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Inhibition by Yeast Killer Toxin-like Antibodies of Oral Streptococci Adhesion to Tooth Surfaces in an Ex Vivo Model

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Abstract

Background: Monoclonal (KTmAb) and recombinant (KTscFv) anti-idiotypic antibodies, representing the internal image of a yeast killer toxin, proved to be microbicidal in vitro against important eukaryotic and prokaryotic pathogens such as *Candida albicans*, *Pneumocystis carinii*, *Mycobacterium tuberculosis*, *Staphylococcus aureus*, *S. haemolyticus, Enterococcus faecalis*, *E. faecium,* and *Streptococcus pneumoniae*, including multidrug-resistant strains. KTmAb and KTscFv exerted a strong therapeutic effect in well-established animal models of candidiasis and pneumocystosis.

Streptococcus mutans is the most important etiologic agent of dental caries that might result from the metabolic end products of dental plaque. Effective strategies to reduce the disease potential of dental plaque have considered the possibility of using antibiotics or antibodies against oral streptococci in general and *S. mutans* in particular. In this study, the activity of KTmAb and KTscFv against *S. mutans* and the inhibition and reduction by KTmAb of dental colonization by *S. mutans* and other oral streptococci in an ex vivo model of human teeth were investigated.

Materials and Methods: KTscFv and KTmAb were used in a conventional colony forming unit (CFU) assay against a serotype C strain of *S. mutans*, and other oral streptococci (*S. intermedius*, *S. mitis*, *S. oralis*, *S. salivarius*). An ex vivo model of human teeth submerged in saliva was used to establish KTmAb potential of inhibiting or reducing the adhesion to dental surfaces by *S. mutans* and other oral streptococci.

Results: KTmAb and KTscFv kill in vitro *S. mutans* and other oral streptococci. KTmAb inhibit colonization of dental surfaces by *S. mutans* and oral streptococci in the ex vivo model.

Conclusions: Killer antibodies with antibiotic activity or their engineered derivatives may have a potential in the prevention of dental caries in vivo.

Introduction

Streptococcus mutans has long been considered to be the principal etiologic agent of dental caries (1–4). Caries may be induced by the metabolic end products of dental plaque. Dental plaque formation originates by the adherence of oral bacteria to the acquired pellicle from the saliva that coats the tooth's surface (5–12). Strategies to reduce the disease potential of dental plaque have hypothesized the use of antibiotics active against *S. mutans* in particular as well as other antimicrobial agents (13–16). Antibiotics that may be given orally or systemically for the prevention of caries or treatment of different infections may enter the oral cavity via saliva and gingival crevicular fluid and lead to an imbalance in the oral microbiota. Beside host side effects, however, prolonged treatments with antibiotics suppress the resident bacterial population resulting in induction and overgrowth of antibiotic-resistant bacteria and other opportunistic pathogens such as *Candida albicans*. Thus, prevention of caries based on antibiotic administration, even though effective in theory, is unfeasible for microbiological and economic reasons.

Secretory IgA antibodies (SIgA), the predominant Ig in whole saliva, are considered to be the main specific defense mechanism in the oral cavity (17). SIgA help to limit microbial adherence to epithelial cells and tooth surfaces. It is thought that naturally occurring SIgA may play an important role in the homeostasis of oral resident microbiota and in preventing caries (18,19). To gain an insight into the role of SIgA in the control of the resident oral microbiota, the potential of passive immunization has been considered. One of the most intriguing questions concerning the role of SIgA in oral microbial ecology is whether antibodies may have a direct effect on the indigenous oral microorganisms, and *S. mutans* in particular.

In a previous study, we showed that microbicidal SIgA, representing the internal image of a killer toxin produced by the yeast *Pichia anomala* (KT) characterized by its wide spectrum of antimicrobial activity, may be induced and detected in the secretions of animals and patients experimentally or naturally infected by microorganisms presenting specific KT cell wall receptors (KTR) (20). Notably,

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KT-like monoclonal (KTmAb) or recombinant in the single chain format (KTscFv) anti-idiotypic antibodies were produced against a KT-neutralizing mAb, which represents the internal image of KTR. KTmAb and KTscFv were shown to act as antibiotics exerting a microbicidal activity in vitro and being therapeutic in vivo in an animal model of mucosal infection (21,22).

In this study, we report that KTmAb and KTscFv are able to kill in vitro *S. mutans* and that administration of KTmAb in an ex vivo model of teeth submerged in saliva may prevent or reduce the colonization by *S. mutans* or other oral streptococci (*S. intermedius*, *S. mitis*, *S. oralis*, and *S. salivarius*) normally occurring in human saliva.

Materials and Methods

Bacterial Strains

In this study a reference strain of *S. mutans* (NCTC 10449) of serotype C, isolated from the dental plaque of a patient in Guy's Hospital in London, was used. Oral streptococci (*S. intermedius*, *S. mitis*, *S. oralis*, and *S. salivarius*) isolated from the saliva of a volunteer donor were also studied. The different bacterial colonies grown on Mitis Salivarius agar with 1% Bacto-Chapman Tellurite solution (Difco Laboratories, Detroit, MI, USA) were identified by the Vitek System (Gram Positive Identification Card, bioMérieux sa, Marcy l'Etoile, France).

Monoclonal and Recombinant KT-like Anti-idiotypic Antibodies

The KT-like anti-idiotypic rat IgM monoclonal antibody K10 (mAb K10) was obtained according to procedures described elsewhere (22). The secreting hybridoma was grown in RPMI 1640 medium (Sigma, St. Louis, MO, USA) with 15% fetal calf serum (Sigma). The supernatant of the hybridoma culture was partially purified by precipitation with ammonium sulfate and dialysis against phosphate-buffered saline (PBS). Antibody concentration was determined by capture ELISA by means of a pair of mouse monoclonal antibodies against μ heavy chain of rat Ig (LO-IMEX, Brussels, Belgium).

The KT-like anti-idiotypic recombinant antibody in the single chain format (scFv H6) was obtained according to the procedures previously described by using the commercial Recombinant Phage Antibody System (Pharmacia Biotech AB, Uppsala, Sweden) (21). The concentration of the affinity chromatography purified scFv H6 was determined by evaluating optical density at 280 nm.

Evaluation of the In Vitro Bactericidal Activity of mAb K10 and scFv H6 Against Streptococcus mutans

The in vitro microbicidal activity of mAb K10 and scFv H6 against the reference strain of *S. mutans* was evaluated by a conventional colony forming unit (CFU) assay. Briefly, the bacterial strain was grown on Muller Hinton (Difco) agar plates for 24 hr at 37°C in a 5% CO_2 atmosphere. Isolated colonies were used to prepare a bacterial suspension in sterile saline at an optical density of 0.5 McFarland that was diluted 1:2000. Ten microliters of the diluted suspension, containing approximately 2–3 $\times$ 10^2 viable bacterial cells, were added to vials containing: i) filter sterilized human saliva, collected from a healthy volunteer donor prior the use, ii) glucose (0.2%), and iii) 10 μg of mAb K10 or 10 μg of scFv H6, to a total volume of 100 μl. As a control, the same bacterial inoculum was treated, in the same conditions, with the same amount of an isotype-matched, commercially available murine IgM (Mouse IgM, kappa, ABPC 22, Sigma) or of an irrelevant scFv (23). The specificity of the killer activity of KT-like antibodies was ascertained by adding the same amount of bacterial cells to mAb K10 and scFv H6 preincubated overnight at 4°C with 100 μg of KT-neutralizing mAb KT4. After 5 hr of incubation at 37°C with the respective reagents, the bacterial cells were distributed in Petri dishes containing Muller Hinton agar. The plates were then incubated at 37°C for 48 hr and the number of colonies that grew out in each plate was recorded. Each test was carried out in triplicate and repeated for confirmation in independent experiments. The statistical significance was assessed by the two-tailed Student *t* test.

Evaluation in an Ex Vivo Model of Inhibition and Reduction of Dental Surface Streptococcal Colonization by mAb K10

The effect of inhibition and reduction of dental surface streptococcal colonization by mAb K10 was evaluated in an ex vivo experimental model utilizing healthy human teeth extracted for orthodontic needs and sterilized by autoclaving. Two different experimental protocols were performed using the reference *S. mutans* strain.

Two antagonist teeth obtained from the same donor, which were submerged in human sterile saliva (1 ml total volume) with 0.2% glucose, were exposed to an appropriate inoculum of *S. mutans* ($\sim$1 $\times$ 10^4 bacterial cells). In the first protocol, one was treated with 100 μg of mAb K10 and the other with the same amount of the commercial isotype-matched mAb as a control. These preparations were incubated at 37°C in a 5% CO_2 atmosphere. One hundred micrograms of mAb K10 or irrelevant commercial isotype-matched mAb were added to the relative vials after 24 and 48 hr. The evaluation of teeth colonization was carried out by determination of CFUs after 3 days of incubation. In the second protocol, one tooth was treated thrice with 100 μg of mAb K10 and the other with the same amount of the irrelevant mAb every 24 hr starting from the third day after exposure to *S. mutans* inoculum. The CFU assay was carried out 24 hr after the last treatment (after 6 days of incubation).

For the CFU assay, each tooth was carefully washed with sterile saline (10 ml) and then energetically brushed for 6 min using 6 ml of sterile saline.

All the operations were carried out under sterile conditions. One hundred microliters of appropriate dilutions of the cell suspensions obtained after brushing were inoculated onto the surface of Petri dishes containing Muller Hinton agar. After 48 hr of incubation at 37°C, the number of colonies grown in each plate was recorded. Each assay was carried out in triplicate for statistical purposes and repeated in independent experiments also by exchanging the antagonist human teeth with regard to their interaction with mAb K10 or irrelevant mAb, respectively.

The same experimental model was used for the evaluation of the inhibition by mAb K10 of the dental adhesion of oral streptococci physiologically occurring in human saliva. For this purpose, two antagonist teeth were submerged in human saliva diluted 1:10,000 in the same filter sterilized saliva, to which had been added glucose (0.2%), mAb K10 (100 μg), or an equal amount of the irrelevant mAb as a control. The teeth were incubated at 37°C in 5% CO_2 atmosphere and treated after 24 and 48 hr with additional amounts (100 μg) of mAb K10 and irrelevant mAb, respectively. The evaluation of teeth colonization was carried out on the third day of incubation by inoculating 100 μl of proper dilutions of the cell suspensions obtained after brushing, as previously described, onto the surface of Petri dishes containing Mitis Salivarius agar. After 48 hr of incubation at 37°C the colonies were counted. Each test was carried out in triplicate for statistical purposes and repeated in independent experiments for confirmation.

The in vitro bactericidal activity of mAb K10 against the oral streptococci isolated from dental surface was carried out, in comparison with the irrelevant mAb, by a CFU assay according to the procedure previously described for *S. mutans*.

Results

The in vitro activity of mAb K10 and scFv H6 against the reference *S. mutans* strain, expressed as percent inhibition in comparison with appropriate controls, was 91.2 ± 1.76% and 48.28 ± 9.39%, respectively (Fig. 1). In representative experiments, the CFU numbers were 112.55 ± 15.63 for mAb K10-treated and 1096.4 ± 45.68 for irrelevant mAb-treated bacterial cells, 169.8 ± 42.57 for scFv H6-treated and 323.72 ± 35.59 for irrelevant scFv-treated bacterial cells ($p < 0.05$). The bactericidal activity of mAb K10 and scFv H6 was proven by the lower CFU number of treated microorganisms in comparison with the number of viable cells constituting the inoculum. Significantly the bactericidal activity was reduced of more than 50% by mAb KT4.

MAb K10, used in the ex vivo model adopted in this study in comparison with the irrelevant mAb, significantly reduced the colonization of *S. mutans* on tooth surfaces. As demonstrated in the CFU assay carried out after tooth brushing, the percentage of

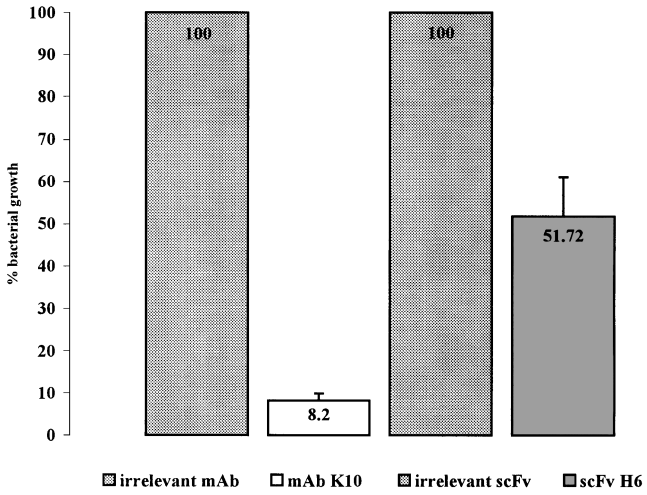

Fig. 1. In vitro bactericidal activity of yeast killer toxin-like monoclonal (mAb K10) and recombinant (scFv H6) anti-idiotypic antibodies against the *S. mutans* reference strain.

inhibition of bacterial growth was 99.95 ± 0.02 (i.e., in a representative experiment 30 ± 21.21 CFU/ml [mAb K10-treated] versus 65.7 ± 7.39 × 10^3 CFU/ml [control]) ($p = 0.05$) (Fig. 2A).

In the same model, a significant reduction of the dental colonization by *S. mutans* was also demonstrated following three treatments by mAb K10 at 24-hr intervals starting 3 days after bacterial inoculum. Under these experimental conditions, the percentage of inhibition determined by the CFU assay in comparison with the irrelevant mAb proved to be 93.53 ± 0.27 (in a representative experiment mAb K10-treated CFU 119.99 ± 9.43 × 10^3/ml, control CFU 1936.66 ± 42.43 × 10^3/ml; $p < 0.01$) (Fig. 2B).

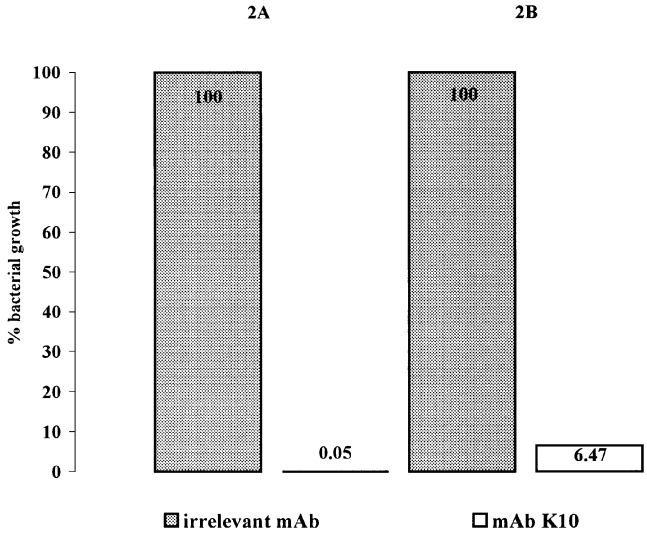

Fig. 2. Inhibition **(A)** and reduction **(B)** of *S. mutans* colonization of human teeth in the ex vivo model by yeast killer toxin-like monoclonal anti-idiotypic antibody (mAb K10).

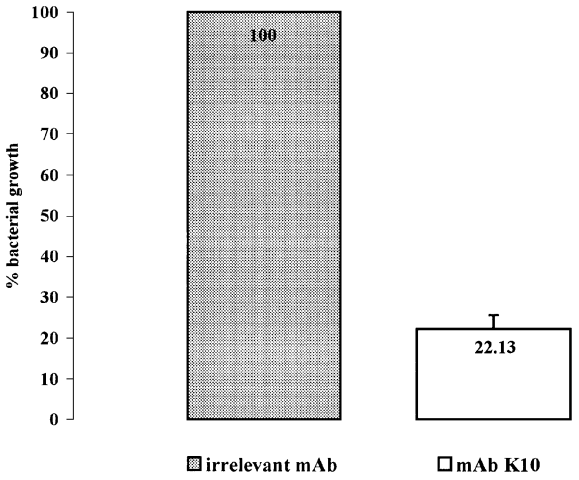

Fig. 3. Inhibition of oral streptococci colonization of human teeth in the ex vivo model by yeast killer toxin-like monoclonal anti-idiotypic antibody (mAb K10).

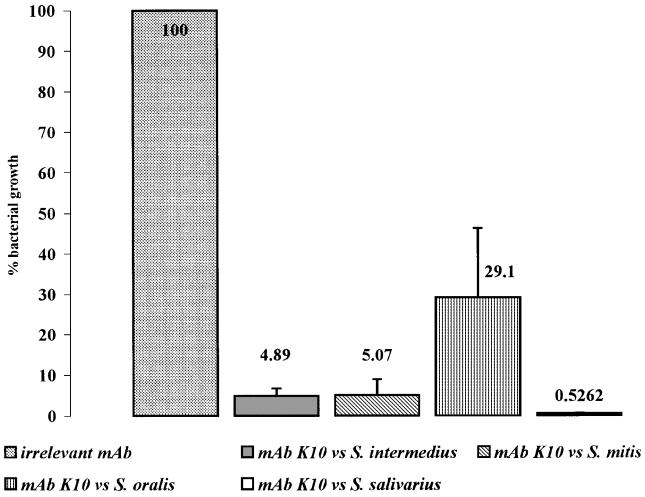

Fig. 4. In vitro bactericidal activity of yeast killer toxin-like monoclonal anti-idiotypic antibody (mAb K10) against human oral streptococci.

Moreover, mAb K10 significantly inhibited adhesion to the surface of the teeth by oral streptococci occurring in a sample of physiologic human saliva. The reduction of bacterial colonization was 77.87 ± 3.37% (in a representative experiment mAb K10-treated CFU 14.95 ± 3.46 × 10^3/ml, control CFU 75.63 ± 4.50 × 10^3/ml; $p < 0.01$) (Fig. 3).

The streptococcal colonies grown on the selective medium were identified as *S. intermedius*, *S. mitis*, *S. oralis,* and *S. salivarius*. The in vitro bactericidal activity of mAb K10 evaluated by the CFU assay as percent reduction in comparison with the irrelevant mAb proved to be 95.11 ± 1.85 against *S. intermedius* (in a representative experiment mAb K10-treated CFU 91.66 ± 28.43, control CFU 3024.17 ± 1195.18; $p = 0.05$), 94.92 ± 3.83 against *S. mitis* (in a representative experiment mAb K10-treated CFU 283.33 ± 103.24, control CFU 22830.83 ± 5788.34; $p < 0.05$), 70.90 ± 17.16 against *S. oralis* (in a representative experiment mAb K10-treated CFU 61.66 ± 32.53, control CFU 516.66 ± 196.55; $p < 0.05$), and 99.47 ± 0.11 against *S. salivarius* (in a representative experiment mAb K10-treated CFU 51.67 ± 5.77, control CFU 10621.67 ± 4169.66; $p < 0.05$) (Fig. 4).

Discussion

The initial step of dental plaque formation is represented by the adhesion of bacteria to the acquired salivary pellicle that physiologically coats the surface of teeth (24,25). This process involves hydrophobic and ionic bonds as well as interactions similar to those exerted by lectins and occurring between bacterial adhesins and receptors sterically complementary on host surfaces (26,27). Efficient adhesins with different specificities have been identified on the cell wall of *S. mutans*, the main causative agent of caries (1). Promising approaches to the prophylaxis of dental caries have been based on the inhibition of adhesion to hydroxyapatite and colonization of tooth surfaces by *S. mutans*. This goal may be achieved by using compounds such as poly-L-aspartic acid and poly-L-glutamic acid, cellulose ethers that may modify the surface of hydroxyapatite thus reducing bacterial colonization (28–30). For reduction of dental plaque, chitosan or modified chitosans have proven to be potentially effective displaying several protective effects. They may buffer the oral pH to prevent the damage caused by acids to tooth enamel and are also able to stimulate the ordered regeneration of the oral cavity's soft tissues (31–33). It has been demonstrated, moreover, that they exert bactericidal activity against different oral microbial pathogens including *S. mutans* (34). It is recognized that subinhibitory concentrations of some antibacterial compounds may interfere with the expression and production of bacterial adhesins, thus conditioning adhesion and the subsequent colonization of host tissues (35). Considering that subinhibitory concentrations of chitosan may be easily achieved in the oral cavity by using appropriate toothpastes and mouthwashes containing such carbohydrate, studies have been carried out on the capacity of sublethal concentrations of chitosan to reduce the adhesion of *S. mutans* to hydroxyapatite (36). Of particular value for prophylactic and therapeutic applications in the oral cavity may be considered the use of chlorhexidine as mouth rinse, or in form of gels or varnishes (37). Alternative strategies have been envisaged to reduce the potential pathogenicity of dental plaque by using antibiotics or immunoglobulins directed against *S. mutans* in particular (15,16,19). The problems intrinsically related to the use of these prophylactic agents are referable to the selection of resistant mutants for the antibiotics and the difficulty to individualize prejudicial cellular targets for SIgA owing to the multifactorial mechanisms of adherence of oral bacteria.

In this study, we present a comprehensive conceptually new approach involving immunoglobulins characterized by their antibiotic activity. Our results clearly show a marked bactericidal effect exerted, in vitro, by mAb K10 and scFv H6 against *S. mutans*. Significantly, their effect was neutralized by the anti-KT mAb KT4, thus attesting to the specificity of their biological activity. The potentiality of KT-like immunologic derivatives, such as mAb K10 or its engineered products, for the reduction of dental plaque by *S. mutans* or other oral streptococci has been corroborated in the ex vivo model of infection utilizing human teeth submerged in human saliva.

When the teeth had been repeatedly treated with mAb K10 in conjunction with or after experimental infection with *S. mutans*, dental colonization showed a dramatic reduction, as demonstrated by the CFU assays carried out following vigorous brushing. The results on the inhibition of streptococcal colonization obtained by using, in the same ex vivo model, human physiologic saliva, suggest that this approach warrants further clinical investigations.

The opportunity to obtain synthetic killer mimotopes derived from the sequence of the variable region of scFV H6, moreover, could make available economical, stable, and safe therapeutic agents of clinical interest in oral pathology.

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