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Induction of immune response to *Streptococcus pneumoniae* by administration of oral viridans streptococci via phosphorylcholine determinant

Yoko Miwa, Hiro-O Ito *, Masakazu Inoue, Takahiko Oho

Department of Preventive Dentistry, Kagoshima University, Graduate School of Medical and Dental Sciences, 8-35-1 Sakuragaoka, Kagoshima 890-8544, Japan

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Abstract

Expression of the phosphorylcholine (PC) epitope was examined in 48 viridans streptococcal strains, including *Streptococcus pneumoniae* R36a as the positive control, and their immunogenicity to induce an *S. pneumoniae*-cross-reactive response was evaluated in mice. Thirteen strains were found to express the PC epitope, while no obvious association was found between the taxonomic categories and PC expression. Serum antibody responses to *S. pneumoniae* cells were induced in mice by intraperitoneal injection of the PC-positive, but not PC-negative, strains. The cross-reactive antibodies induced by non-pneumococcal oral streptococci were readily inhibited by free hapten PC. IgM was the sole isotype of the anti-pneumococcal and anti-PC antibodies, and the phenomenon of immunological memory was not observed. Since the anti-PC antibody is critically important for resistance against pneumococcal infection in mice, the present results indicate the possibility that PC-expressing oral commensal bacteria have a significant influence on the hosts' responsiveness to *S. pneumoniae*.

Keywords: Phosphorylcholine; *Streptococcus pneumoniae*; Viridans streptococci; Cross-reactive immunity; Oral flora

1. Introduction

Streptococcus pneumoniae is the most common cause of bacterial pneumonia, and is also the major cause of bacterial meningitis and otitis media [1]. A significant number of mortalities, especially in the age extremes, i.e., <1 and >60 yr, are due to this organism, even in this era of antibiotics in developed countries [2,3].

Phosphorylcholine (PC) is the immunodominant epitope found on the cell wall of *S. pneumoniae* [4,5]. The humoral immune responsiveness to the PC epitope is known to be critical for resistance against lethal pneumococcal infections in mice [4]. The PC determinant is also found on a variety of microorganisms other than *S. pneumoniae* [6]. Information about PC epitope expression on oral bacteria has been accumulated in recent years [7–11]; several gram-positive and gram-negative bacteria have been shown to possess this epitope. Gillespie et al. [8] have reported the PC epitope in members of the *Streptococcus mitis* group, in which *S. pneumoniae* and *S. oralis* are included, but not in representative strains of other oral streptococcus species. Gmür et al. [9], on the other hand, have found the epitope in some strains of the *S. milleri* group (presently called the *S. anginosus* group), in addition to the *S. mitis* group and many species of the genus *Actinomyces*,

* Corresponding author. Tel.: +81 99 275 6182; fax: +81 99 275 6188.

E-mail address: dearkids@dentb.hal.kagoshima-u.ac.jp (Hiro-O Ito).

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except *A. naeslundii*. Schenkein et al. [11] have reported that approximately one third of bacterial cells in human dental plaque are positively stained for PC in an immuno-fluorescence analysis.

The anti-PC antibody response is critically important for resistance against pneumococcal infection in mice [4]. It is therefore conceivable that PC-expressing oral bacteria elicit an immune response cross-reactive to the dominant epitope on *S. pneumoniae*, and consequently they have a significant influence on the susceptibility of normal human individuals to *S. pneumoniae*. This hypothesis is supported by the finding that anti-PC antibody levels are elevated in sera from patients with periodontal diseases [11], and that anti-PC monoclonal antibodies (mAbs) were frequently generated as by-products when mAbs to certain oral strains were produced [9]. However, the relationship between levels of PC epitope expression and their capacity to elicit immune responses towards *S. pneumoniae* has not been directly examined yet.

The aim of the present study was to determine that PC-expressing oral bacteria induce an immune response to *S. pneumoniae*, via the PC determinant. We found the PC epitope on several strains of oral streptococci, including the *S. mitis* and the *S. milleri* groups, in agreement with the observation of Gmür et al. [9]. Immunization with the oral strains induced an antibody response cross-reactive to *S. pneumoniae* in mice, which was inhibitable with free PC molecules in the solutions.

2. Materials and methods

2.1. Bacterial strains and growth conditions

A total of 48 viridans streptococcal strains were used. These included 18 type or reference strains obtained from the international type culture collections, i.e., *S. mitis* ATCC 6249, *S. oralis* ATCC 35037, 10557, *S. gordonii* ATCC 10558, *S. sanguinis* ATCC 9811, *S. parasanguinis* ATCC 15909, 15911, *S. anginosus* ATCC 33397, *S. constellatus* ATCC 27823, NCTC 10708, *S. intermedius* ATCC 27335, *S. salivarius* ATCC 13419, *S. mutans* ATCC 33535, *S. sobrinus* ATCC 6715, *S. cricetus* ATCC 19642, *S. rattus* ATCC 19645, *Granulicatella adiacens* ATCC 49175, *Abiotrophia defectiva* ATCC 49176, and *S. pneumoniae* ATCC 27336 (R36a), and 30 isolates from human oral cavities. Some characteristics of the *S. milleri* group (*anginosus* group) strains [12] and the nutritionally variant streptococci (NVS) strains [13] have been described elsewhere. Cells of all the strains were cultured at 37 °C overnight in Todd-Hewitt broth (BBL Microbiology Systems, Cockeysville, MD, USA), or the medium supplemented with 0.001% pyridoxal–HCl for the NVS strains, as described previously [13]. The cultures were centrifuged and the cells were washed twice in phosphate buffered saline (PBS; 10 mM PO_4, 150 mM NaCl, pH 7.4), and resuspended in 0.05 M carbonate buffer (pH 9.6) at 1.0 of optical density at 600 nm (OD_{600}), corresponding to approximately 10^9 cells ml^{-1}. In some experiments, bacterial cells were washed three times with distilled water and lyophilized.

2.2. Detection of the PC epitope on bacterial cells

Expression of the PC epitope on bacterial cells was determined using an enzyme-linked immunosorbent assay (ELISA) and a TEPC15 (T15), a representative mouse anti-PC mAb [5]. Serially diluted bacterial cell suspensions of known densities were added to the wells (40 μl/well) of polystyrene ELISA plates (96-well) (Greiner, Frickenhausen, Germany). The wells were dried out in a dry incubator at 37 °C, then treated with absolute ethanol, and dried out again. A serial dilution of *S. pneumoniae* R36a was included in each plate to construct a reference-binding curve. The plates were washed three times with PBS containing 0.02% Tween 20 (PBST), and any uncoated surfaces were saturated with 2% non-fat dry milk. T15 (IgA, κ, kindly donated by Dr. Nobuo Sakato) was added to each well at 5 μg ml^{-1} and incubated at 25 °C for 1 h. After washing 3times with PBST, the wells were incubated with an alkaline phosphatase (AP)-conjugated goat anti-mouse IgA antibody (Southern Biotechnologies, Birmingham, AL, USA) for 1 h at 25 °C. After washing 4× with PBST, the plates were finally incubated with the AP substrate *p*-nitrophenylphosphate, and the OD of each well was measured at 405 nm in a microtiterplate reader.

The binding curves of T15 to *S. pneumoniae* R36a and other bacterial strains were drawn by 4-parameter logistic equations, and 50% effective concentrations (EC_{50}) of the reactions were calculated. The level of PC expression of each strain was expressed as percentage relative to the EC_{50} value of *S. pneumoniae* R36a.

The specificity of binding of T15 to bacterial cells was verified in a competitive ELISA using PC and its analogues, acetylcholine (ACh) and phosphorylethanolamine (PE). ELISA plate wells were coated with 10^7 bacterial cells per well and incubated with T15 (5 μg ml^{-1}) in the presence of various concentrations of PC and its analogues. After a 1 h incubation, unbound mAb was washed out with PBST, and T15 that had bound with the bacterial cells was detected using the same method as described above.

2.3. Animals and immunization

Specific pathogen-free, female BALB/c mice (CLEA Japan Inc., Tokyo, Japan) of 6- to 8-weeks old were injected intraperitoneally (i.p.) with bacterial cells (100 μg