Multivalent Choline Dendrimers Increase Phagocytosis of *Streptococcus pneumoniae* R6 by Microglial Cells

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**Conclusions:** Multivalent dendrimers containing choline end groups are promising antimicrobial agents for the management of pneumococcal diseases.

**Introduction**

*Streptococcus pneumoniae* (Spn) causes life-threatening infections, including pneumonia, septicaemia and meningitis [1]. The dissemination of multidrug-resistant strains in conjunction with current vaccines that do not protect against all pneumococcal serotypes calls for novel therapeutic approaches to combat this pathogen threat [2, 3]. Choline-binding proteins (CBPs) are cell surface virulence factors [for a review, see ref. 4] common to all pneumococcal serotypes, so they are potential drug development targets [5]. Blocking pneumococcal CBPs would indeed reduce bacterial dissemination and prevent the release of bacterial products responsible for the inflammatory response which in pneumonia and bacterial...
meningitis contribute to the development of tissue damage [6]. Trapping CBPs by the addition of synthetic dendrimer structures containing choline end groups has been successfully achieved in vitro with specific inhibitory effects on pneumococcal division and autolysis [7], pointing to these nanostructures as possible novel antimicrobials.

Microglia, the macrophages of the brain, respond rapidly to any disturbance of the central nervous system homeostasis [8]. Upon recognition of well-conserved microbial structures through pattern recognition receptors, they can shift into an activated state [9]. The activation of microglia occurs in both cerebral and systemic infections probably as a mechanism to increase the resistance of the brain against the entry of pathogens [10, 11]. In vitro, microglia can phagocytose non-encapsulated and encapsulated strains of Spn at a low rate [12]. The phagocytic activity of microglia can be enhanced by the addition of agonists of Toll-like receptors [12] alone or in combination with previous pneumococcal opsonization with an anti-phosphorylcholine monoclonal antibody [13]. Here, we investigated whether short- and long-term co-incubation of Spn with a synthetic choline dendrimer might influence the capacity of microglial cells in primary cultures to phagocytose bacteria.

Methods

Preparation of Bacteria and Dendrimers

Spn strain R6 (non-encapsulated, serotype 2) was grown in tryptic soy broth at a temperature of 37°C. Growth was monitored by quantitative plating of viable bacteria. Ten-fold dilutions were performed in sterile saline and plated and quantified on blood agar plates. Polypropylene imine (PPI) dendrimers containing 8 choline end groups (g2-cho) or their non-functional counterparts (g2-NH₂) were synthesized as described (fig. 1a) [7]. In co-incubation experiments, dendrimers were diluted in phosphate buffer and added to the pneumococcal culture at defined times and concentrations.

Primary Cultures of Microglial Cells

Primary microglia were prepared from brains of newborn C57BL/6N mice and cultured as previously described [12]. Briefly, brains of mice aged 1–3 days were mechanically dissociated and suspended in Dulbecco’s modified Eagle’s medium with Gluta-max, supplemented with 10% heat-inactivated fetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco, Karlsruhe, Germany). Cells were plated at a density of two brains per T75 culture flask (Corning Costar, Wiesbaden, Germany) and maintained at 37°C in a humidified atmosphere with 5% CO₂. Microglial cells were isolated from the mixed glial cultures by shaking 200 times/min for 30 min and plated (20,000 cells/well) in 96-well plates.

Effects of Co-Incubation of Spn R6 and Dendrimer Compounds

The choline dendrimer (g2-cho) and its non-functional counterpart (g2-NH₂) were added to pneumococcal cultures at two different times according to the growth kinetics of Spn R6. Both compounds were tested at 0.1, 1, 10 and 100 μM. Spn R6 incubated with phosphate buffer served as control. Co-incubation lasted for 2 or 14 h at 37°C and 5% CO₂.

The effects of short and long periods of co-incubation were assessed in terms of pneumococcal growth morphology and bacterial uptake by murine microglial cells.

In the phagocytosis studies, Spn R6 was incubated with the two dendrimer preparations for 2 or 14 h and then added to the microglial cells (multiplicity of infection of about 50 bacteria per microglial cell) for 30 min at 37°C and 5% CO₂. During this time, microglia were allowed to phagocytose Spn R6. Then, supernatants were removed and cells were incubated for 1 h in culture medium containing 100 μg/ml gentamicin (Sigma-Aldrich, St. Louis, Mo., USA) to kill extracellular bacteria. After washing and cell lysis with distilled water, the intracellular bacteria were enumerated by quantitative plating of the lysates on blood agar plates. To ensure compatibility of different experiments, phagocytic rates (%) of different groups of microglia (MG) were calculated as follows:

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\frac{\text{phagocytosed bacteria (CFU/well) by X-treated MG}}{\text{bacterial inoculum added to X-treated MG}} \times 100
\]

where control microglia are defined as cells exposed to Spn R6 plus phosphate buffer.

In selected experiments, the supernatants, after 30 min of phagocytosis, were subjected to microscopy analysis. Supernatants were directly plated on glass coverslips and dried at 37°C. Thereafter, samples were stained with Löffler’s methylene blue solution (Merck, Darmstadt, Germany) for 5 min. After washing and heat fixation, images were acquired with an Olympus BX1 microscope (Olympus, Tokyo, Japan).

Results

Effects of g2-cho and g2-NH₂ on Bacterial Growth

We first determined the growth kinetics of Spn R6 in tryptic soy broth (fig. 1b). Then we evaluated the effect of long (addition from the beginning of bacterial growth, 0–14 h) and short (addition at the late exponential growth phase, 12–14 h) dendrimer incubation on Spn growth. To assess the effect of choline dendrimer, the second-generation g2-cho was chosen as it shows virtually the same affinity for CBPs as higher-order particles [7], but it is less likely to produce undesirable side effects in possible therapeutic uses due to its lower positive net charge. Trapping CBPs by addition of 100 μM g2-cho inhibited daughter cell separation at the end of cell division, causing the for-
Fig. 1. **a** Chemical structures of the PPI dendrimers containing 8 choline end groups (g2-cho) or their non-functional counterparts (g2-NH2). **b** Growth kinetics of Spn R6. Media and growth conditions are described above. The density of viable bacteria [colony-forming units (CFU)/ml] was determined by 10-fold serial dilution and plated and counted on blood agar plates. Data are shown as means. Error bars represent standard deviations. Solid and dashed arrows indicate the addition times of dendrimer compounds corresponding to the early and late exponential growth phases, respectively. **c**–**e** Spn R6 (×100) after long (0–14 h; **c**) and short (12–14 h; **d**) incubation with 100 μM choline-functionalized dendrimer containing 8 choline end groups (g2-cho) or after long (0–14 h; **e**) incubation with the control dendrimer g2-NH2.
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Fig. 2. Phagocytosis of bacteria by microglial cells after incubation of Spn R6 with g2-cho dendrimers. Bacteria were incubated with different concentrations of either choline-functionalized PPI dendrimers (g2-cho) or the non-functional second-generation amine dendrimers (g2-NH2) from the beginning of the experiment (0–14 h; a) or in the late exponential growth phase (12–14 h; b). Then, bacteria were added to the microglial cells for 30 min. The ratio of phagocytosed bacteria to added bacteria of the control group (microglia that were co-incubated with bacteria and phosphate buffer) was defined as 100%. Data are shown as medians. Error bars represent the 25th/75th percentiles. Asterisks indicate statistically significant differences to the control group of microglial cells (Kruskal-Wallis test followed by correction for repeated testing by Dunn’s multiple comparison test, * p < 0.05; *** p < 0.001).

Discussion

CBPs are essential for pneumococcal function including cell-wall division, adhesion to the host and autolysis with subsequent release of bacterial pro-inflammatory compounds [4]. Mutants defective in CBPs showed significantly reduced colonization of the infant rat nasopharynx upon intranasal challenge [14]. Therefore, CBPs might constitute a new target for the development of novel antimicrobials. Inhibition of CBPs by the addition of choline and choline analogs (atropine and ipratropium) blocked cell separation and the characteristic autolysis of Spn, thereby inducing the formation of long chains or phagocytosed bacteria to added bacteria of the control group (microglia that were co-incubated with bacteria and phosphate buffer) was defined as 100%. Data are shown as medians. Error bars represent the 25th/75th percentiles. Asterisks indicate statistically significant differences to the control group of microglial cells (Kruskal-Wallis test followed by correction for repeated testing by Dunn’s multiple comparison test, * p < 0.05; *** p < 0.001).
even preventing bacterial growth [5, 15]. However, this interaction was weak, requiring high concentrations (in the millimolar range) to achieve a therapeutic effect. Functionalized dendrimers have the capacity of increasing the affinity of a determined ligand for its cognate target by multivalent effects [16]. The recent development of choline-functionalized poly(propylene imine) dendrimers containing different numbers of choline end groups maximized the binding efficiency to the pneumococcal cell wall [7], so we decided to assess their influence on microglia-mediated phagocytosis of Spn.

We selected two time points when dendrimer compounds were added to the bacterial suspension according to the growth kinetics of Spn R6. Phagocytosis of bacteria by microglial cells was substantially increased in both cases, although to a different extent, depending on the interval of dendrimer-bacterial co-incubation, probably reflecting the different chain lengths induced in each condition. This is in accordance with previous results showing that the potency of the effect exerted by choline and its analogs depends on the metabolic state of the bacterium [5]. Addition of the g2-cho dendrimer at the beginning of the pneumococcal growth probably reflects its use as a prophylactic agent to prevent colonization while its addition at the end of the logarithmic phase reflects the conditions of manifest infection. Our results support the concept of increasing the host’s resistance to infection by modifying bacterial morphology as an alternative to affecting bacterial growth. We suggest that phagocytosis assays should be included into screening programs for new anti-infective drugs and reinforce the potential of choline dendrimers as promising starting points for the development of novel anti-pneumococcal agents.

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References