Biologic testing of leachable aromatic compounds from denture base materials

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The aromatic compounds phenyl benzoate (PB), phenyl salicylate (PS), and biphenyl (BP), which have previously been found to leach from poly(methyl methacrylate) denture base materials, were tested for cytotoxicity and biologic effects by L929 cells in culture. The octanol–water partition coefficient (log P_{ow}), a descriptor for the lipophilicity, was determined for the compounds. Cytotoxicity was evaluated by total cell growth and the plating efficiency test, and biologic effects by the total fatty acid composition of L929 cells. The commonly used tests, total cell growth and plating efficiency, did not show any significant changes of the cells due to the compounds. On the other hand, BP and PS, in particular, induced changes in the total fatty acid composition of L929 cells. The problem of bioavailability of aromatic compounds in cell culture assays and the relation to lipophilicity was addressed. □ *Acrylic resins; biologic effects; cytotoxicity; denture bases; leaching*

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Biocompatibility of dental materials has been evaluated by cell culture assays, animal tests, and studies in humans (1–4). Cell cultures are valuable tools to acquire knowledge about the mechanisms whereby dental biomaterials produce pathologic reactions at a cellular level (5, 6). Cytolysis, cell growth, and membrane changes have previously been used in cell culture systems to reveal cytotoxic effects from chemical compounds.

Because of the extensive use of poly(methyl methacrylate) (PMMA) materials in the medical and dental field, compounds leaching from these materials should be tested for potential biologic effects (7). Heat-cured PMMA is widely used for complete dentures (8). The cytotoxic effects of PMMA denture base materials have been evaluated by cell cultures (9–11); however, the specific compound or compounds causing responses have not been identified. Recent studies have shown that aromatic compounds leach from PMMA denture base materials (12–14). Phenyl benzoate (PB), phenyl salicylate (PS), and biphenyl (BP) are released, and an inverse relation has been shown to exist between the amount of compounds released and the processing temperature of the PMMA materials (12–14). The formation of PB, PS, and BP results from free-radical intermediates initiated by the decomposition of benzoyl peroxide (14).

Biologic testing of lipophilic compounds represents special problems because they are not readily bioavailable in cell culture assays. The biologic activity of lipophilic compounds is related to their physicochemical properties, mainly their lipophilicity (15, 16). The effects of lipophilic compounds are closely related to their accumulation in the cell membrane (17), where they affect the fatty acid composition of cells (18).

The aim of the present study was to estimate the lipophilicity of and to evaluate in vitro cytotoxic and biologic effects of the previously detected aromatic compounds phenyl benzoate, phenyl salicylate, and biphenyl, which leach from PMMA denture base materials.

Materials and methods

Cells and growth medium

Mouse fibroblast cells, L929 (Flow Laboratories, Rickmansworth, UK) were grown as a monolayer in Eagle's minimum essential medium (MEM) (Whittaker Bioproducts, USA), containing Earle's balanced salt solution, 1% L-glutamine, 2% penicillin/streptomycin, and 5% inactivated fetal bovine serum (½ h at 56°C). The cells were grown in polystyrene petri dishes (5 cm diameter; Nunc, Denmark) or tissue culture flasks (50 ml, Nunc). An atmosphere of 5% CO_2 in air and a relative humidity of 95% were used for incubation.

Plating efficiency

Petri dishes were seeded with 100 cells and incubated for 17–18 h before treatment. The growth medium (5 ml) was replaced on the 1st, 3rd, and 6th day with medium containing solutions of phenyl benzoate (PB), phenyl salicylate (PS), or biphenyl (BP) (purity > 98% GC, Fluka Chemie AG, Switzerland) in acetone (Riedel-de Haën AG, Germany) in concentrations of 0, 0.01, 0.1, 1.0, and 10.0 µg/ml. The final concentration of acetone in the medium was always 0.5%. Five dishes

were used for each concentration. Ten days after seeding, cells were washed with 0.9% NaCl, fixed with ethanol, and stained with crystal violet. Colonies were manually counted, and colony-forming ability was compared with the controls (0.5% acetone). Results from the plating efficiency test was expressed as numbers of colonies in percentage of the controls (acetone only).

Growth curve analyses

Petri dishes were seeded with 3000 cells. PB, PS, and BP were added to the growth medium in a concentration of 0.1 µg/ml in acetone. The final concentration of acetone in the medium was 0.5%. The medium was replaced after 7 days. Each day for 16 succeeding days two parallel dishes from each group were trypsinized (Flow Laboratories, Scotland), and the number of cells was counted in an electronic counter (Coulter Counter Model ZM, Coulter Electronics Ltd., UK).

The growth rate constant K, was calculated in accordance with the formula

$$K = \frac{\log n_2 - \log n_1}{t_2 - t_1}$$

for the period between days 3 and 6. n_1 and n_2 denote number of cells at time t_1 and t_2 (t = number of days).

Fatty acid analysis

A number of $2.3 \cdot 10^5$ cells was transferred to cell tissue flasks. Four parallel samples were used for each compound. The standard MEM medium was replaced by medium containing solutions of 0.01 µg/ml PB, PS, or BP in acetone. The final concentration of acetone was 0.5%. Twenty-four hours later cells were transferred to 15-ml tubes, washed three times in 0.9% NaCl, and pelleted by centrifugation. The cell pellets were resuspended in 3 ml of saline and mixed with 0.5 ml of 3 mol/l HCl in methanol (Supelco Inc., USA). The fatty acid C19:0 (2 µg/ml) in methanol was used as an internal standard. The samples were methanolyzed at 100°C for 2 h; 0.5 ml distilled water was added to each tube, and fatty acid esters were extracted three times with 1 ml of hexane (high-performance liquid chromatography (HPLC) grade, Rathbun Chemicals Limited, Scotland). The samples were evaporated to 100–200 µl under a stream of nitrogen and transferred to sample vials, and the fatty acid esters subjected to gas chromatography. A gas chromatograph (GC) (Hewlett-Packard 5890, USA) equipped with a flame-ionization detector and an autosampler (Hewlett Packard 7673) was used. The fused silica capillary column used (DB 23) was 30 m × 0.32 mm inside diameter, with 0.25-µm film thickness. The temperature program was as follows: 2 min at 60°C, 30°C/min to 145°C, 1 min at 145°C, 2.8°C/min to 220°C, and then a final hold time of 3 min. Splitless injection was used, and the injector and the detector temperatures were 300°C. Peak areas were calculated, and retention time was registered by a laboratory computer system (Multichrom, VG Data Systems Ltd, UK).

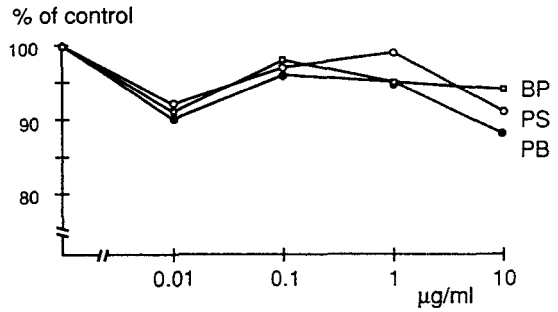

Fig. 1. Relative plating efficiency after treatment with 0, 0.01, 0.1, 1.0, and 10.0 µg/ml of phenyl benzoate (PB), phenyl salicylate (PS), and biphenyl (BP). Results are expressed as the number of colonies in percentage of controls 10 days after seeding with 100 cells/dish. Each point represents the median value of five dishes of cells.

Fatty acid methyl esters were identified by comparing retention time with a standard solution of fatty acid methyl esters (Nu Check Prep Inc., USA).

Determination of lipophilicity (log P_{ow})

The log P_{ow} values were measured with a standard HPLC method (19). The test substances were dissolved in a 3:1 methanol/water solution, about 0.4 mg/ml. A suitable wavelength for UV absorbance of test solutions by HPLC was initially determined by means of a spectrophotometer (CARY) with a 1-cm quarts cell.

The log P_{ow} of test substances was subsequently determined by two injections of 10–50 µl test substance solution on an HPLC (Constametric I) with a 25-cm column (Merck LiChrosper100 RP-18). The detector was a variable-wavelength UV detector and/or an RI detector (LDC Spectromonitor III). The test was carried out at room temperature. The dead-volume retention was 2 min, as determined with a thio-urea standard. The calibration curve was based on seven standard components with log P_{ow} values ranging from 1.5 to 4.7.

Presentation of results and statistics

The absolute amounts of fatty acids were determined in micrograms per milliliter with the fatty acid C19:0 as an internal standard.

The number of peaks in chromatograms from cell samples was counted by the chromatography software, indicating the number of compounds in the samples. A log P_{ow} value of 0 denotes an equal partition between water and octanol, and a log P_{ow} value of 1 denotes 10 parts more of the compound in octanol than in water.

The Mann–Whitney two-sample test and the Wilcoxon signed-rank test were used to test for statistical significance. A significance level of $p < 0.05$ was chosen.

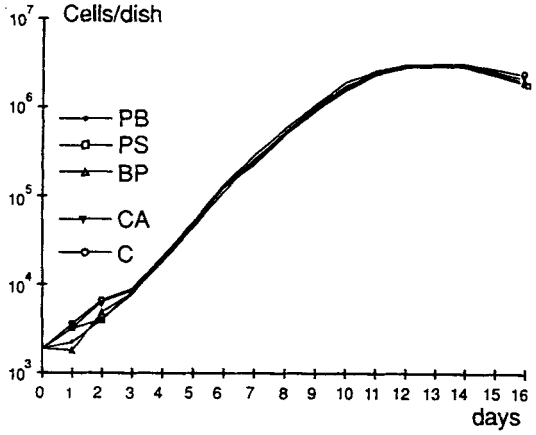

Fig. 2. Effects of 0.1 μg/ml of phenyl benzoate (PB), phenyl salicylate (PS), and biphenyl (BP) on L929 cell growth. CA = control with acetone; C = control without acetone (medium only). Each point represents the median value of three dishes of cells.

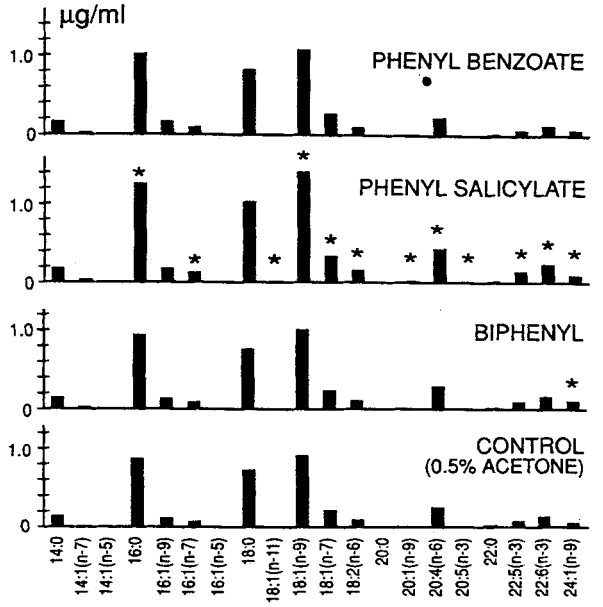

Fig. 3. The concentration of saturated and unsaturated fatty acids in L929 cells after exposure to 0.01 μg/ml of phenyl benzoate, phenyl salicylate, or biphenyl for 24 h. Each value represents the median of four samples. An asterisk denotes statistically significant differences compared with controls (medium containing acetone).

Results

The lipophilicity, expressed as log P_{ow} values, of the compounds was 3.4 for PB, 3.7 for PS, and 4.0 for BP. A high number indicates higher lipophilicity.

The plating efficiency test showed that all the aromatic compounds, especially in the concentration 0.01 μg/ml, tended to reduce the colony count, although not statistically significantly (Fig. 1). The colonies from exposed cells seemed to be smaller than the control colonies.

In the growth curve study the effects of the aromatic compounds were not discernible from the results of the controls (Fig. 2). Between days 3 and 6 the growth rate constant varied between 0.38 and 0.40 for these compounds.

Changes in the total fatty acid composition of L929 cells was monitored to determine the effect of PB, PS, and BP (Fig. 3). The number of peaks, counted from the chromatograms, was lowest for controls (0.5% acetone only) and highest for the PS-exposed cells. The fatty acid content of L929 cells after a 24-h exposure did not differ from the controls for PB, whereas BP-exposed cells had a significantly higher amount of the fatty acid C24:1 (n-9). PS caused a considerable change in the total fatty acid composition of L929 cells—that is, significantly higher levels of 12 of the 20 fatty acids analyzed. Eleven of these fatty acids were unsaturated, five polyunsaturated, and six monounsaturated. In addition, the concentration of one saturated fatty acid had increased (C16:0) (Fig. 3).

Discussion

Previous reports have shown that organic compounds leach from denture base materials in vitro and in vivo (20–26). In recent studies we have reported on the gas-chromatographic/mass-spectrometric detection of the lipophilic compounds PB, PS, and BP, from PMMA denture base materials (12–14).

Log P_{ow} is considered to be related to biologic responses (27). The recorded log P_{ow} values were at the same level as naphthalene, which has a relatively high lipophilicity (17). In biomembranes, which contain a biomolecular phospholipid layer, the lipophilicity is an essential factor in the membrane penetration of organic substances (28).

When cell cultures are used to test for biologic and cytotoxic effects, the lipophilicity of test compounds should be addressed. A water-soluble compound may simply be added to the cell culture medium, whereas test procedures are more complicated when lipid-soluble compounds are involved (29). The problem in testing lipophilic compounds has been proposed to arise from their low water solubility and slow rate of metabolism (30).

We have used acetone instead of the alternative dimethyl sulfoxide (DMSO) to dissolve the lipophilic compounds (31, 32). DMSO has been shown to interact with cell membranes in a manner that might obscure the effects of test compounds (33, 34). Because of the high vapor pressure of acetone it will be lost from the medium during incubation, and, hence, its adverse effects are negligible (31).

It has been demonstrated by quantitative structure-

activity relationship studies (QSAR) that a linear correlation exists between cytotoxicity and log P_{ow} for many kinds of lipophilic compounds (35–38). The relations often fit better when QSAR analysis includes properties other than lipophilicity (that is, electronic, size, and steric contribution) (32). However, the small differences between log P_{low} values and the low number of compounds tested in our study make it difficult to correlate exactly the degree of lipophilicity with the cytotoxic effects.

Plating efficiency tests and total cell growth tests are methods commonly used to study in vitro cytotoxicity. The fact that we found no statistically significant difference compared with controls when using these techniques could possibly be attributed to solubility-related factors, which have been proposed to explain the toxicity cut-off in aromatic compounds with high lipophilicity (39).

Accumulation in the lipid regions of cells, where important enzymes and transport systems are located, has been proposed as a mechanism of toxicity associated with lipophilic compounds (36). Compared with the plating efficiency test and the cell growth test, the fatty acid composition of the cells should be more directly relevant to the mechanism of toxic action of lipophilic compounds. The latter point has also been stressed by Phillips et al. (40).

With regard to possible effects on the cell membrane lipids by compounds leaching from PMMA denture base materials, Al-Nazhan & Spångberg (41) have shown that the cell membranes of L929 cells may be affected when they are exposed to extracts from acrylic polymers. Our method for extracting the total amount of fatty acids allowed small samples of cells (2.3×10^5) (42). PS induced pronounced changes in the total fatty acid pattern of L929 cells, compared with BP and PB (Fig. 3). Mainly the unsaturated fatty acids were affected by PS, which is in accordance with the results from Dutta et al. (18). The degree of unsaturation of fatty acids in phospholipids has been shown to be the main factor regulating membrane fluidity and may influence membrane-bound enzymes and receptors in cell membranes (43). A higher water solubility of PS (44), compared with BP and PB, could possibly explain the effect on total fatty acid pattern.

In conclusion, the effects on L929 cells of PB, PS, and BP seemed to be related to the compounds' bioavailability in the cell culture system, which is a matter of partition between water, lipids, and proteins. Our data suggest that at least PS affects the cell membrane composition. Lipophilic compounds need to be evaluated for the mechanism of cytotoxicity. Compared with the plating efficiency test and the total cell growth test, change in fatty acid composition appears to be a more specific monitor of the effects of lipophilic compounds. Thus, leachable aromatic compounds from PMMA denture base materials need to be further evaluated for their long-term effects. In addition to the response from PS, PB, and BP, the biologic influence resulting from the possible release of free-radical intermediates by the degradation of benzoyl peroxide should be taken into consideration in denture patients.

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