Effect of chlorhexidine on the release of lysosomal enzymes from cultured macrophages

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Exposure of rat's peritoneal macrophages to chlorhexidine at concentrations up to 0.01 per cent resulted in the release of lysosomal enzymes, e.g. chloride-dependent arginine aminopeptidase and ß-D-glucuronidase into the medium from resting cells. The activity of extracellular lactate dehydrogenase increased up to the concentration of 0.01 % and then decreased with increasing chlorhexidine concentration. This could indicate a certain type of «mummifying» of the cells and/or their membranes. The incorporation of trypan blue was found in all cells after a rinse with 0.05 per cent or higher concentrations of chlorhexidine. The direct effect of chlorhexidine on these enzyme activities showed a slight activation of ß-D-glucuronidase and lactate dehydrogenase, but an inhibition of arginine aminopeptidase. Also the binding of chlorhexidine to cells was found. Low chlorhexidine concentrations caused an additional enzyme release from phagocytizing cells.

Key-words: Chlorhexidine; macrophages; phagocytes; enzyme activities

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Chlorhexidine has been reported as a useful and safe drug in the prevention and treatment of periodontal disease (2, 6, 10, 13, 15). However, it has been shown that the penetration of chlorhexidine occurs in a small degree through the healthy oral mucosa of guinea pig (4) and hamster (9). The penetration is increased, especially when chlorhexidine has been used after surgical procedures or in connection with epithelial lesions.

Previous studies have shown that chlorhexidine has toxic effects on epithelial cells (5), fibroblasts (3), and leukocytes (7). It can also produce changes in microcirculation (9), granulation tissue formation (14), and wound healing (11). The safe use of this antimicrobial agent presupposes that the concentrations which induce side effects should be well known. A previous report of the influence of chlorhexidine on leukocytes has been carried out by using 0.2 and 2.0 per cent solution. These concentrations are hardly present in *in vivo* situations. The purpose of this study was to assess the effects of different concentrations of chlorhexidine on cell viability and lysosomal enzyme release of the resting macrophages as well as during the phagocytosis of these cells.

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MATERIAL AND METHODS

Material

Three months old female Long Evans rats, weighing about 200 g were used as donors of macrophages. Eagle's medium (with Earle's salts) and HEPES (1 M) came from Gibco Bio-Cult. Ltd. (Paisley, Scotland). Streptomycine sulphate and penicillin were obtained from Hoechst AGC Frankfurt, Germany). Sterile saline from Leiras (Turku, Finland) and heparin (5000 UI/ml) from Medica (Helsinki, Finland) were used. p-Nitrophenyl-ß-D-glucuronide and zymosan were obtained from Sigma (St. Louis, MO, USA). Zymosan was prepared for use according to Weissman et al. (19). Trypan Blue was from Allied Chemical (Morris-town, NJ, USA). Chlorhexidine gluconate and chainlabelled (^{14}C)-chlorhexidine (specific activity 10.25 µCi/mg and purity of 98.8 %) were supplied by Imperial Chemical Industries Ltd., Pharmaceuticals Division Macclesfield, Cheshire, United Kingdom (4).

All other chemicals were of analytical grade and their sources have been reported earlier (12).

Cell incubations

Peritoneal macrophages were harvested with 10 ml of sterile saline containing 20 UI heparin as an anticoagulant. The cells were washed twice (500 x *g*, 10 min, + 20 °C) with the incubation medium (Eagle's medium supplied with 20 mM HEPES, 20 mM $NaHCO_3$, 50 µg/ml of streptomycin sulphate and 100 U/ml of penicillin) and finally suspended into the same medium (2 x 10^6 cells/ml). The cells were adhered to the bottom of petri dishes (Falcon®, 10 x 35 mm) for two hours at 37 °C in an Assab CO_2-incubator T/303 (Assab Medicin AB, Stockholm, Sweden) with a gas-phase of 95 % air and 5 % CO_2 and 100 % humidity.

The media were discarded and the cells treated with varying concentrations of chlorhexidine by rinsing the cells for two minutes with media containing chlorhexidine, after which the incubation was continued in media without added chlorhexidine. The specific activity of labelled chlorhexidine in the media was 1 µCi/mg. The incubation time was three hours. The incubations were ended by removing the media. The media were used for enzyme determinations. The cells were washed once with 20 ml of the incubation medium, scraped from the petri dishes and suspended in 0.8 ml of distilled water. Disruption of the cells was performed by a 3-second sonication (MSE Ultrasonic Disintegrator, + 4 °C, amplitude 3µ, and diameter of the prope 3 mm). 200 µl of a tissue solubilizer Soluene-100 (LKB-Wallac, Turku, Finland) was added to each sample and the samples were left standing in room temperature for two hours before counting in ACS scintillation cocktail (Amersham, Buckinghamshire, England) in an Ultrobeta 1210 liquid scintillator (LKB-Wallac, Turku, Finland). The cells were counted and identified as previously described (17).

Enzyme assays

ß-D-glucuronidase was determined with p-nitrophenyl-ß-D-glucuronide (5). Aminopeptidase activity was determined by using N-L-aminoacyl-2-naphtylamines (-2NA) as substrates in the presence of 0.2 M NaCI and without added salt (12). Aminopeptidase activity was assayed in reaction mixtures (0.6 ml) containing 0.3 ml of 0.05 M Na-phosphate buffer pH 7.2, with or without 0.4 M NaCl; 0.1 ml of 10^{-3} M substrate solution; 0.1 ml of water and 0.1 ml of enzyme solution. For both en-

zyme determinations the velocity of the hydrolysis of the substrates was measured and expressed as μmol of hydrolysis product formed per minute. Lactate dehydrogenase activity was assessed according to Wroblewski & La-Due (20).

RESULTS

An exposure of macrophages in culture to 0.001 – 0.01 per cent chlorhexidine solutions for two minutes caused an enhanced release of the chloride-dependent arginine aminopeptidase from non-phagocytizing peritoneal macrophages into the media (Fig. 1). A similar effect on ß-D-glucuronidase release was also seen (Fig. 2). The higher concentrations (0.05 and 0.1 per cent) of chlorhexidine decreased arginylaminopeptidase activities in the media compared to that obtained with 0.01 per cent concentration. The activity of ß-D-glucuronidase, however, stayed almost at the same level. During the phagocytosis the exposure of 0.001 per cent chlorhexidine solution caused a slight additional release of both enzymes into the media. The higher concentrations of chlorhexidine did not change ß-D-glucuronidase activity and decreased arginyl aminopeptidase activity.

The release of the cytoplasmic enzyme, lactate dehydrogenase, into the media with both the non-phagocytizing and phagocytizing cells was increased with an increased concentration of chlorhexidine up to 0.01 per cent (Fig. 3). The two higher concentrations decreased the release of lactate dehydrogenase. The incorporation of trypan blue was found after a rinse with 0.001, 0.05 and 0.1 per cent chlorhexidine in 10, 100 and 100 per cent respectively. The untreated cells showed no incor-

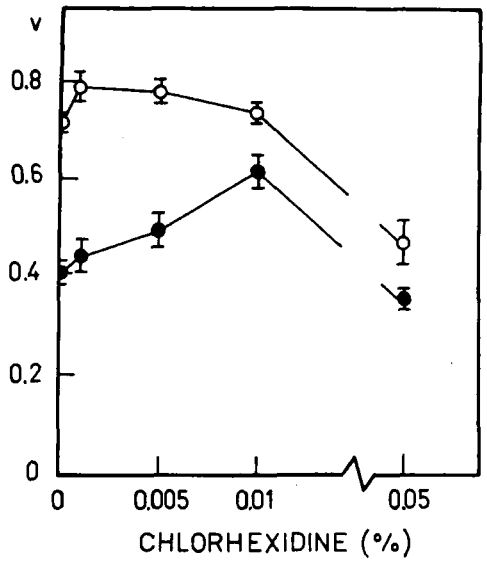

Fig. 1. The effect of different chlorhexidine concentrations on the release of arginine aminopeptidase (v = μmol x min^{-1} x 10^{-5}) from resting (•) and phagozytizing (o) macrophages expressed as the mean value and the range of three different experiments.

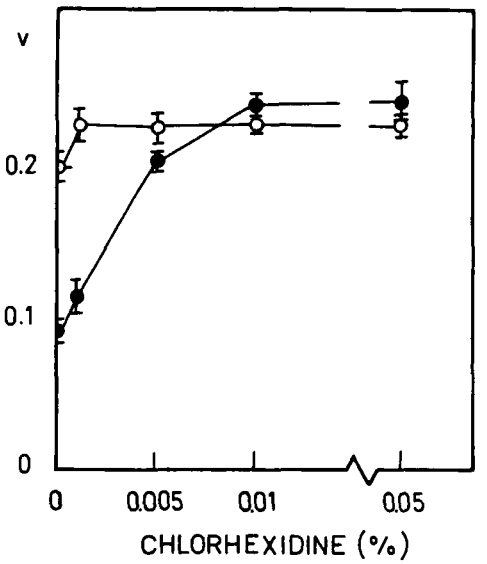

Fig. 2. The effect of different chlorhexidine concentrations on the release of ß-D-glucuronidase (v = μmol x min^{-1} x 10^{-4}) from resting (•) and phagocytizing (o) macrophages expressed as the mean value and the range of three different experiments.

poration. The direct effect of chlorhexidine on these enzyme activities showed a slight activation of ß-D-glucuronidase and lactate dehydrogenase, but a strong inhibition of the chloride-dependent arginine aminopeptidase (Fig. 4). Chlorhexidine did not, however, affect the amount of chloride activation.

The binding of chlorhexidine to macrophages was studied with (^{14}C)-chlorhexidine. This showed a similar binding to the phagocytizing and non-phagocytizing cells. About 10 per cent of the total counts present in the media were bound to the cells.

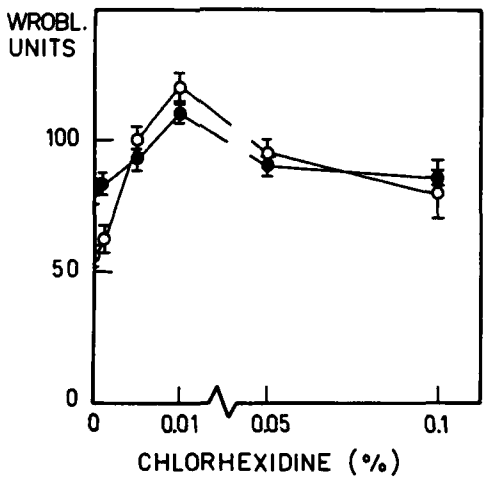

Fig. 3. Lactate dehydrogenase activity in the medium after exposure of resting (•) and phagocytizing (o) macrophages to different concentrations of chlorhexidine.

DISCUSSION

The present results confirmed earlier findings (3, 5, 16), which showed a cytotoxic effect of chlorhexidine concentrations of 0.005 – 0.01 per cent. In a previous study Goldschmidt (3) pointed out differences in sensitivity among ^{3}H-leucine and trypan blue incorporation and ^{51}Cr release. The finding that fibroblasts did not release much more than half of their ^{51}Cr label agrees with the results obtained for the release of intracellular lactate dehydrogenase from macrophages. Rinses with chlorhexidine concentrations up to 0.01 per cent caused the release of a small part of lactate dehydrogenase and higher concentrations inhibited the release. The enzymatic properties of lactate dehydrogenase were not markedly affected by the chlorhexidine concentrations used (Fig. 4), which indicated that with these concentrations a certain type of «mummifying» of the cells and/or their membranes must have taken place. Also a similar effect of chlorhexidine on capillary walls was seen in experimental inflammation (8).

The release of lysosomal enzymes as ß-D-glucuronidase and the chloride-dependent arginine aminopeptidase with 0.001 and 0.005 per cent concentrations from non-phagocytizing cells compared to phagocytizing cells did not indicate a summative effect of chlorhexidine and zymosan. Although

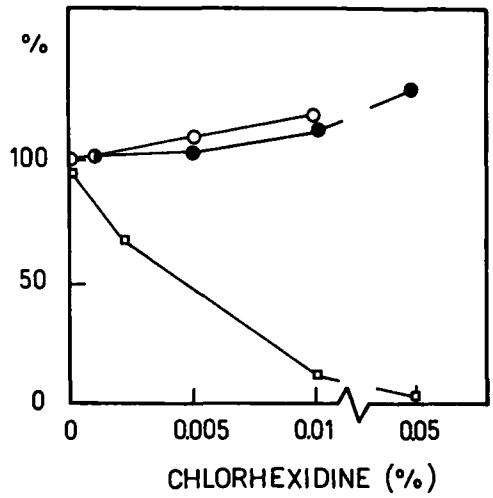

Fig. 4. The effect of different chlorhexidine concentrations on the activities of arginine aminopeptidase (□), ß-D-glucuronidase (o) and lactate dehydrogenase (•)

the 0.001 per cent concentration slightly increased, the release in the presence of zymosan, the above conclusion can be drawn on the basis of earlier results (18) concerning the release of these enzymes from phagocytizing macrophages. The comparison of phagocytizing and resting cells also supports the idea of «mummifying» effect of chlorhexidine on membranes. The obtained results support earlier findings of cytopathological effects of chlorhexidine and show the harmful effects on cultured macrophages with 0.005 – 0.01 % concentrations.

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