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Technetium-99m labeling and fibronectin binding ability of Corynebacterium diphtheriae

Marcação de Corynebacterium diphtheriae com Tecnécio-99m e avaliação da capacidade de ligação à fibronectina de plasma humano

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Abstract
The use of radionuclides has permitted advances in areas of clinical and scientific knowledge. Several molecules and cells have been labelled with Technetium-99m (^{99m}Tc). The stannous chloride ($SnCl_2$) has a significant influence on the labeling and stability of ^{99m}Tc radiotracers. The frequent risk of diphtheria epidemics has intensified interest in the virulence factors of *Corynebacterium diphtheriae.* Although studies have looked at potential adhesins including haemagglutinins and exposed sugar residues, the molecular basis of mechanisms of adherence remains unclear. Adherence of pathogens to mammalian tissues may be mediated by fibronectin (FN) found in body fluids, matrix of connective tissues, and cell surfaces. In the present study we evaluated the binding ability to human plasma FN by ^{99m}Tc labeled-*C.diphtheriae*. Due to adverse effects of stannous ions, microorganisms were submitted to survival and filamentation induction assays. Data showed a dose dependent susceptibility to $SnCl_2$ bactericidal effects. Cell filamentation was observed for concentrations of $SnCl_2 \geq 110$ μg/ml. Adherence levels of ^{99m}Tc labelled 241 strain to coverslips coated with 20 μg/ml FN were higher ($P = 0.0037$) than coated with bovine serum albumin. FN binding by the sucrose fermenting 241 *C. diphtheriae* strain ($8.9\% \pm 2.6$) was significantly lower ($P=0.0139$) than *Staphylococcus aureus* Cowan I strain ($34.1\% \pm 1.2$). Therefore, bacterial ^{99m}Tc labeling represents an additional tool that may contribute to the comprehension of *C. diphtheriae* interactions with host receptors such as FN that act as biological organizers by holding bacterial cells in position and guiding their migration.
***Keywords*:** *Corynebacterium diphtheriae,* Human plasma Fibronectin, Microbial Labeling, Stannous Chloride, Technetium.

Resumo
O uso de radionuclídeos permitiu avanços em áreas de conhecimento clínico e científico. Diversas células e moléculas foram marcadas com o nuclídeo Technetium-99m (^{99m}Tc). O cloreto de estanho ($SnCl_2$) exerce significativa influência no processo de marcação e na estabilidade do radiotraçador ^{99m}Tc. O risco de pandemia de difteria intensificou o interesse relativo aos fatores de virulência do Corynebacterium diphtheriae. Hemaglutininas e resíduos de açúcar expostos na superfície bacteriana parecem contribuir para a aderência do bacilo diftérico a substratos diversos. Entretanto, os aspectos moleculares do mecanismo de aderên-

cia ainda não foram totalmente esclarecidos. A aderência de diversos patógenos aos tecidos de mamíferos pode ser mediada por moléculas de fibronectina (FN) encontradas nos fluídos orgânicos, matriz de tecidos conjuntivos, e nas superfícies celulares. No presente estudo foi avaliada a eficiência de marcação do C. diphtheriae pelo ^{99m}Tc e a capacidade de interação com FN plasmática humana. Ensaios de viabilidade e filamentação celular foram realizados para investigar os efeitos adversos do íon estanho. O efeito bactericida foi proporcional a dose utilizada de $SnCl_2$. A indução de filamentação celular só ocorreu nos ensaios realizados com concentrações de $SnCl_2 \geq 110$ μg/ml. A amostra 241 marcada com ^{99m}Tc foi capaz d'e aderir mais avidamente às lamínulas de vidro recobertas com 20 μg/ml FN (P=0.0037) do que com albumina de soro bovino. A capacidade de aderência a FN plasmática da amostra 241 fermentadora de sacarose de C. diphtheriae (8.9% + 2.6) foi significativamente menor (P= 0.0139) quando comparado com a da amostra Staphylococcus aureus Cowan I (34.1% + 1.2). A marcação bacteriana pelo ^{99m}Tc pode ser utilizada como ferramenta adicional nos ensaios de interação do C. diphtheriae com moléculas receptoras humanas como a FN, que atuam como organizadores biológicos capazes de permitir a fixação bacteriana em determinados sítios e orientar a disseminação pelos tecidos do hospedeiro.

Palavras-chave: *Corynebacterium diphtheriae, Cloreto estanoso, Fibronectina de plasma humano, Marcação bacteriana, Tecnécio.*

Introduction

The labeling of microorganisms with radionuclides has many applications in biological research, including quantitation of microbial clearance and adherence to host cells. Several radionuclides with different physical properties have been used to label microorganisms (Kishore, 1981). Gamma emitter isotopes present some advantages over beta emitters as radioisotopic microbial labels especially due to short half-lives that cause less environmental impact and fewer disposal problems than do long-lived isotopes. However, only a few studies have been reported about radiolabeling of bacteria with gamma emitters (Perin *et al.*, 1997) such as ^{99m}Technetium (^{99m}Tc). Bacterial labeling with ^{99m}Tc seems to be a very promising tool for quantifying bacteria and analyzing their functional properties ^{99m}Tc has been used to label gram-negative species including *Klebsiella pneumoniae* (Bernardo-Filho *et al*., 1986), *Pseudomonas aeruginosa* (Plotkowski *et al*., 1987), *Escherichia coli* (Arden *et al*., 1993) and *Salmonella abortusovis* (Perin *et al*., 1997).

The ^{99m}Tc fixation depends on reducing conditions frequently obtained by the use of stannous chloride ($SnCl_2$). The pertechnetate ion is efficiently incorporated by bacterial cells after reducing from its +7 state to a lower valence. The concentrations of stannnous ion have a marked effect on *in vitro* labeling efficiency of cells, and it has been shown that optimum yields are achieved with concentrations of 0.03-0.15 μg/ml. Any deviation from this range is likely to cause a decrease in labeling efficiency of up to 20%. The cells take up the tin of the $SnCl_2$ solution and the reduction of the pertechnetate takes place on or in the cells. When ^{99m}Tc is added to the cellular suspension already in the reduced form, it is not capable of passing through the cell membrane. (Bernardo-Filho *et al.*, 1994; Braga *et al.*, 2000).

The survival fraction of the culture is not substantially altered by the presence of stannous ion and ^{99m}Tc generally used in the described technique (Bernardo-Filho *et al.*, 1986; Plotkowski *et al.*, 1987). The ^{99m}Tc labeling process did not modify microbial physicochemical properties of *P. aeruginosa* such as surface charge, hydrophobicity, adherence to buccal epithelial cells and phagocytosis by human leucocytes (Plotkowski *et al.*, 1987). Inhibitory effect on growth of *Staphylococcus epidermidis* and *K. pneumoniae* was observed only with ^{99m}Tc activities above 250 mCi (Stathis *et al.*, 1983; Bernardo-Filho *et al.*, 1986). Stannous ion, as a chloride salt, can exert a genotoxic effect by inducing and/or producing DNA lesions (Bernardo-Filho *et al.*, 1994; Souza *et al.*, 2003). Previous studies with *E. coli* suggested the participation of reactive oxygen species (ROS), in the lethal effect induced by $SnCl_2$ (Dantas *et al.*, 1996).

Although diphtheria is thought to be declining in Brazil, the disease remains endemic in most states through the last two decades with a case-fatality range of 5% to 10% (Funasa, 2002; Mattos-Guaraldi *et al*., 2003). Changes in the circulating toxigenic *C. diphtheriae* strains may be responsible for episodic diphtheria epidemic waves. However, the microbial factors that distinguish epidemic from endemic diphtheria bacilli have not been identified. Immunized patients may also develop bacteremia and endocarditis due to *C. diphtheriae* (Mattos-Guaraldi *et al*., 1998). Although studies have looked at different potential adhesins of both sucrose fermenting and non-fermenting biotypes (Mattos-Guaraldi *et al*., 2000; Hirata Jr *et al*., 2002), further characterization of adherence mechanisms and function of adhesins are necessary for a better understanding of the pathogenicity of diphtheria bacilli.

Fibronectin (FN) is ubiquitous mammalian glycoprotein with diverse functions which binds to certain bacteria but not to others. Microbial adhesion to FN correlated closely with the propensity of bacterial species to produce endocarditis. Microorganisms with a high isolation frequency from endocarditis cases

(*S. aureus*) bound significantly better to FN *in vitro* than others (*P. aeruginosa*) rarely implicated in this disease (Scheld *et al*., 1985; Stanislawski and Sorin, 1991). The influence of FN in the pathogenesis of diphtheria and in invasive *C. diphtheriae* infections have not been established.

We report here the validation of the ^{99m}Tc-labeling technique applied to *C. diphtheriae* and bacterial binding activity to immobilized plasmatic FN.

Materials and Methods

Bacterial strains and growth conditions

Toxigenic sucrose fermenting *C. diphtheriae* 241 strain isolated from throat of diphtheria patient previously identified in Laboratório de Difteria e Corinebacterioses, Faculdade de Ciências Médicas, UERJ, Rio de Janeiro, RJ, Brazil, and *Staphylococcus aureus* Cowan I (ATCC 12598) positive control strain (Vercellotti *et al.*,1984) were used in this study. Stock cultures were maintained in 10% skim milk containing 25% glycerol at -80 ºC. Microorganisms were previously grown in Luria Bertani medium (LB; Difco Lab., Detroit, MI, USA) for 24 / 48h at 37 °C, washed twice and resuspended at a concentration of 1-2 x 10^8 bacteria/ml in 0.9 % NaCl solution.

Reagents

Solutions of the stannous chloride reducing agent ($SnCl_2$; Sigma Chemical Co., USA) were prepared in deionized ultra pure water to minimize metal contamination. As the $SnCl_2$ in solution is rapidly oxidized, it was freshly prepared in vacuum tubes for each assay. The ^{99m}Tc solution (sodium pertechnetate) was obtained from a ^{99}Mo^{99}Tc generator (IPEN-TEC, Brazil) and ^{99m}Tc activity was determined in scintillation counter with sodium-iodide-thallium crystal (Clinigamma, gamma counter, LKB, Wallac, Finland).

Survival experiments

Bacterial suspensions were incubated with $SnCl_2$ solution in order to reach 0.11µg/ml, 11µg/ml, 44 µg/ml, 110 µg/ml and 220 µg/ml concentrations. Aliquots of bacterial suspensions collected immediately after addition of $SnCl_2$ were diluted and plated on LB agar for determination of viable cells/ml (CFU/ml) (Bernardo-Filho *et al.,* 1994).

Filamentation induction assays

Exponentially growing cultures in LB medium subjected to various $SnCl_2$ concentrations were observed for morphological aspects by light microscopy at 1000 X magnitude (Bernardo-Filho *et al.,* 1994).

Bacterial labeling

Bacterial labeling procedures were based on methods previously described (Plotkowski *et al.,* 1987). In each vacuum tube, 2.5 ml of bacterial suspensions were added of 0.5 ml $SnCl_2$ solution in order to provide final concentration of 0.10 µg/ml). After incubation for 1 h at 37^{0}C, ^{99m}Tc solution prepared in saline was added to $SnCl_2$-treated bacterial suspensions in order to provide 4 mCi/ml final activity. After incubation 10 min at 37^{0}C, ^{99}Tc-labelled bacteria were harvested by centrifugation, washed twice and resuspended in saline for FN-binding assay.

Binding of ^{99}Tc-labeled bacteria to immobilized FN

Human plasma FN (Sigma) was diluted in phosphate buffered saline 0.01M, pH 7.2, to 5, 10 and 20 µg/ml. Thermanox coverslips (13mm diammeter) coated with 50 ml FN were incubated overnight at 4^{0}C. FN coated coverslips were washed three times and reincubated with PBS-1% bovine serum albumin (BSA; fraction V, Sigma) for 2 h at 22^{0}C. Fifty microlitters of ^{99m}Tc labeled-bacterial suspensions were dispensed over FN coated coverslips and incubated 1 h at 37ºC. Coverslips were washed three times for radioactivity determination. Percentage of radioactivity (% ATI) was calculated as previously described (Braga *et al.,* 2000). Binding experiments were also done in the presence of 20 mg/ml BSA.

Statistical analysis

Values were expressed as mean plus standard deviation of four experiments. Student's t test was used to compare the adherence of strains to different substrata and F test was used to analyze variance between bacterial species.

Results and Discussion

Radionuclides represent an additional tool in biomedical sciences and have contributed to the comprehension of many biological phenomena. Considering the radionuclides that are employed to label biomolecules and cells, ^{99m}Tc has characteristics that justifies its increasing use, such as 6h half-life, low radiation dose emitted by decay, low cost and relatively simple handling of waste disposables. Although $SnCl_2$ is widely used as reducing agent in the ^{99m} Tc-labeling process, some deleterious effects of this substance have been described.

Results of investigations on bactericidal effects of stannous ion to *C. diphtheriae* are presented in Figure 1. Initial number of viable cells (1.4 x 10^8 CFU/ml) was reduced to 1.1 x 10^7 CFU/ml) when used 44 μg/ml $SnCl_2$ ($P < 0.001$). However, microscopic examination of 241 bacterial cells submitted to different concentrations of $SnCl_2$ demonstrated cellular filamentation only within 110 and 220 μg/ml $SnCl_2$ (data not shown).

The pre-tinning step followed by incubation of bacterial suspension with ^{99m}Tc previously used in direct labeling of *P. aeruginosa* cells (Plotkowski *et al.*, 1987) was found to be effective for *C. diphtheriae*. Data demonstrated adherence of ^{99m}Tc labeled *C. diphtheriae* to immobilized plasmatic FN (Figures 2 and 3). In Figure 2, results indicated 6.53% ± 0.78 , 7.53% ± 1.02 and 8.9% ± 0.97 adherence of 241 strain to 5, 10 and 20 μg/ml FN, respectively. Results in Figure 3 showed that adhesion of ^{99m}Tc labeled-241 strain to coverslips coated with 20 μg/ml FN was significantly higher ($P=0.0037$) than coverslips coated with ^{99m}Tc labeled-bovine serum albumin (BSA). However, the adhesion of ^{99m}Tc labeled-*C. diphtheriae* to 20 μg ml^{-1} FN (8.9% ± 2.6) was significantly lower ($P = 0.0139$) than the ^{99m}Tc labeled-*Staphylococcus aureus* Cowan I control strain (34.1% ± 1.2).

Naturally occurring mediators of bacterial adherence, such as wound fluid, serum and fibronectin may exert effects on the adherence of diphtheria bacilli to cutaneous-mucosal surfaces. The adherence of bacterial pathogen to wounded skin may be an essential step in *C. diphtheriae* skin infections. The ability to identify what affects the binding of bacteria to wounded surfaces can lead to a better understanding of wound infection (Mertz *et al.*, 1987; Romero-Steiner *et al.*, 1990). FN may favor bacterial adhesion by establishing a bridge between bacteria and epithelial cell receptors, especially during infection of healing injured epithelium (Plotkowski *et al.*, 1992).

C. diphtheriae was found to adhere to glass surfaces coated with FN as previously observed with *S. aureus* (Vercellotti *et al.*, 1984) and *P. aeruginosa* (Mohammad *et al.*, 1988). It was noted that a greater number of bacteria adhered to glass surfaces coated with FN whereas surfaces treated with BSA showed reduced bacterial adhesion. Adhesive properties to inert surfaces had been related with carbohydrate production of microorganisms, including corynebacteria species (Christensen *et al.*, 1982; Bayston *et al.*, 1994). *C. diphtheriae* were shown to be encased in an extracellular matrix when subjected to electron mi-

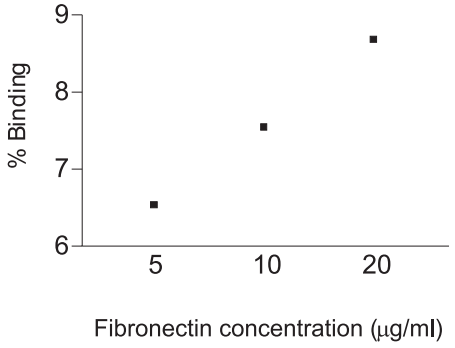

Figure 2. Percentage of adherence of ^{99m}Tc-labeled *C. diphtheriae* 241 to human plasma fibronectin at different concentrations. Each value represents the mean of four samples.

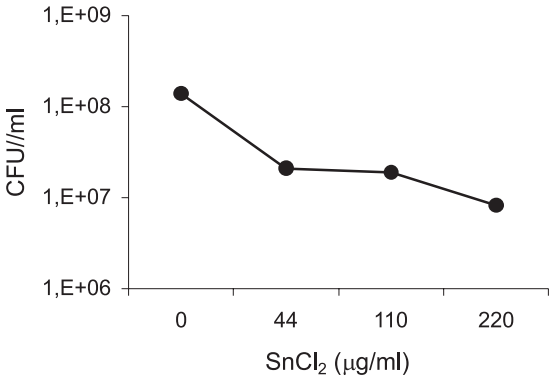

Figure 1. Surviving fractions of *C. diphtheriae* 241 strain submitted to varied concentrations of $SnCl_2$.Values are means of four isolated experiments. Standard deviation did not exceed 15%.

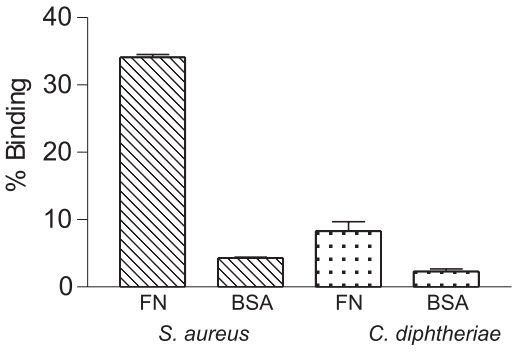

Figure 3. Percentage of adherence to human plasma fibronectin (FN) and bovine serum albumin (BSA) of *C. diphtheriae* 241 and *Staphylococcus aureus* Cowan I strains submitted to ^{99m}Tc- labeling. Each value represents the mean ± SD of four samples.

croscopy (Reyn *et al.,* 1966). Differences in degrees of adherence to glass were related to the expression of saccharides on surfaces of *C. diphtheriae* cells (Mattos-Guaraldi *et al.,* 1999). Bacterial adherence to biomaterials can be significantly influenced by the composition of the adsorbed proteins at the interface. These observations suggest a potential ability of *C. diphtheriae* to develop biofilms as demonstrated with other human pathogens such as *S. aureus* and *P. aeruginosa* (Donlan, 2002).

Native valve endocarditis is mainly caused by biofilm associated microorganisms ((Vercellotti *et al*., 1984; Scheld *et al*., 1985). Plasma hyperfibronectinemia may be manifested during bacterial sepsis (Kiener *et al.,* 1986). Microbial adhesion to the constituents of non-bacterial thrombotic endocarditis (NBTE) was described as an important early event in the pathogenesis of infective endocarditis. FN, expressed on the surface of NBTE, may mediate adherence of circulating microorganisms and favor bacterial colonization during the early pathogenesis of infective endocarditis (Scheld *et al*., 1985). Data suggest that FN-binding properties may also corroborate to development of sepsis and endocarditis by *C. diphtheriae*. Additional studies are necessary in order to demonstrate if microbial adhesion to FN *in vitro* correlates with production of *C. diphtheriae* endocarditis in animal models. ^{99m}Tc labeling of whole cell or surface components of bacteria represents an additional tool during investigations *in vivo* and *in vitro* focused on the effects of adherence on pathogenesis of *C. diphtheriae* infections.

Conclusion

In addition to the validation of the ^{99m}Tc-labeling technique applied to diphtheria bacilli, the present research also demonstrated reactivity of *C. diphtheriae* to immobilized human plasma FN.

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