Archs oral Biol. Vol. 33, No. 12, pp. 881–885, 1988
Printed in Great Britain.

0003-9969/88 $3.00 + 0.00

EFFECTS OF PROPRIETARY ORAL RINSES CONTAINING CHLORHEXIDINE, HEXETIDINE AND BENZYDAMINE ON THE PROLIFERATION OF HUMAN BUCCAL EPITHELIAL CELLS IN CULTURE

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(*Accepted* 19 *July* 1988)

Summary—Cell cultures were established from small samples of buccal tissue, using a 3T3 fibroblast feeder-layer technique. After exposure to increasing dilutions of three proprietary oral rinses for 22 h or 2 h, the effects upon cell proliferation were studied by measurement of [^{3}H]-thymidine incorporation into cellular DNA. Cell membrane damage was assessed by measurement of lactate dehydrogenase content. Cultures exposed to hexetidine-containing or chlorhexidine-containing rinses for 22 h at dilutions of 250-fold or lower showed almost complete inhibition of [^{3}H]-thymidine incorporation. Cultures treated with benzydamine-containing rinse at the same dilutions showed no significant inhibition of incorporation. Exposure to the same dilutions of hexetidine- and chlorhexidine-containing rinses for 2 h resulted in 65% and 20% inhibition of incorporation, respectively. Lactate dehydrogenase content decreased to negligible levels after exposure to the rinse containing hexetidine at a 250-fold dilution, but was unaffected by the other two rinses. Thus dividing buccal epithelial cells *in vitro* may be adversely affected by exposure to certain commercial oral rinses.

INTRODUCTION

Antiseptic or anti-inflammatory mouthwashes are widely used as an aid to oral hygiene, in the treatment of minor infections, for the promotion of healing after surgery, and in the management of recurrent ulceration. Little is known of the direct effects of such preparations on human oral mucosa. The development of a cultured human oral-cell system may provide a useful model to investigate any such effects. The maintenance of oral epithelia in culture has been described by several investigators (Jepsen, 1974; Mlinek and Buchner, 1975; Hill and Williams, 1980), mostly employing outgrowth from tissue explants as the source of epithelial cells. The technique of Rheinwald and Green (1975) for the *in vitro* cultivation of skin keratinocytes employs inactivated 3T3-fibroblast feeder layers and allows the rapid production of sheets of confluent keratinocytes from fragments of enzymatically dissociated skin. This technique has been applied to the cultivation of human gingival and buccal epithelia as described by Taichman, Reilly and Garant (1979), who used 7–10-day epithelial outgrowth from tissue explants as a source of oral epithelial cultures.

We have now used the Rheinwald and Green technique to cultivate buccal epithelia from several adult sources using trypsin-dissociated epithelial layers from small biopsies. The effects of two antiseptic and one anti-inflammatory proprietary oral rinses on these cells were investigated.

MATERIALS AND METHODS

Procedures for establishment of buccal cell cultures

Small samples of buccal tissue (less than 1 cm^2) were removed from patients undergoing oral surgery. The epithelial layer was separated from the underlying connective tissue after treatment with trypsin. The dispersed cell suspension was inoculated into 75 cm^2 tissue-culture flasks containing inactivated 3T3-fibroblast layers, as described by Rheinwald and Green (1975). The inoculation density was approx. 5×10^5 to 10^6 epithelial cells per flask. The cells were maintained in complete medium composed of Dulbecco's Minimal Essential Medium (DMEM) and Ham's F12 Medium at a 3:1 ratio, containing 10% fetal calf serum (Imperial Labs) and supplements as described by Wu *et al.* (1982). Confluent or nearly confluent cells were subcultured and stored, resuspended in complete medium containing 10% glycerol, in liquid nitrogen.

Intra-oral preparations

The two antiseptic rinses contained hexetidine (2.94 mmol/l, Oraldene, Warner–Lambert Health Care, Eastleigh, Hants, England) and chlorhexidine gluconate (2.23 mmol/l, Corsodyl, ICI, Ltd, Macclesfield, Cheshire, England). The anti-inflammatory rinse contained benzydamine–HCl (4.34 mmol/l, Difflam, Riker Laboratories, 3M Health Care, Loughborough, Leics, England). Each is recommended for use undiluted in the mouth. Cultured buccal cells were exposed to these rinses at dilutions ranging from 50 to 1000-fold in the culture medium. Pure samples of the active principles were obtained from the manufacturers, and were added to the cultures at concentrations which corresponded to those given by the appropriate dilutions of each oral rinse.

[^{3}H]-thymidine incorporation into cultures

The effects of the intra-oral preparations on the cultured buccal cells were investigated using cells

derived from 3 male patients under the age of 25. For each set of experiments, the frozen cells were rapidly thawed, diluted to the appropriate volume with complete medium, and seeded into 25 cm^2 tissue-culture dishes containing inactivated 3T3 cells. The cultures were maintained at 37°C for 2–3 days until obvious colonies of epithelium were apparent, but before confluence was reached (about 50% confluence). The 3T3 cells were then removed prior to each experiment by a brief exposure to 0.25% trypsin/0.01% EDTA (less than 1 min) followed by rapid pipetting. 5 ml of complete medium were added to each dish, and duplicate cultures were treated with each oral preparation, or its active agent, as described above. Media for control cultures (triplicate dishes) received diluent only. After exposure to the preparations for periods of 22 h, or 2 h, the cultures were labelled with [methyl-^{3}H]-thymidine (37 kBq/ml, sp. act. 185 GBq/mmol, Amersham International, High Wycombe, Bucks, England) for 4.5 h. The cultures were thoroughly rinsed with phosphate buffered saline (PBS) and harvested by scraping into 1.0 ml of PBS. Each cell suspension was dispersed by sonication, and radioactivity in acid precipitable DNA was determined using a Beckman LS1801 scintillation counter.

In order to investigate whether cells recovered from the effects of treatment, cultures exposed for a period of 22 h, together with untreated controls, were rinsed and fresh medium added. The cultures were then maintained at 37°C for 48 h before addition of [^{3}H]-thymidine and harvesting as described above.

Protein, DNA and lactate dehydrogenase (LDH) measurement

Cellular protein content for use in the calculation of enzymatic specific activity was determined as described by Lowry *et al.* (1951). Cellular DNA content was determined by a fluorimetric method (Downs and Wilfinger, 1983). Lactate dehydrogenase activity in sonicated cell suspensions was assayed by the spectrophotometric method of Wroblewski and La Due (1955). Units of enzymatic activity are defined as μmol of pyruvate converted to lactate/min per mg protein at 22°C. The effects of the three oral rinses on the catalytic activity of lactate dehydrogenase were investigated using both porcine muscle LDH (Sigma Chemical Co., Poole, Dorset, England) and human cellular LDH.

Statistical analysis

Significance of differences from control values at each dilution of rinse, or concentration of active principle was assessed using the Mann–Whitney U test. A value of $p < 0.05$ on a two-tailed test was considered to show a significant difference.

RESULTS

The results of exposure to the three oral rinses and their active principles for 22 h prior to radioactive labelling are shown in Fig. 1. Data are derived from experiments on cultures initiated from three separate frozen cell isolates. In order to make all experiments comparable, the incorporation of [^{3}H]-thymidine into cellular DNA was expressed as a percentage of the control cultures exposed to diluent only.

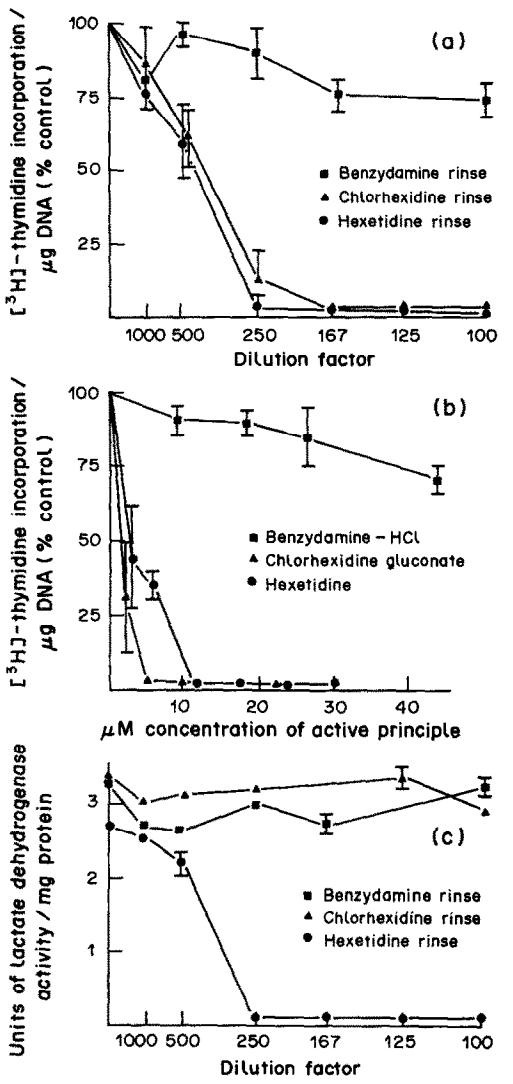

Fig. 1. (a) Incorporation of [^{3}H]-thymidine into cellular DNA after 22 h exposure of cultures to diluted oral rinses, and (b) to the active principles in each rinse. (c) Lactate dehydrogenase activity in cultures exposed for 22 h to diluted oral rinses. Points represent the mean value obtained from at least six culture dishes. Bars represent the range of values obtained. Range bars are not shown where variation was less than 3%, or there was no detectable enzymatic activity.

Cultures exposed to hexetidine-containing or chlorhexidine-containing mouthwash showed almost complete inhibition of incorporation of [^{3}H]-thymidine at dilutions of 250-fold or lower (Fig. 1a). Significant inhibition of incorporation was also observed at 500- and 1000-fold dilutions ($p < 0.05$).

The benzydamine-containing mouthwash was the least inhibitory to cell growth. Cultures treated with dilutions of 250-fold or greater showed no significant inhibition of incorporation of [^{3}H]-thymidine relative to untreated control cultures ($p > 0.05$). However, at

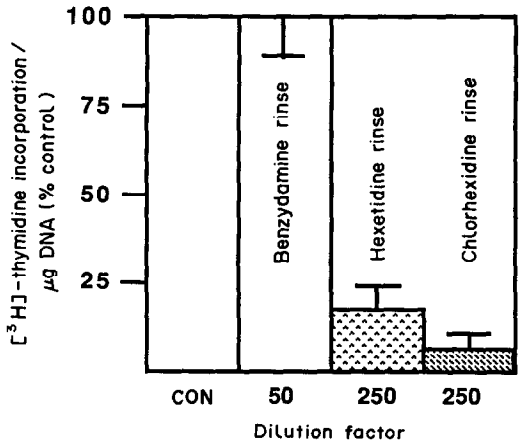

Fig. 2. [^{3}H]-thymidine incorporation into cellular DNA in cultures exposed for 22 h to diluted oral rinses as shown, followed by a 48 h recovery period in fresh medium. Values shown as means and ranges (see Fig. 1).

dilutions of lower than 250-fold, significant inhibition of the order of 25% was observed ($p < 0.05$).

Cultures exposed to the active agents from the three rinses showed similar inhibition of [^{3}H]-thymidine incorporation to those exposed to the complete formulations (Fig. 1b).

The specific activity of LDH in the cells after exposure to the rinses is shown in Fig. 1c. After treatment with hexetidine-containing mouthwash at dilutions of 250-fold or less, specific activity decreases sharply to undetectable levels. In contrast, treatment with chlorhexidine-containing mouthwash at the same dilution had no significant effect, even though this dilution was sufficient to cause greater than 90% inhibition of [^{3}H]-thymidine incorporation. No significant reduction of enzyme specific activity was seen after exposure to benzydamine-containing mouthwash. All three agents, at concentrations in excess of those used to treat cells, had no direct effect upon the catalytic activity of LDH.

Cultures allowed a 48 h recovery period in fresh medium after treatment with growth-inhibitory concentrations of hexetidine-containing or chlorhexidine-containing mouthwashes did not recover their ability to incorporate [^{3}H]-thymidine into DNA (Fig. 2). Cells treated with benzydamine-containing mouthwash, however, recovered growth activity similar to that of the untreated control cultures.

When cultures were exposed to growth-inhibitory concentrations of the oral rinses for a shorter time (2 h) prior to labelling, 250-fold dilutions of hexetidine- and chlorhexidine-containing mouthwash in the culture medium caused 65% and 20% inhibition of incorporation relative to the untreated control respectively (Fig. 3a). After exposure to benzydamine-containing mouthwash for 2 h the levels of inhibition of incorporation were approximately the same as those caused by a 22 h exposure. LDH specific activity (Fig. 3b) was decreased after a 2 h exposure to hexetidine-containing mouthwash (100-fold dilution), but unaffected by such exposure to the other two mouthwashes.

DISCUSSION

The cultured oral-cell system has the advantage that, once the cultures are established, the cell isolates may be stored and used for further investigations as required. Hence patients or volunteers in a study are not subjected to repeated biopsy in order to obtain tissue for biochemical or histological investigations. However, it may be argued that such *in vitro* systems do not mimic the mouth *in vivo*, which is lined by a stratified squamous epithelium forming an effective structural barrier to the external environment (Squier and Johnson, 1975). Our experiments were conducted on actively dividing non-stratified buccal cells in culture; these lack the permeability barrier present *in vivo* in the upper stratum of intact epithelia containing membrane-coating granules. However, our results may be relevant to the use of oral rinses in healing or ulcerated mucosa when this superficial barrier zone may be disrupted or lost.

Exposure of the cultured buccal epithelia to the three oral preparations clearly caused inhibition of cell proliferation over the times investigated. This inhibition (Fig. 1b) was due to the active principles of the rinses and not to any flavouring or colouring of the preparations. The reason for this effect on cell growth is unknown, but inhibition of DNA replication, or interference with oxidative metabolism are possible explanations.

It is clear from the measurement of cellular LDH content that only hexetidine has a disruptive or damaging effect upon the cell membrane, permitting leakage of this soluble cytoplasmic enzyme into the culture medium. Hexetidine is an oil almost insoluble in water and non-ionizing. It is possible that this compound is preferentially soluble in the lipid component of the cell membrane, thus disrupting membrane integrity. Chlorhexidine and benzydamine–HCl, however, are ionizable and water-soluble and hence are unlikely to enter the lipid phase of the cell membrane (Jain, 1972).

When used in practice, the undiluted oral preparations are held in the mouth for short periods of the order of 1 min. Exposure of the cultured cells for 22 h may be considered excessive by comparison with recommended clinical exposure times. However, some indication that the effects may be brought about more rapidly is found in the outcome of 2 h incubations (Fig. 3). The hexetidine-containing mouthwash caused a substantial inhibition of growth within this shorter time whereas the effect of chlorhexidine-containing mouthwash was time-dependent, requiring longer to exert its inhibitory effect. After the 2 h exposure to hexetidine-containing mouthwash the LDH specific activity again decreased to negligible levels, this effect requiring a higher concentration (100-fold dilution) than the 22 h exposure (250-fold dilution). LDH specific activity in cells treated with chlorhexidine-containing mouthwash was unaffected relative to the untreated control. With benzydamine-containing mouthwash, the levels of inhibition of [^{3}H]-thymidine incorporation after a 2 h exposure were approximately the same as those caused by a 22 h exposure, indicating that its inhibitory effect was independent of time over the period investigated. Therefore the effect of the

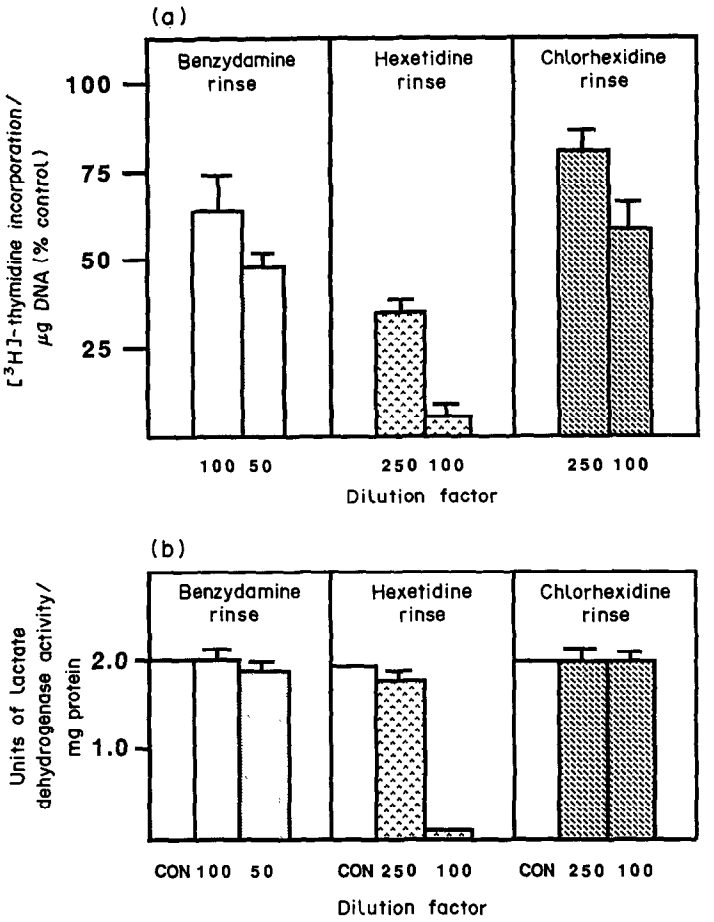

Fig. 3. (a) [^{3}H]-thymidine incorporation, and (b) lactate dehydrogenase specific activity in cultures exposed to two dilutions of each oral rinse for a 2 h period prior to radioactive labelling. Values shown are means and ranges (see Fig. 1).

mouthwashes upon the cells relates both to concentration and duration of exposure, and may be related to the mechanism of uptake of the active agents. It should also be noted that recovery of growth activity was observed only in those cells that had been treated with the benzydamine-containing mouthwash.

It thus appears that, in the absence of a protecting squamous layer, agents intended to promote healing in the mouth may inhibit growth of the cells of the buccal mucosa *in vitro*. However, it is necessary to compare the potential for damaging effects *in vitro* with the potential for beneficial anti-bacterial or analgesic properties *in vivo*. Benzydamine-containing mouthwash appears to be an effective analgesic and anti-inflammatory agent (Simard-Savoie and Forest, 1978). The anti-microbial properties of chlorhexidine-containing and hexetidine-containing mouthwashes have been well documented (Grenby and Saldanha, 1984; Ashley, 1984), and studies on the long-term use of chlorhexidine have shown no pathological changes in the stratum corneum of the intact oral mucosa (Mackenzie *et al.*, 1976) or in glycolytic enzymes of the gingival mucosa (Nuki *et al.*, 1976). However, where the integrity of the oral mucosa is compromised, or there are extensive healing lesions, it may be necessary to consider carefully the use of these agents *in vivo*.

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