vehicle. In the second experiment, we used 3 groups of infected BALB/c mice, with each group given RC-527 at 3 different concentrations. There were 3 to 6 mice per group in the first experiment and 6 mice per group in the second experiment.

**RESULTS**

**RECOGNITION OF RC-527 BY TLR4**

To confirm that RC-527 affects TLR4, we performed an experiment in the uninfected TLR4 complex-deficient C3H/HeJ and wt C3H/HeOuJ mice. There was a significant increase in the numbers of total CD11b⁺, Gr1⁺, CD3⁺, CD4⁺, and CD8⁺ T cells as measured by flow cytometry in the RC-527-treated group 2 days after single-drug administration in the wt C3H/HeOuJ mice, but not in the TLR4 complex-deficient C3H/HeJ mice (Figure 1). When these mice were infected after treatment with RC-527, the RC-527-treated group of wt C3H/HeOuJ mice had significantly more cells and tended to have less \textit{S pneumoniae} than the vehicle-treated group, but the latter difference did not reach statistical significance (Figure 2).

**NASAL CULTURES**

Mice were sedated with a respiratory-failure dose of 120 mg/kg of pentobarbital sodium (Nembutal) given by intraperitoneal injection, and nasal lavage was performed with 200 µL of phosphate-buffered saline solution. The lavage liquid was then serially diluted (neat, 1:10, 1:100, 1:1000, and 1:10 000), and each dilution was plated onto Columbia sheep's blood agar plates. The plates were incubated for 24 hours, and then the number of colonies was counted.

**STATISTICAL ANALYSIS**

Logarithmic conversion for normalization was performed on the flow cytometric and culture data. We compared differences by means of 1-way analysis of variance, followed by Tukey multiple comparison tests. We considered $P \leq 0.05$ to indicate statistical significance.

**TISSUE HARVESTING AND PROCESSING**

Flow cytometry was used for quantifying cells present in the sinuses. The mice were killed, and the skull was bisected sagittally for exposure of the sinuses. The tissue from the sinuses was treated with a preparation containing ketamine hydrochloride (50 mg/kg) and xylazine hydrochloride (5 mg/kg). We placed 50 µL of RC-527 intranasally (25 µL per nostril) in mice 24 hours before infection. In the first experiment, we
ROLE OF RC-527–INDUCED IMMUNE RESPONSE IN PNEUMOCOCCAL CLEARANCE

We next treated BALB/c mice with RC-527, because BALB/c mice, with their tendency to form a Th2-mediated immune response, are less able to clear an *S pneumoniae* infection than are C37Bl/6 mice with a Th1 background (T.L., V.K., J.K., P.K., K.T., and R.M.N., unpublished observation, March 2004). Treatment with RC-527 decreased the bacterial count in the BALB/c mice compared with the vehicle-treated group in the first few days. There was a statistically significant difference between the group treated with 1 µg of RC-527 and the vehicle-treated group at postinoculation day 2 (Figure 3). Stimulation with RC-527 resulted in a significant increase in the numbers of CD11b⁺, GR1⁺, CD4⁺, and CD8⁺ cells in the group treated with 10 or 1 µg of RC-527 compared with vehicle and/or between groups in uninfected and infected wt C3H/HeOuJ mice. Asterisk indicates *P*<.01 compared with vehicle; dagger, *P*<.05 compared between treatment groups; double dagger, *P*<.01 compared between treatment groups; section mark, *P*<.05 compared with vehicle; and horizontal bars, means.
in an increase in inflammatory cells, which was demonstrated by an increase in GR1<sup>+</sup>, CD11b<sup>+</sup>, CD3<sup>+</sup>, CD4<sup>+</sup>, and CD8<sup>+</sup> T cells (Figure 4). The effects were significantly different at day 2 and had gradually decreased at day 5, until there were no significant differences at day 14.

**EFFECT OF TLR4 MUTATION ON THE RESPONSE TO S PNEUMONIAE**

To investigate the role of TLR4 in *S pneumoniae* infection, we inoculated *S pneumoniae* intranasally at 5 × 10<sup>7</sup> CFU in TLR4 complex–deficient C3H/HeJ and wt C3H/HeOuJ mice and evaluated bacterial cultures from nasal lavage and cell counts in sinuses from flow cytometric analysis at postinoculation days 2 and 21. There were no significant differences in the bacterial count and the total CD11b<sup>+</sup>, GR1<sup>+</sup>, CD3<sup>+</sup>, CD4<sup>+</sup>, and CD8<sup>+</sup> T cells in sinus tissue between wt C3H/HeOuJ and TLR4 complex–deficient C3H/HeJ at postinoculation days 2 and 21 (Figure 5 and Figure 6).

**COMMENT**

Aminoalkyl glucosaminide 4-phosphates such as RC-527 have been reported to induce protective innate immune responses when administered before some bacterial or viral challenges of mice. In our study, at 1- and 10-µg doses, we found that RC-527 induced significant inflammation in BALB/c mice. The effect on the infection was much less than that which occurs after administration of an antibiotic, and the effect did not lead to faster resolution of the infection or inflammation.

In the present study, we demonstrated that RC-527 can induce an early immune response. The effect is clearly mediated by TLR4, because there was an increased influx of phagocytic cells after challenge with RC-527 in uninfected wt mice, whereas there was no such effect in TLR4 complex–deficient C3H/HeJ mice. The effect was observed in BALB/c mice at days 2 and 5 after drug admin-
istration, but not at day 14. Together, these studies show that TLR4 complex is present in the upper respiratory airway, which agrees with the findings of Wang and colleagues and Claeys and colleagues. Our study demonstrated an early significant increase of CD3^+^, CD4^+^, and CD8^+^ T cells in both infected and uninfected wt C3H/HeOuJ mice treated with RC-527, but not in TLR4 complex-deficient C3H/HeJ mice at day 2 and/or day 5.

Immunity enhanced by TLR4 agonists, however, showed a limited additional effect in eradicating gram-positive pneumococcal infection of the sinuses. Branger and colleagues suggested that the role of TLR4 in pneumococcal pneumonia in mice was relatively limited, providing incomplete protection only after infection with low bacterial doses (6 × 10^3^ CFU) in wt mice, whereas TLR4 had a more important effect on the immune response in *Klebsiella pneumoniae*, providing protection after infection with low or high doses of bacteria. If we had used smaller inocula, we might have shown the protective effect seen in the experimental pneumonia infection, but low doses produce inconsistent infections in our model. Most studies, however, have shown the importance of TLR4 in host defense against gram-negative but not gram-positive bacteria.

Although TLR4 is important for the recognition of gram-negative bacteria, TLR2 is important in the recognition of gram-positive bacteria through cell wall and membrane components such as lipoteichoic acid, lipoprotein, and peptidoglycan. In a study by Knapp and colleagues, survival did not differ between TLR2^−/−^ and wt mice after infection with a high (10^5^ CFU) or with a low (5 × 10^3^ CFU) bacterial inoculum of *S pneumoniae* in the lungs, and there was no difference in bacterial clearance of the lungs 48 hours after inoculation, suggesting a limited role of the innate immune response to pneumococcal pneumonia. A modest protective effect of TLR2 was also reported in a study by Echchammaoui et al. Taken together, TLR2 and TLR4 contribute minimally to the elimination of pneumococcal infections of the airway in mice.

Many studies have shown that the pneumococcus can interact with the initial inflammatory response to inhibit some components of the host defense and hence continue its multiplication without being eliminated. Lysed pneumococcal populations release pneumolysin into the tissues, which has a wide range of cytotoxic and inhibitory effects on host tissue and immune cells. Pneumolysin interacts through the TLR4 complex. The virulence and multiple function of pneumolysin, especially in early stages of infection by pneumococci, are crucial to pneumococcal colonization of a host.

Furthermore, the study of Dallaire and colleagues demonstrated the enhanced survival effect of LPS (a TLR4 agonist) on mice infected with 5 × 10^6^ CFU of *S pneumoniae* and the decreased effect when LPS injection was delayed 24 hours after the onset of infection. Because the host defense against microbial infection depends on the rapid clearance of the organisms from the site of infection, it might improve the initial clearance of microorganisms by increasing the early inflammatory response and thus have a beneficial effect on survival. In our study, pretreatment with a TLR4 agonist reduced some bacterial load in the early pneumococcal infection, but the amount of reduction was insufficient to clear the infection.

In conclusion, a synthetic AGP, RC-527, can stimulate the immune response through TLR4 for about 5 days. However, there was a limited beneficial response to the infection with gram-positive pneumococci. Furthermore, deficiency of the TLR4 complex does not significantly hinder the response to *S pneumoniae* infection.

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**Figure 6.** Numbers of total CD11b^+^ (A), GR1^+^ (B), CD4^+^ (C), and CD8^+^ (D) cells after infection with *Streptococcus pneumoniae* in wild-type C3H/HeOuJ and TLR4 complex-deficient C3H/HeJ mice at postinoculation days 2 and 21. There was no significant difference in total numbers of CD11b^+^, GR1^+^, CD4^+^, and CD8^+^ cells between the 2 strains at postinoculation days 2 and 21 (n=4 per time point). Horizontal bars indicate means.