Cytotoxicity of chlorhexidine digluconate to murine macrophages and its effect on hydrogen peroxide and nitric oxide induction

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Abstract

Chlorhexidine, even at low concentrations, is toxic for a variety of eukaryotic cells; however, its effects on host immune cells are not well known. We evaluated in vitro chlorhexidine-induced cytotoxicity and its effects on reactive oxygen/nitrogen intermediate induction by murine peritoneal macrophages. Thioglycollate-induced cells were obtained from Swiss mice by peritoneal lavage with 5 ml of 10 mM phosphate-buffered saline, washed twice and resuspended (10⁶ cells/ml) in appropriate medium for each test. Cell preparations contained more than 95% macrophages. The cytotoxicity was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide assay and the presence of hydrogen peroxide (H₂O₂) and nitric oxide (NO) by the horseradish peroxidase-dependent oxidation of phenol red and Griess reaction, respectively. The midpoint cytotoxicity values for 1- and 24-h exposures were 61.12 ± 2.46 and 21.22 ± 2.44 µg/ml, respectively. Chlorhexidine did not induce synthesis or liberation of reactive oxygen/nitrogen intermediates. When macrophages were treated with various sub-toxic doses for 1 h (1, 5, 10, and 20 µg/ml) and 24 h (0.5, 1, and 5 µg/ml) and stimulated with 200 nM phorbol myristate acetate (PMA) solution, the H₂O₂ production was not altered; however, the NO production induced by 10 µg/ml lipopolysaccharide (LPS) solution varied from 14.47 ± 1.46 to 22.35 ± 1.94 µmol/l and 13.50 ± 1.42 to 20.44 ± 1.40 µmol/l (N = 5). The results showed that chlorhexidine has no immunostimulating activity and sub-toxic concentrations did not affect the response of macrophages to the soluble stimulus PMA but can interfere with the receptor-dependent stimulus LPS.

Introduction

Chlorhexidine is an antibacterial agent widely used for skin disinfection, preoperative whole-body disinfection, and prevention and treatment of oral disease because of its broad-spectrum efficacy, retention by the skin and mucosa, and low risk of allergic complications (1). In recent years, vaginal washing with this compound before or during delivery has been suggested to prevent the development of both maternal and neo-
Studies have shown that small amounts of chlorhexidine are absorbed through the skin and vaginal mucosa (3,5) and that, even at low concentrations, this compound has toxic effects on a variety of eukaryotic cells (6). However, these effects on host immune cells are not well known.

Macrophages are widely distributed in different tissues and play an essential role in the development of the specific and nonspecific immune response. These cells can be activated by a variety of stimuli such as bacterial components, cytokines and chemicals. Once activated, macrophages produce and release numerous secretory products including several cytokines, inorganic reactive radicals, reactive oxygen intermediates (ROI) and reactive nitrogen intermediates (RNI), all having biological activity (7). Hydrogen peroxide (H2O2) and nitric oxide (NO) are important in cell signaling and are effector molecules for the microbicidal and cytotoxic response of macrophages after stimulation (8). If ROI and RNI may be considered as beneficial intermediates (with respect to their microbicidal and tumoricidal activities), they can also become destructive for the host tissue under certain conditions (9). There is insufficient information about the effect of chlorhexidine on macrophage function.

The purposes of the present study were to determine in vitro the cytotoxicity of chlorhexidine digluconate to murine peritoneal macrophages and investigate whether this compound induces the production of ROI and RNI as well as to determine if chlorhexidine treatment affects macrophage functions in the presence of stimulation with phorbol myristate acetate (PMA) and lipopolysaccharide (LPS).

Material and Methods

Chemicals

Chlorhexidine digluconate was obtained from Henri Pharma Produtos Farmacêuticos (São Paulo, SP, Brazil). Dextrose, horseradish peroxidase (HRP), LPS, penicillin, phenol red, PMA, RPMI-1640 and streptomycin were purchased from Sigma (St. Louis, MO, USA). Brewer thioglycollate medium was purchased from Difco (Detroit, MI, USA). Fetal bovine serum (FBS) was obtained from Cultilab (Campinas, SP, Brazil). 2-Mercaptoethanol, sodium hydroxide and sodium chloride were purchased from Merck (Darmstadt, Germany).

Animals

Swiss mice (6-8 weeks old, weighing 18 to 25 g), supplied by the Faculdade de Ciências Farmacêuticas, Universidade Estadual Paulista, Araraquara, SP, Brazil, were maintained in a polycarbonate box (at 23 ± 1°C, 55 ± 5% humidity, 10-18 circulations/h and a 12-h light/dark cycle), with water and food available ad libitum.

Peritoneal macrophages

Resident and thioglycollate-elicited peritoneal exudate cells were obtained from mice following intraperitoneal injection of 3 ml thioglycollate medium (3.0 g/100 ml) and lavage of the peritoneal cavity with 5 ml of 10 mM phosphate-buffered saline (PBS), pH 7.2, 3-4 days later. The proportion of macrophages in the peritoneal exudate was determined by cell staining with May-Grünwald-Giemsa. Cell preparations contained more than 95% macrophages. The cells were washed twice with PBS and resuspended in appropriate medium for each test.

Cytotoxicity assay

Macrophages (2 x 10⁶ cells/ml) were suspended in RPMI-1640 containing 5% heat-inactivated FBS, 100 IU/ml penicillin, 100 µg/ml streptomycin and 50 mM 2-mercaptoethanol. The suspension (100 µl) was added
to each well of a 96-well tissue culture plate and the cells were incubated at 37°C in a humidified atmosphere containing 5% CO₂. After 1 h, the wells were washed and adhering cells exposed to different concentrations of chlorhexidine for 1 and 24 h. The test was accompanied by a viability positive control (medium + cells) and negative control (medium + chlorhexidine). Finally, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay (10) was performed and the absorbance at 540 nm determined with a microplate reader (Spectra III Classic, Tecan Austria Ges.m.b.H, Salzburg, Austria.

**Hydrogen peroxide**

H₂O₂ was measured by the HRP-dependent oxidation of phenol red (11). Macrophages (2 x 10⁶ cells/ml) were suspended in 10 mM potassium phosphate buffer containing 140 mM NaCl, 5.5 mM dextrose, 0.56 mM phenol red and 0.01 mg/ml HRP, pH 7.4. Briefly, 100 µl of this suspension was added to each well of a 96-well tissue culture plate and exposed to different concentrations of chlorhexidine for 1 h (time for the H₂O₂ assay) at 37°C in a 5% CO₂ atmosphere. The reaction was stopped by the addition of 10 µl of 1 N NaOH and the absorbances were read at 620 nm. The results are reported as nmol/ml calculated from a hydrogen peroxide standard curve. The test was also performed with macrophages previously treated with sub-toxic concentrations of chlorhexidine for 1 and 24 h, washed twice with RPMI-1640 and stimulated with LPS. Each experiment was accompanied by a positive control (medium + cells + 10 µg/ml LPS solution) and a negative control (medium + cells).

**Statistical analysis**

The midpoint cytotoxicity value, the concentration of chemical agent needed to reduce the spectrophotometric absorbance to 50%, was determined by linear regression analysis with 95% confidence limits. H₂O₂ and NO concentrations are reported as means ± SD. Statistical difference between groups was determined by one-way analysis of variance (ANOVA; P < 0.05). All experiments were carried out in triplicate and repeated at least three times.

**Results**

**Effect of chlorhexidine on cell viability**

Concentration-response cytotoxicity
curves for 1- and 24-h exposures of macrophages to chlorhexidine are shown in Figure 1. Initial toxicity, i.e., a statistically significant reduction in the viability of chlorhexidine-exposed cells relative to unexposed controls, occurred at >20 µg/ml chlorhexidine with 1-h exposure and at >5 µg/ml with 24-h exposure. Concentrations below these were designated as sub-toxic. The midpoint cytotoxicity values were 61.12 ± 2.46 and 21.22 ± 2.44 µg/ml.

**Effect of chlorhexidine on H$_2$O$_2$ and NO induction**

To evaluate the ability of chlorhexidine to induce the production and liberation of ROI and RNI, murine macrophages were exposed to various concentrations of the test substance for 1 and 24 h (time for the H$_2$O$_2$ and NO assay, respectively). At none of the concentrations tested did chlorhexidine induce the production of H$_2$O$_2$ or NO by macrophages (Table 1).

**Production of H$_2$O$_2$ and NO by chlorhexidine-exposed macrophages**

To determine the effect of chlorhexidine on H$_2$O$_2$ and NO production by macrophages, these cells were treated with sub-toxic concentrations of chlorhexidine for 1 and 24 h and stimulated with both PMA and LPS. Table 2 shows that the H$_2$O$_2$ production was not altered in macrophages exposed to chlorhexidine for 1 and 24 h but the NO production induced by LPS was reduced when macrophages were exposed to sub-toxic doses of chlorhexidine (Figure 2).

**Discussion**

Macrophages have been used for immunocytotoxicity testing because they permit the measurement of the cytotoxic response directly in the cell culture and because of their ability to maintain immunological functions in the presence of many different chemical agents (13). The cytotoxicity of chlorhexidine has been assayed using cell lines or primary cultures of mammalian cells (6); however, there is no specific information on the toxicity of this substance to immune cells. In the present study, we demonstrated that macrophages are as sensitive to the toxic...
effects of chlorhexidine as other cell types and that the cytotoxic potential of the substance is dependent on the concentration and time of exposure (14). It is important to note that chlorhexidine is toxic to macrophages in vitro at concentrations 100 times below those used in clinical practice, usually 0.2-0.5% (15).

In response to antigens or inflammatory signals generated at sites of tissue injury, macrophages undergo a process of cellular “activation” which is associated with morphological, functional, and biochemical changes in the cells. One prominent characteristic of activated macrophages is their increased capacity to release pro-inflammatory and cytotoxic mediators, which aid in antigen destruction (16).

The present data show that chlorhexidine does not induce the production of ROI/RNI by macrophages. When macrophages were treated with chlorhexidine (sub-toxic concentrations) for 1 and 24 h and stimulated with PMA there was no alteration in H$_2$O$_2$ production. However, NO production induced by LPS was decreased in macrophages previously treated with sub-toxic doses of this substance. These observations are probably relevant to the mechanism underlying chlorhexidine-induced cytotoxicity.

Chlorhexidine may adversely affect the eukaryotic plasma membrane by nonspecific electrostatic binding to negative moieties (protein and phospholipid components), causing alteration in the structure of the cell membrane and in the osmotic equilibrium of the cell (6,14). PMA is a soluble stimulus that acts directly on protein kinase C (present in cytoplasm) activating the production of the superoxide anion, which is subsequently converted to H$_2$O$_2$ (17). PMA-induced stimulation can be mediated by specific receptors, and also via insertion into the membrane due to its hydrophobic nature (18). Probably, chlorhexidine does not prevent the entry of PMA into the cell, since this substance also acts by a receptor-independ-

tent mechanism.

Several types of receptors have been reported to be present on the macrophage surface. The inhibitory effect of several chemicals (ligands) on NO production induced by LPS would appear to be derived from the inhibition of LPS binding to the receptor through ligand-LPS complex formation or ligands may compete for LPS receptor (19). However, in our study, the reduction in the

<table>
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<th>Chlorhexidine (µg/ml)</th>
<th>1 h (nmol/ml)</th>
<th>24 h (nmol/ml)</th>
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<tbody>
<tr>
<td>Control</td>
<td>7.73 ± 0.84</td>
<td>5.30 ± 0.34</td>
</tr>
<tr>
<td>0.5</td>
<td>-</td>
<td>5.07 ± 0.65</td>
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<tr>
<td>1.0</td>
<td>6.75 ± 0.70</td>
<td>5.55 ± 0.57</td>
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<tr>
<td>5</td>
<td>6.90 ± 0.73</td>
<td>5.32 ± 0.37</td>
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<tr>
<td>10</td>
<td>7.91 ± 1.20</td>
<td>-</td>
</tr>
<tr>
<td>20</td>
<td>6.96 ± 1.00</td>
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Data are reported as the mean ± SD for at least three independent experiments carried out in triplicate.

Figure 2. NO production by macrophages treated with chlorhexidine for 1 h (A) and 24 h (B) and stimulated with LPS. Data are reported as the mean ± SD for at least three independent experiments carried out in triplicate. *P < 0.05 compared to control (ANOVA).
NO production by chlorhexidine-treated macrophages is likely the result of the action of this substance on plasma membrane. Chlorhexidine may interact or damage membrane receptors interfering in the binding receptor-LPS.

The products of the oxidative burst are used to kill phagocytic pathogens and also for the extracellular destruction of other cells. Based on the results obtained here we conclude that chlorhexidine does not produce an immunostimulatory effect. However, this substance may have an immunosuppressive effect on exposed macrophages which is related to receptor-dependent particulate stimuli.

References