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Original article

The encapsulated strain TIGR4 of *Streptococcus pneumoniae* is phagocytosed but is resistant to intracellular killing by mouse microglia

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Abstract

The polysaccharide capsule is a major virulence factor of *Streptococcus pneumoniae* as it confers resistance to phagocytosis. The encapsulated serotype 4 TIGR4 strain was shown to be efficiently phagocytosed by the mouse microglial cell line BV2, whereas the type 3 HB565 strain resisted phagocytosis. Comparing survival after uptake of TIGR4 or its unencapsulated derivative FP23 in gentamicin protection and phagolysosome maturation assays, it was shown that TIGR4 was protected from intracellular killing. Pneumococcal capsular genes were up-regulated in intracellular TIGR4 bacteria recovered from microglial cells. Actual presence of bacteria inside BV2 cells was confirmed by transmission electron microscopy (TEM) for both TIGR4 and FP23 strains, but typical phagosomes/phagolysosomes were detected only in cells infected with the unencapsulated strain. In a mouse model of meningitis based on intracranic inoculation of pneumococci, TIGR4 caused lethal meningitis with an LD_{50} of 2×10^2 CFU, whereas the LD_{50} for the unencapsulated FP23 was greater than 10^7 CFU. Phagocytosis of TIGR4 by microglia was also demonstrated by TEM and immunohistochemistry on brain samples from infected mice. The results indicate that encapsulation does not protect the TIGR4 strain from phagocytosis by microglia, while it affords resistance to intracellular killing.

Keywords: *Streptococcus pneumoniae*; Capsule; Microglia; Phagocytosis; Intracellular survival

1. Introduction

Streptococcus pneumoniae (the pneumococcus) is a human pathogen that causes otitis media, pneumonia, sepsis and meningitis, especially in infants and the elderly [1]. It is the most important etiological agent of community acquired pneumonia in adults and the second most frequent cause of bacterial meningitis after *Neisseria meningitidis* [2]. Pneumococcal meningitis induces high morbidity and mortality, with serious neurological sequelae in about 25% of cases [3].

S. pneumoniae produces a plethora of virulence factors, including the polysaccharide capsule, several surface-located

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proteins and the toxin pneumolysin [4,5]. The capsule is the major virulence determinant due to its strong anti-phagocytic properties. In the non-immune host, it prevents the activation of the alternative complement pathway and the interaction of C3b surface-bound molecules with receptors on phagocytic cells, thereby inhibiting bacterial uptake by phagocytes [6–8]. In addition, the capsule may prevent the activation of the classical complement cascade by hindering surface antigens from binding to natural IgM [9]. Several lines of evidence indicate that the capsule also influences pneumococcal colonization, survival, and dissemination in the host. Indeed, the transparent phenotypes, which produce smaller amounts of capsular polysaccharides (CP) compared to the opaque ones, are more efficient in colonizing the mucosal surface of the nasopharynx, whereas the opaque phenotypes are more virulent in systemic infections [10,11]. On the basis of differences in CP structure, *S. pneumoniae* can be divided into more than 90 serotypes. The biochemical structure of CP can influence the degree of encapsulation of different serotypes, their virulence, as well as their prevalence [12]. The brain is a compartment isolated from immunosurveillance, where adaptive immunity is limited; however, it possesses its own innate immune system which plays a crucial role in eliminating invading pathogens, as well as cell debris [13]. Innate immune glial cells are represented by microglia, perivascular cells, astrocytes, epithelial cells of the choroid plexus and ependyma [13]. Microglial cells, comprising up to 15% of brain cells, are located within the brain parenchyma, just behind the brain blood barrier (BBB) and constitute the main phagocytic population of CNS [13–15]. Ramified microglial cells have been referred to as "resting", while activated cells generally have an ameboid macrophage-like morphology. Concurrently, morphological changes are associated with acquisition of effector functions, such as phagocytosis, inflammatory responses and antigen presentation [13–15]. In spite of being in a resting state, microglia is a highly dynamic surveillant of the brain parenchyma; it interacts with other cells, removes catabolites and/or cell debris and promptly responds to invading pathogens and/or tissue lesions [14,16]. Microglia is also a major source of cytokines/chemokines that trigger inflammatory responses, including the recruitment of peripheral leukocytes into the CNS [13–15]. Recent studies have suggested that the C-type lectin SIGN-R1, or its rat homologue CD209b, is expressed on microglia, but not on neurons or astrocytes, and is able to mediate the uptake of capsular polysaccharides of *S. pneumoniae* [17].

In the present study, we have employed the type 4 TIGR4 strain and its unencapsulated mutant FP23 to investigate the role of the capsule in the interaction between pneumococci and microglia both *in vitro* and *in vivo*.

2. Materials and methods

2.1. Pneumoccocal strains, growth conditions and labeling of bacteria

The capsulated TIGR4 strain [18] and its isogenic unencapsulated mutant FP23 [19,20] were used throughout the experiments. Preliminary phagocytosis assays were also performed with the type 3 HB565 strain [20,21] and its unencapsulated derivative FP33 [20]. *S. pneumoniae* was cultured in Tryptone Soy Broth (TSB, Oxoid, Hampshire, UK) at 37 °C with 5% CO_2. Solid culture media were obtained by addition of 1.5% agar and 3% defibrinated horse blood (Biotec Snc, Grosseto, Italy) to TSB. Bacteria were frozen in glycerol (15% v/v) and stored at −80 °C. The number of bacteria in frozen stocks was determined by viable counting of serial dilutions in sterile PBS (EuroClone, Whethereby, UK) and plating onto blood agar plates. For *in vitro* assays, bacteria were thawed, washed, harvested by centrifugation and suspended at the desired concentrations. When required, staining of *S. pneumoniae* was performed by incubating bacteria with 2 μg/ml of Oregon green 488 (Og) (Molecular Probes, Eugene, OR, USA) in the dark at 37 °C for 1 h, as previously described [22]. After labeling, bacteria were washed twice with PBS and then resuspended in RPMI 1640 medium (Gibco, Grand Island, NY, USA) at the desired concentration.

2.2. Microglial cells

The previously established murine microglial cell line BV2 [23] was maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (hiFBS) (Defined Hyclone, Logan, UT, USA), gentamicin (50 μg/ml; Bio Whittaker, Verviers, Belgium) and L-glutamine (2 mM; EuroClone, Milan, Italy), hereafter referred to as "complete medium". Cells were detached by vigorous shaking biweekly, and fresh cultures were started at a concentration of 5×10^5/ml.

2.3. In vitro phagocytosis assays

Two different assays were performed.

2.3.1. Giemsa-stained cytospin assay

Phagocytosis assays were performed as previously described [23]. Microglial cells were incubated with either TIGR4 or FP23 bacteria at 37 °C for different time periods at the effector to target (E:T) ratio of 1:10 (10^6 cells: 10^7 bacteria). Experiments were carried out in RPMI medium containing L-glutamine (2 mM), and 10% of either normal FBS (nFBS) or hiFBS. After incubation, excess of microorganisms was removed by centrifugation of the cell suspension on a Ficoll cushion at 300×*g* for 10 min. Microglial cells at the interface were recovered and used for cytospin preparations. Bacterial uptake was evaluated following Giemsa-staining of the cytospin. A minimum of 200 macrophages were scored and the percentage of phagocytic cells was calculated according to the formula:

$$\text{Phagocytic cells}(\%) = \frac{\text{Number of cells containing} \geq 1 \text{ bacterium}}{\text{Total number of cells examined}} \times 100$$

whereas the phagocytosis index (PI) was calculated as the number of phagocytosed bacteria/total number of phagocytic cells.