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Light and Electron Microscope Study of the Morphological Changes in L 929 Cells and Human Periodontal Ligament Fibroblasts Exposed to Toxic Dental Material.

Saad Abdulaziz Al-Nazhan

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CHANGES IN L 929 CELLS AND HUMAN PERIODONTAL LIGAMENT
FIBROBLASTS EXPOSED TO TOXIC DENTAL MATERIAL

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D.D.S., King Saud University, Saudi Arabia, 1982

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LIGHT AND ELECTRON MICROSCOPE STUDY OF THE MORPHOLOGICAL CHANGES IN L 929 CELLS AND HUMAN PERIODONTAL LIGAMENT FIBROBLASTS EXPOSED TO A TOXIC DENTAL MATERIAL

Presented by
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The University of Connecticut
1987
TO MY PARENTS, WIFE AND FAMILY
ACKNOWLEDGEMENTS

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INTRODUCTION

In medical and dental clinical practice various alloplastic materials are commonly used. These include cardiac valves, artificial joints, dental materials and container materials to store biological tissues and fluids. These materials are directly or indirectly placed in contact with living tissue. To prevent unnecessary adverse reactions, the biocompatibility of these materials must be determined before they are used.

Recommended standard methods to evaluate the biocompatibility of dental materials and their safety (in vivo and in vitro) have been published by the Federation Dentaire Internationale (FDI) in 1980 and by the American Dental Association (ADA) in 1979.

One method of evaluation of the toxic effect of dental materials is to place the material directly into the prepared cavities in animal or human teeth or to implant them into animal tissues. The material and the surrounding tissue are subsequently removed and examined microscopically to determine the tissue response. In such in vivo experimental models, however, variables such as variations between and within species, microbial contamination, and microleakage are difficult to control. Furthermore, inadequacy of current quantitative histological methods limits the validity of the results (Tyas & Browne 1977).
As an alternative to the in vivo methods, in vitro techniques have been widely used as preliminary screening tests to evaluate the biocompatibility of dental materials.

Experiments, in vitro, can be carried out under controlled conditions, the results are reproducible, and are relatively easy to quantitate. A major goal of in vitro assays is to develop methods that are relevant to in vitro conditions.

The radioactive chromium (\(^{51}\text{Cr}\)) release method is one of the commonly used screening tests approved by the FDI and ADA. \(^{51}\text{Cr}\) has been used widely in vitro and in vivo by many investigators to measure the volume of circulating red cells (Sterling & Gray 1950), to tag and measure red blood cells' survival (Gray & Sterling 1950, Ebaugh et al. 1953, Sutherland et al. 1954), to study the life span of leukocytes (McCall et al. 1955), and to study hemolysis (Weinrach et al. 1958). In addition, this method has been used for quantitative and kinetic studies of immunologically induced lysis of nucleated cells (Sanderson 1964, Wigzell 1965) and for material toxicity evaluation (Spangberg 1973).

\(^{51}\text{Cr}\), as an isotonic solution of \(\text{Na}_2\text{CrO}_4\), noncovalently binds to proteins and other cell constituents (Gray & Sterling 1950). This method has the following advantages:

1) The labelling of the cells is very simple and rapid.

2) The half-life of \(^{51}\text{Cr}\) is short (about 27 days).

3) In contrast to many other isotopes, such as \(^{3}\text{H}\)-thymidine and \(^{32}\text{P}\), the released \(^{51}\text{Cr}\) cannot be reutilized by the cells.

4) Nonspecific leakage of the isotope is very low.

5) Quantitative cytotoxicity assays are accurate because of minimum handling.

6) Only a small amount of $^{51}$Cr is required which causes no damage to the cells.

7) It is not expensive.

In addition to the radiocrhromium release method, several other techniques, such as quantitative enzyme cytochemistry methods (Meryon & Browne 1983), vital stain exclusion (Guess et al. 1965), filter technique using Millipore filters (Wennberg et al. 1979), and hanging drop cultures (Kawahara et al. 1968), have been used to study cellular changes caused by dental materials. These methods are all indirect in character, as they do not require the detailed observation of the reacting cells. Direct light microscopic evaluation of cultured tissue for the evaluation of biocompatibility has only been reported in a few instances (Kawahara et al. 1968, Autian 1970). Little attention has been paid to the morphological alteration of the test cells in tissue culture. Electron microscopic evaluation of cell injury caused by dental materials has not yet been reported.
PURPOSE OF THE STUDY

The purposes of the present investigation are:

1. To study the morphological changes that occur as a result of recorded cell injury during a standardized cytotoxicity evaluation in vitro. Light and electron microscopy will be used to correlate cellular structural changes with the radiochromium release.

2. To compare the cellular responses of two different fibroblast cell types exposed to the test material. A primary culture of human periodontal ligament (PDL) fibroblasts and a heteroploid cell line (L 929) derived from mouse skin fibroblasts, will be compared.

3. To evaluate the morphological effects of the $^{51}$Cr labeling on the cultured cells.

4. To study cell recovery after injury.
REVIEW OF THE LITERATURE

Methods Used For In Vitro Cytotoxicity Testing

A number of methods have become established as being rapid, relatively inexpensive, reliable and reproducible screening tests of materials suggested for use in patient care. FDI approved methods (1980) include monolayer assays as well as the hemolysis test method. These methods are briefly described below.

The Monolayer Methods

Agar Overlay Method

In this method, a confluent monolayer of cells is established in petri dishes and the cells are stained with a vital stain (Neutral Red) before the experiment. The culture medium is then decanted and replaced by a layer of a nutrient agar (Guess et al. 1965, Powell et al. 1970, Autian 1974, Imai et al. 1982). The test material is placed directly on the top surface of the agar layer and the culture is incubated. After various incubation periods, the cell monolayer is examined microscopically. In this method, cellular death is assumed to occur when the cells lose the vital stain.

Although an approved FDI and ADA method, the agar overlay assay has many disadvantages including the following: there is no direct contact between the test material and the cells as the cells are separated from the test material by the agar layer. Furthermore, the thickness of the agar layer, the molecular size of the toxic
component, water solubility of the test material and whether or not the test material reacts with the agar gel are factors which might affect the diffusion rate and, thus, alter the cell-material interaction.

**Millipore Filter Method**

A cell monolayer is established on top of a 0.45 um Millipore filter. The filter is placed on nutrient agar with the monolayer facing the agar. The test material is then placed on top of the filter. After various incubation periods, the material is removed and the cell monolayer is stained cytochemically for succinate dehydrogenase activity (Tronstad et al. 1978, Wennberg et al. 1979, Meryon et al. 1983).

The problem with this method is similar to the agar overlay method; namely, the Millipore filter separates the test material from the cells preventing a direct contact. Also, inverting the filter may alter the gas and nutrient supply to the cells resulting in adverse cell responses not related to the test material itself.

**Chromium Release Method**

This method, developed by Spangberg (1973), uses a cell monolayer prelabeled for 20 hours with $^{51}$Cr in sodium chromate. The test material is prepared in culture chambers and a suspension of labeled cells is added. After various incubation periods, $^{51}$Cr released from the cells into the media and values obtained from cells exposed to the material is measured and compared with those of the controls. The reference counts of the original chromium incorporated
into the cells is used to compare results. The following formula is
used to calculate the percentage of the $^{51}$Cr release:

$$\% \text{ Release} = \frac{^{51}\text{Cr in test materials or controls}}{^{51}\text{Cr in reference samples}} \times 100$$

This method measured changes in cell membrane permeability. Unlike the previous methods, this method provides complete cell-
material contact.

Other Monolayer Methods

1) In a method described by Spangberg in 1969, based on the
Bergman method (1963), a suspension of cells and test solution is
mixed in a culture chamber (glass ring fixed to a glass slide). At
the end of the experiment, the attached cells are fixed in Carnoy's
fixative and stained with Ehrlich's haemotoxylin. Cells attached to
the glass slide and the frequency of mitosis per field of vision are
counted microscopically.

2) Wennberg (1976) exposed cell monolayers established on the
bottom of glass vials to toxic substances. The incorporationof
tritiumlabeled thymidine ($^{3}\text{H}$-thymidine) into the cells is measured
after 30 minutes using a liquid scintillation counter.

The problem of using $^{3}\text{H}$-thymidine is that the thymidine can be
reutilized by other cells after cell lysis. This limits the accuracy
of this method.

3) Munaco et al. (1978) used ground particles of set materials
stored in saline up to five months. The supernatant fluid was drawn
off and replaced every two weeks. A suspension of these particles was
added to confluent bovine pulp cell monolayers in T-flasks. After an incubation period, the cells were fixed in neutral buffered formalin and stained using a dichrome technique. Areas of cellular death were measured between one and five days using an ocular square grid.

This method is problematic as the size of the particles is not controlled resulting in variable dispersion of materials. During such a long storage time, variable material extraction and microbial contamination may also be a problem. In this study, it is also to be expected that the toxic products of the materials would be removed as the toxicity of the materials are evaluated every other week when a suspension of the material had been washed.

4) Kasten et al. (1982) placed three test materials 1 mm from each other in a plastic culture dish and then added a cell suspension. After the incubation period, they counted the attached viable cells in four microscopic fields per sample at three different distances (3, 6 and 9 mm) from the test materials with the aid of a micrometer disc and phase optics.

Such a method is limited to the study of water soluble components. It depends on the subjective judgement of the investigator as the zone of damage and lysis are not always clearly defined. There is also no clear criteria for the determination of cell death. The differentiation of toxic concentration between several samples may also be difficult to separate as the toxic material will diffuse in an unpredictable manner due to turbulence in the culture medium.
The Red Blood Cell Method (Hemolysis Test)

The test material is placed in a test tube containing saline solution, and fresh rabbit blood is added. After incubation, the solution is centrifuged and decanted, and the optical density (OD) of the supernatant is determined using a spectrophotometer. Hemolytic activity on the basis of hemoglobin release is determined after certain time intervals (Dillingham et al. 1975, Autian 1974). The following formula is used to calculate the percentage of hemolysis:

\[
\text{% Hemolysis} = \left( \frac{\text{OD of test sample} - \text{OD of negative control}}{\text{OD of positive control} - \text{OD of negative control}} \right) \times 100
\]

This method assesses injury to cell membranes only, and its relevance is difficult to determine.

The Explant Method

In this method the test material is placed near a tissue explant (usually a connective tissue) and the migration and transformation of fibroblasts from the explants are observed by phase contrast microscopy. The relative outgrowth value obtained by measuring areas of enlargement of fibroblastic outgrowth from the explants is used as a toxic index (Kawahara et al. 1960 and 1963, Kellner et al. 1965).

As this method is dependent on diffusion of toxic products and the turbulence in the culture medium, substantial differences in response leading to ambiguity may occur.
The Metabolic Activity Method

**Oxygen Uptake**

This method measured the changes in oxygen uptake of cultured cells when exposed to toxic materials. Spangberg (1969b) measured the oxygen uptake of HeLa cells using a Warburg's apparatus and direct technique. The gas and temperature are equilibrated and recorded before starting the experiment (control) and after exposing the cells to the test material. The oxygen uptake is expressed relative to the respiration of the culture measured before adding the test material. A decrease in oxygen consumption is related to cell injury and toxicity. With this time consuming method, only liquid materials can be tested.

**DNA Analysis**

The materials to be tested are prepared in the form of a disk and fixed to the bottom of a plastic petri dish with silicone grease. A cell suspension is added and the culture incubated for 1, 3 and 6 days without changing the culture medium. After incubation, the cells are washed and detached from the surface of the materials by trypsin. The suspended cells are treated with ice-cold 5% trichloracetic for 20 minutes to remove low molecular weight components and again for 15 minutes in a boiling water bath.

The DNA content is measured in the hot acid extract (Leirskar & Helgeland 1972). They observed that the amount of DNA measured was lower in the experimental culture compared to the control.

This method is limited to the liquid material only.
Simulated Cavity Method

The simulated cavity method is a modification of the Millipore filter method introduced by Meryon et al. (1982). In this method a layer of compacted dentin powder or a slice of dentin is placed between the filter and the test material in order to reproduce a more realistic simulation of teeth in vivo. This method provides a reasonable approach for the evaluation of dental restorative materials as the technique brings into play the modifying effect of dentin on the toxicity of materials. The method requires an excessive amount of culture medium in the system reducing the dentin filtered toxic products of the filling material to a nondetectable level. Substantial improvement of this method is required before it will have a practical value.

Criteria of Cytotoxicity Evaluation

Evaluation of dental material cytotoxicity in vitro has been performed by many investigators using systems of cultured cells. The following criteria have been used.

Cell Counting

Mitotic inhibition and cell death occurs as a result of exposure to a toxic material. This leads to a reduction in cell numbers compared to control cultures. Therefore, the difference between the experimental and the control cell count is used as an indicator of cytotoxicity (Kawahara et al. 1968, Spangberg 1969a, Meryon et al. 1983). Such a criterion gives no indication of whether the cells are dead or alive. Furthermore, there are materials containing substances
known to act as fixatives that will affect the quantitation. Also, a reduction of the rate of cell mitosis is not necessarily an indicator of cell injury.

**Vital Staining**

The use of vital staining (Guess et al. 1965) either positively with neutral red stain, which is taken up by normal functioning cells (Mohammad et al. 1978), or negatively by trypan blue, which is taken up only by dead cells (Goldschmidt et al. 1976), have been used to differentiate between living and dead cells.

The disadvantage of using vital staining is that vital cells may stain with trypan and therefore be recorded as dead cells.

**Cell Morphology**

Light microscopic inspection of alteration of cell morphology, cell surface modifications and cell lysis were used as indicators of cell damage (Kawahara 1968 and 1979, Autian 1970).

**Metabolic and Enzyme Activity**

Spangberg (1969b) used a Warburg apparatus to record oxygen uptake by cells after being exposed to the test materials. Helgeland and Leirskar (1972) measured the amount of glucose utilized and lactate formed in the culture media and related them to cell growth, while Meryon and Riches (1982) measured the enzyme activities of beta-galactosidase (fluorimetrically) and lactate dehydrogenase (spectrophotometrically) in both media and cell containing fractions. On the other hand, Tyas (1977) and Meryon et al. (1983) measured the staining density of the cells (acid phosphatase for lysosomal enzyme or succinic dehydrogenase for mitochondrial enzyme) using a scanning
and integrating microdensitometer so any changes in metabolic activity would be accompanied by alterations in enzyme activity. Feigal et al. (1985) assessed the stain intensity visually using a dissection microscope.

Radioactive Markers

Changes of membrane permeability was measured by the $^{51}$Cr-release (Spangberg 1973) after prelabeling the cells with sodium chromate and exposing them to the dental materials. The $^{51}$Cr-release into the medium was quantitated and used as an indication of cell injury.

Changes in DNA synthesis by the cells is measured by recording the tritium-labeled thymidine ($^3$H-thymidine) uptake (Wennberg 1976). These changes are utilized as a criterion of toxic influence. The released thymidine can be reutilized by the cells which may introduce experimental errors.

Colony Formation

In this method, cell cultures are incubated with the test solution medium for 30 minutes, washed with fresh medium, and reincubated for seven days. The culture is stained with 2% crystal violet in 20% ethanol and the number of cell colonies are counted with an automated colony counter (Hensten-Pettersen & Helgeland 1981).

Cell Types Used in In Vitro Cytotoxicity Testing

Many sources of cells (aneuploid or diploid) have been used by investigators to evaluate the toxicity of dental materials using in
**vitro** techniques. L 929 and HeLa cells are the most commonly used. The cells listed below represent examples of cell types which have been commonly used.

### Established Cell Lines

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Type</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>L 929</td>
<td>Mouse skin fibroblast</td>
<td>Guess et al. 1965</td>
</tr>
<tr>
<td>L 60T</td>
<td>Mouse fibroblasts</td>
<td>Peters et al. 1972</td>
</tr>
<tr>
<td>3T3</td>
<td>Swiss mouse fibroblasts</td>
<td>Hanks et al. 1981</td>
</tr>
<tr>
<td>RT</td>
<td>Rat osteogenic sarcoma</td>
<td>Peters et al. 1972</td>
</tr>
<tr>
<td>BHK 21(C-13)</td>
<td>Syrian hamster kidney fibroblasts</td>
<td>Meryon et al. 1983</td>
</tr>
<tr>
<td>BHK 21/4</td>
<td>Baby hamster kidney cell line</td>
<td>Vander Wall 1972</td>
</tr>
<tr>
<td>VX2</td>
<td>Rabbit carcinoma</td>
<td>Peters et al. 1972</td>
</tr>
<tr>
<td>NCTC 2544</td>
<td>Human skin epithelium</td>
<td>Helgeland &amp; Leirskar 1972</td>
</tr>
<tr>
<td>HEL-199</td>
<td>Human embryonic lung</td>
<td>Vander Wall et al. 1972</td>
</tr>
<tr>
<td>HeLa</td>
<td>Human cervical carcinoma</td>
<td>Kapsimalis 1960</td>
</tr>
<tr>
<td>Raji</td>
<td>Human Burkitt lymphoma</td>
<td>Koskinen et al. 1981</td>
</tr>
<tr>
<td>Chang</td>
<td>Human liver epithelium</td>
<td>Potter &amp; Matrone 1977</td>
</tr>
<tr>
<td>KB</td>
<td>Human oral carcinoma</td>
<td>Antrim 1976</td>
</tr>
<tr>
<td>F/L &amp; WISH</td>
<td>Human amnion</td>
<td>Sisca et al. 1967</td>
</tr>
<tr>
<td>ca 9.22</td>
<td>Human gingival carcinoma</td>
<td>Imai et al. 1982</td>
</tr>
</tbody>
</table>

### Primary Culture

<table>
<thead>
<tr>
<th>Culture</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chick embryo liver fibroblasts</td>
<td>Verne 1954</td>
</tr>
<tr>
<td>Chick embryo heart fibroblasts</td>
<td>Verne 1954</td>
</tr>
<tr>
<td>Chick embryo kidney fibroblasts</td>
<td>Verne 1954</td>
</tr>
<tr>
<td>Chick embryo nervous tissue</td>
<td>Verne 1954</td>
</tr>
<tr>
<td>Human skin fibroblasts</td>
<td>Spangberg 1969c</td>
</tr>
<tr>
<td>Human gingival epithelium</td>
<td>Jacobsen 1977</td>
</tr>
</tbody>
</table>
Established cell lines have a heteroploid chromosome pattern and their response to toxic constituents may not reflect that of comparable cells in vivo which have a normal diploid chromosome pattern. As an example, established fibroblast cell lines in vitro do not secrete extracellular collagen indicating that their metabolism is different from that of fibroblasts in vivo.

Different cells exhibit varying susceptibility to a given toxic component. Different culture conditions, methods and media have been employed in experiments evaluating toxicity of dental materials, thereby influencing the outcome of the results.

Cell lines may have different abilities to metabolize toxic components of materials thereby rendering it less toxic leading to variability.

Direct comparisons of cell behavior and responses to dental materials have been made between established and primary cell lines. Hanks (1981) reported that established cell lines are more sensitive, whereas Meryon and Riches (1982) found that primary cell lines are more sensitive to the toxic effects of dental materials. Sisca et al. (1967) reported no differences in the results when two permanent cell lines (F/L & WISH cells) were used to test the toxic effect of dental
materials. On the other hand, Spangberg (1969b) compared the effect of root canal filling materials on HeLa cells and human embryonic fibroblasts and found that, in general, the fibroblasts were slightly less sensitive. Feigal et al. (1985) reported that normal diploid human pulp cells were significantly more sensitive when compared to L 929 cells. Leirskar and Helgeland (1972) compared the effect of different filling materials on L 929 and NCTC 2544 cells and found no difference. In another study by Hensten-Pettersen and Helgeland (1981), no difference was obtained when NCTC 2544 cells were compared with human gingival epithelium and fibroblasts under the same conditions. Sensitivity of the cells varied according to sera selection (Feigal et al. 1985) and the material tested (Spangberg 1973).

The toxicity of dental materials has been extensively studied; however, information about morphological changes of the injured cells due to material toxicity are rare.

The most commonly reported changes as a result of cell injury during cytotoxicity evaluation is cell detachment (Kawahara et al. 1979, Meryon et al. 1983). Cell shape is also altered. The spindle shape of fibroblasts change to a round appearance as a result of cytoplasmic shrinkage. These morphological changes have been described in the literature using phase contrast microscopy. Electron microscope results describing the ultrastructural changes in the cells in vitro, as a result of material injury, have not yet been reported.

Because of the lack of information in this area, it is of interest to compare the responses of human dental fibroblasts and the commonly used cell line (L 929) to dental materials.
**Human Dental Fibroblasts**

The development of the dental pulp begins at the 8th week of fetal life as a connective tissue condensation, the dental papilla, under the dental epithelium. This mesenchymal cell population will give rise to all of the structures in the tooth except the enamel, which is formed by the ameloblasts.

The dental papilla (the future dental pulp) controls early tooth formation. The structural specificity for tooth germ shape has been shown by Kollar and Baird (1969, 1970) to reside in the mesenchyme, which directs the morphodifferentiation of tooth patterns. The mesenchyme surrounding the outside of the developing tooth, the dental sac, condenses and becomes more fibrous.

The dental sac cells will form the tissues of the periodontium, which consists of the periodontal ligament (PDL), cementum, and alveolar bone.

**Cytology of Dental Fibroblasts**

Fibroblasts are the basic cells of the dental pulp and the periodontal ligament. Their morphology is similar to connective tissue fibroblasts seen elsewhere in the human body and they synthesize collagen. They are large and irregularly shaped cells with abundant cytoplasm. Characteristically, fibroblasts have numerous processes resulting in fusiform, spindle, polygonal or stellate shapes. The cell membrane is smooth and the nucleus is oval or kidney-shaped with small indentions and delicate evenly distributed chromatin. The nuclei contain one or more nucleoli (Movat & Fernando 1962, Engel et al. 1980).
In the dental papilla during development, the fibroblasts are large and rounded or polyhedral with well-defined nuclei that stain deeply with basic dyes and lightly staining cytoplasm which appears homogenous. As the pulp matures, the cells take on a stellate-shape. The multiple processes of the cells extend to adjacent cells and establish intracellular junctions or cell to cell contacts (Engel et al. 1980). The cells of the inner layer of the dental follicle, that is derived from and continuous with with dental papilla around the cervical loop, have the characteristics of undifferentiated fibroblasts, and they give rise to periodontal ligament fibroblasts and alveolar bone (Freeman & Ten Cate 1971).

**Ultrastructure of Dental Fibroblasts**

According to Griffin & Harris (1966), fibroblasts isolated from dental pulp of unerupted human teeth are recognized in vivo by the dilated profiles of the rough-surfaced endoplasmic reticulum, intracellular filaments, swollen mitochondria with irregular bodies. The cell bodies of the early differentiated fibroblasts are polygonal in shape and contain a number of vesicles that appear on the surface of the endoplasmic reticulum. When pulpal fibroblasts mature, the cell bodies become stellate in shape with irregular processes (Han et al. 1965). Three types of vesicles can be seen:

1) Pinocytotic vesicles described by Goldberg & Green (1964) and Engel et al. (1980) are seen only within the cell and appear to arise from invaginations of the plasma membrane.

2) Secretory vesicles which are smaller than pinocytotic vesicles appear to be derived from the Golgi complex. They contain
amorphous, moderately electron-dense material, and can be seen adjacent to the plasma membrane externally and intracellularly.

3) Cisternal vesicles derived from the dilated sacs of the rough-surfaced endoplasmic reticulum contain coiled, irregularly beaded filaments (Griffin & Harris 1966, Engel et al. 1980).

Variable numbers of ribosomes (rows and clusters) can be seen scattered freely throughout the cytoplasm during the early stage of differentiation and later between the intracellular fibrils.

The Golgi complex is poorly developed during early differentiation; but, as the cells mature, an extensively developed stack of membranes and a large number of vesicles can be seen.

Mitochondria during the early stages are small and variable in number and mostly of oval to elongated shape with irregular cristae. As the cells grow, the mitochondria become large, numerous, and elongated in shape with straight cristae.

The cytoplasmic ground substance is dense and contains varying numbers of intracellular fibrils. Collagen fibers are present on the cell body and on the processes.

Ultrastructurally, fibroblasts characteristically have large nuclei with one or more prominent nucleoli (Ross 1966, Engel et al. 1980). The nuclei during early differentiation are round to oval in shape and have a smooth contour; but, as the cells grow, the nuclei become oval or elongated in shape with irregular nuclear membranes.

A cilium is frequently found near the nucleus, and an additional centriole may be located perpendicular to the long axis of the cilium (Han et al. 1965, Griffin & Harris 1966). The cilia consist of a basal body, ciliary shaft and sheath.
Membrane-bound lysosome-like bodies containing a highly electron dense, homogeneous or lamellated material are prominent components of fibroblasts (Engel et al. 1980).

**Dental Fibroblasts in Culture**

Cell culture *in vitro* is a technique that maintains specific cell types in culture medium where they form a single layer of cells, a monolayer, on the bottom of the culture dish. Human fibroblasts, *in vitro*, have a finite growth potential. After varying periods of duplication, usually $50 \pm 10$ cell doublings, they eventually cease to divide and die (Hayflick & Moorhead 1961, Hayflick 1965). The causes of this were unrelated to conditions of cell culture, the media composition used, or the presence of mycoplasma or latent viruses (Hayflick & Moorhead 1961, Todaro et al. 1963). Rather, it was directly related to mitotic activity of the cells and to the numbers of subcultivations at a particular split ratio.

Human gingival fibroblasts *in vitro* elaborate banded and unbanded collagen fibrils (Rose & Robertson 1977, Yajima et al. 1980) and preserve their normal morphological structure (DeRenzis & Chen 1983).

Kroeger et al (1961) cultured pulpal cells of newborn mouse molar teeth in plasma clots and classified them into three groups:

1) spindle-shaped cells (fibroblast-like),
2) round cells with or without visible protoplasmic extension, and
3) polygonal cells with a long protoplasmic process (odontoblast-like).
They concluded that pulp cells grown in vitro lose many of their morphological characteristics especially at the glass-clot interface where they tend to flatten and spread out.

Arnold & Baram (1972) cultured an explant of periodontal ligament tissue using chicken plasma clot. Proliferation of fibroblasts from explant was observed after 5 to 20 days. These fibroblasts were maintained for three months by subculturing at weekly or biweekly intervals.

Brunette et al. (1976) cultured an explant of periodontal ligament tissue using culture medium. Outgrowth of cells was seen after 4 to 14 days. The cells were of two morphological types, fibroblast-like and epithelium-like. The fibroblast-like cells were spindle- to roughly trapezoid-shaped.

The fibroblast-like cells tend to take over in mixed culture (Gilbert & Migeon 1975). The reason for this is not understood.

Soder et al. (1979) cultured an explant of attached gingiva and after two weeks cultivation, fibroblast-like cells were observed forming colonies throughout the culture flask (Brunette et al. 1976). These fibroblast-like cells varied from spindle to roughly trapezoid-shape with ovoid-shaped nuclei, circular or rod-like mitochondria, well-developed Golgi complexes of well-preserved cristae, and evenly distributed rough-surfaced endoplasmic reticulum in the cytoplasm. In general, the behavior of human fibroblasts of dental origin in vitro is similar to those of other human tissues.
Mouse Skin Fibroblasts (L 929)

L 929 cell lines in tissue culture have a stellate or spindle shape with round or oval nuclei. They do not secrete extracellular collagen. After about three months in culture, they invariably show evidence of spontaneous transformation; they begin to multiply faster until they eventually grow at a steady fast rate (Paul 1970). The chromosomes double in number after a few passages and then become aneuploid. Most L cells acquired polygonal or rounded shape in dense culture (Domnia et al. 1972). This is possibly due to retraction of the main processes after contact with other cells.

There are two main differences between L cells and normal mouse fibroblasts during early stages of spreading. First, at the stage of initial cell-substratum contact, L cells have numerous micovilli at the cell surface in contact with the smooth surface of normal cells (Willingham & Pastan 1975, Cherny et al. 1975). These microvilli allow the cells to attach to the substrate and to other cells. Second, many L cells form small lamellar cytoplasm which spread in several directions so that the cells acquired a stellate or fusiform shape. Some cells do not form lamellar cytoplasm and have an elongated or spindle shape (Vasiliev & Gelfand 1977).
MATERIALS AND METHODS

Cells

L 929 Cell Line

Three- to five-day old culture of L 929 cells was used (Flow Lab). The cells were suspended in culture medium at a density of 5 x 10^5 cells per milliliter. The culture medium was changed every other day and the day before an experiment. Cells were harvested with 0.02% trypsin in phosphate buffered saline (PBS) by incubation at 37°C and 100% humidity for 5 to 10 minutes.

Human Periodontal Ligament Fibroblasts

Periodontal tissue was obtained from a maxillary premolar tooth extracted during orthodontic therapy from a 14 year old male patient. After extraction, the tooth was stored in PBS. The periodontal ligament tissue was scraped off, washed several times with PBS, and then cut into small pieces with scissors. The tissue fragments were washed again with PBS, suspended in culture medium, and centrifuged at 100xg for 5 minutes. The tissue fragments were transferred into culture flasks and allowed to settle on the bottom of a culture flask using a small amount of medium. The flasks were placed in a humidified incubator at 37°C for 30 minutes to allow the tissue fragments to attach to the plastic substrate. More medium was added, and incubation continued for one week. The fourth subculture was used.
cells were suspended in culture medium at a density of $5 \times 10^5$ cells per milliliter. The cells were grown and harvested in the same manner as the L 929.

**Culture Medium**

Eagle's MEM with Earl's BSS (Flow Laboratory), supplemented with 10% (v/v) fetal calf serum, 2mM L-glutamine and 2.2mg sodium bicarbonate per ml was used. In addition, 100 I.U/ml penicillin and 50 ug/ml streptomycin were added to the culture medium (Spangberg 1973).

**Cell Labeling**

$^{51}$Cr was supplied as sodium chromate in a sterile isotonic solution. The activity of the sodium chromate was 350 to 450mC per mg. The cells were labeled with approximately 2 uC per $10^5$ cells 12 to 20 hours before the experiment. The radiochromium labeling procedure described by Spangberg (1973) was followed.

**Test Material**

Cold-curing acrylic resin (Coldpac, Chicago, IL. Batch #11137) was mixed in a powder/liquid ratio of 2:1 according to the manufacturer's directions. Three grams of this mixture was spread over the whole bottom surface of 55 cm$^2$ culture dishes. Twenty ml of culture medium was added and the dishes were stored refrigerated for 24 hours. The culture medium containing the toxic component of the acrylic resin was collected and stored at +4°C in glass bottles. At the time of experiment, the toxic component of the
acrylic was filtered and diluted with culture medium to the following concentrations: 1, 1/2, 1/4, 1/8, 1/16, 1/32, 1/64, 1/128, 1/256, and 1/512.

**Experimental Procedures**

The labeled cells were harvested with 0.02% trypsin and washed in PBS solution before being suspended in culture medium. The cell suspension was washed and centrifuged in culture medium four times at 500xg. The experiments were performed in plastic tissue culture clusters (Costar, Cambridge, MA, USA) containing 24 wells each with an inner diameter of 16mm. The experimental tissue cultures were divided into two sets. The first set used for the chromium release counting and cell recovery evaluation and the second set for light and electron microscopic examination. One ml of cell suspension was mixed with one ml of various concentrations of the media conditioned by the acrylic resin in the culture well and incubated at 37°C and 100% humidity for two and four hours. At the same time, one ml of the cell suspension and one ml of the culture medium were added to culture wells with no test materials to serve as negative controls. At the end of the incubation period, one ml of culture medium was withdrawn from each culture well and transferred to test tubes. These tubes were centrifuged for 10 minutes at 500xg after which 0.5 ml of the supernatant in each test tube was withdrawn and counted for one minute in a Beckman 5500 gamma particle counter (t-samples). During dispersion of the cells, 0.5 ml samples were withdrawn randomly. These samples (r-samples) were used as reference points for calculating the 51Cr-release in the experiments.
Release \( \% = \frac{2 \times {^{51}}\text{Cr in t-samples}}{ {^{51}}\text{Cr in r-samples}} \times 100 \)

The percentage of \( {^{51}}\text{Cr-release} \) was calculated on the basis of the total amount incorporated in the target cells (Spangberg 1973).

To determine if cells could recover from the toxic effects of the test material, the remaining cultures were washed with fresh medium twice at the end of the incubation period. Two ml of fresh medium were added to each culture and the recovery process was evaluated after 24 hours and 48 hours.

**Microscopic Sample Preparation**

**Light Microscopy**

Cell cultures of L 929 and human PDL fibroblast were examined and photographed in a Zeiss phase contrast microscope.

**Scanning Electron Microscope (SEM)**

The cell cultures (L 929 and PDL fibroblasts) were prefixed in 0.1% glutaraldehyde in tissue culture medium for 5 minutes. The medium was then decanted and replaced with 2.0% glutaraldehyde in 100mM Na-cacodylate buffer (pH 7.2) at room temperature and fixed for half an hour in the same solution. Specimens were dehydrated (5 minutes each) through 50%, 70%, 90%, and 100% ethanol, then critical-point dried with \( \text{CO}_2 \). The tissue culture cluster wells were trimmed, mounted using silver conducting paint, and gold-sputter coated to a thickness of \( \sim 5-7\) nm. Specimens were then examined with a Hitachi H300 equipped with a H3010 scanning attachment, operated at an accelerating voltage of 20Kv.
Transmission Electron Microscopy (TEM)

The cell cultures were prefixed in 0.1% glutaraldehyde in tissue culture medium for 5 minutes. The medium was then decanted and replaced with 2.0% glutaraldehyde in 100mM Na-cacodylate buffer (pH 7.2) at room temperature and fixed for 5 minutes in the same solution. An equal volume of 2.0% osmium tetraoxide in 100mM cacodylate buffer (pH 7.2) was added and the culture fixed for one hour followed by three 5 minute washes with distilled water. The cells were stained with 1% uranyl acetate for 30 minutes, and then washed three times (10 minutes each) with distilled water. The specimens were dehydrated (10 minutes each) in a graded series of 50%, 70%, 90%, 95%, and two 100% ethanol washes. Cells were infiltrated in 1:1 poly Bed:ethanol for one hour, then replaced with fresh poly Bed (Polysciences, Inc., Warrington, PA). The resin was changed again after one hour and left overnight. Finally, the specimens were embedded in fresh poly Bed and cured in a 60°C oven for 24 hrs. Semi-thin sections were cut with a glass knife, stained and viewed with the light microscope to check the general condition and orientation of the specimen and to select areas of interest. Thin sections were cut with a diamond knife, mounted on 300 mesh copper grids, stained with uranyl acetate and lead citrate, and examined with a Zeiss EM10 at 80Kv accelerating voltage.

Unlabeled cells grown under control culture conditions were prepared for light and electron microscopy in the same manner.
RESULTS

Morphology of Normal Cells

PDL Fibroblasts

Figures 1 and 2 illustrate the cytological features of PDL fibroblasts in vitro. Fibroblasts were elongated, spindle-shaped with tapering cytoplasmic extensions (Fig. 1B). The outer surface of the cell was generally smooth but sometimes covered with microvilli (Fig. 1C). The nuclei appeared ovoid with a double membrane. The nucleoli were well developed and sometimes more than one could be seen. Mitochondria were elongated, round or rod-like in shape with double membranes and well-preserved cristae. The rough endoplasmic reticulum comprised a network of narrow cisternae distributed around the nucleus and in the cytoplasm (Fig. 2). Free ribosomes were widely distributed and some attached to the surface of the endoplasmic reticulum. A number of fine filaments or microtubules were distributed in the cytoplasm. Several well-developed Golgi complexes were seen composed of arrays of packed cisternae and numerous small vesicles (Fig. 2). Membrane-bound lysosome-like bodies were prominent components of most cells. Polyribosomes and vacuoles within the cytoplasm were occasionally observed.
L 929 Cells

Figures 3 and 4 illustrate the cytological features of L 929 cells. The cells were elongated, spindle- or fusiform-shaped. Dividing cells appeared round in shape and were observed scattered throughout the culture (Fig. 3A, 3C). The outer surface of the cells were covered with large numbers of hemisphere-shaped projections (blebs). Few microvilli were observed (Fig. 3B, 3C). Mitochondria with double membranes appeared circular or rod-like in shape. Cristae were well preserved. The nuclei occupied most of the cell, had double membranes (Fig. 4), and were elongated or round shaped with more than one nucleoli. The chromatin was evenly distributed in the nucleolus. Lysosomes, polysomes and vacuoles were occasionally observed. A network of rough-surfaced endoplasmic reticulum was distributed in the cytoplasm. Ribosomes were seen on the surface of the endoplasmic reticulum and in the cytoplasm. Golgi complexes were occasionally seen.

Effect of Chromatin Labeling on Cell Morphology

PDL Fibroblast Labeled Cells

Figures 5 through 8 illustrate the effect of $^{51}$Cr on the PDL cell morphology. After two hours of incubation, the labeled control cells were either spherical or irregularly elongated spindle shape and the cell body covered with microvilli (Fig. 5A, 5B).

After an additional two hours of incubation, the cells were spread and exhibit a spindle to elongated shape with numerous microvilli on the cell body (Fig. 7C). Some cells had smooth surfaces (Fig. 7B). Lamellar cytoplasm was seen around the cell body at both 2
and 4 hours of incubation (Fig. 5B, 7B), and the intracellular organells were normal and well developed (Fig. 6, 8).

**L 929 Labeled Cells**

Figures 9 through 12 illustrate the $^{51}$Cr effect on the L 929 cell morphology. After two hours of incubation, the labeled control cells exhibited a fusiform, stellate or spindle-like profile (Fig. 9). A number of rounded cells were seen. Most of the cells had microvilli, and some cells had blebs on the surface. The internal structure of the cells were normal (Fig. 10). Occasionally, mitotic figures, Golgi complexes and vacuoles were observed.

After an additional two hours of incubation, the cells were spindle-shaped and covered with microvilli (Fig. 11). Mitotic figures were seen more often (Fig. 11A), and the intracellular structure was well developed (Fig. 12).

**Effect of the Test Solutions on Cell Morphology**

**PDL Fibroblasts**

Figures 13 through 22 illustrate the effect of the test solution on the PDL cell morphology. The two hour exposure to the full strength acrylic test solution (stock solution) caused the cells to round up and sometimes detach from the substratum (Fig. 13A, 15A). After two hours exposure to the stock solution, the cells had a "fried egg-like" appearance with ruffles covering the cell border (Fig. 13C). Myelin figures, numerous vacuoles, and autophagic vacuoles were observed (Fig. 14). The Golgi complex was difficult to identify.
Mitochondria and lysosomes were occasionally seen. The nuclei and endoplasmic reticulum appeared normal. Cells with preserved normal structure were seen.

When the exposure time was increased to four hours, the destruction of the cells became more severe. Cells were roughly round with blebs covering the cell border (Fig. 18A, B). Rough endoplasmic reticulum was occasionally seen. The Golgi complex appeared to be disorganized and often difficult to identify. The number and size of the vacuoles increased (Fig. 19A). Lysosomes, myelin figures and autophagic vacuoles were observed (Fig. 19B). Mitochondria were occasionally seen. The nuclei appeared normal. Some normal cells similar to the control were observed.

The general morphology of the cells, when exposed to a four time dilution of the test solution, was round and covered with ruffles or blebs (Fig. 15, 20). Many lysosomes and vacuoles were present. More Golgi complexes were seen at two hour exposure (Fig. 16). Nuclei with double membranes and mitochondria appeared normal (Fig. 16, 21A). Endoplasmic reticulum appeared swollen at four hours exposure (Fig. 21B).

When the concentration of the test solution decreased, the damage became less at both exposure times and the cells preserved their normal morphology (Fig. 17, 22).

**L 929 Cells**

Figures 23 through 34 illustrate the effect of the test solution on the L 929 cell morphology. When the L 929 cells were exposed to
the acrylic test solution, a number of changes occurred. In general, the cells rounded up and often detached from the substratum (Fig. 23A, 25A, 29A, 31A).

After a two hour exposure to stock solution, a number of small and large vacuoles and autophagic vacuoles were seen (Fig. 24A). The cytoplasm appeared less dense. Golgi complexes, mitochondria and endoplasmic reticulum were difficult to identify (Fig. 24B). The outer surface of the cells were covered with blebs and microvilli (Fig. 23B, 23C).

The four hour exposure to the stock solution caused greater disturbance of the cell nuclei. Mitochondria were completely disorganized. The Golgi complex and cell membrane had disappeared (Fig. 30A, 30B). The endoplasmic reticulum was swollen. The outer surface of the cells was covered with blebs (Fig. 29C). Very few cells were not affected and few were similar to the control cells (Fig. 30C).

At the four time dilution of the test solution, the damage to the cells was less at both exposure times. The general appearance of the morphology of the cells was round and covered with blebs (Fig. 25B, 31B). Few cells had microvilli (Fig. 25C). Nuclei, mitochondria and endoplasmic reticulum appeared normal (Fig. 26, 32). More vacuoles were observed at a four-hour exposure.

When the concentration of the test solution decreased further, the damage became less at both exposure times and the cells preserved their normal morphology (Fig. 27, 28, 33, 34).
**Chromium Release**

The results are summarized in Tables I and II.

**PDL Fibroblasts**

The spontaneous release in the control values was 3.6 ± 0.6 percent after two hours and 6.6 ± 0.3 percent in the four hour experiments. The amount of $^{51}$Cr-release at two and four hours was approximately 15 percentage points above the control cultures when the stock solution was tested. For the remaining concentrations, the $^{51}$Cr-release percent was equal to the control (Table I).

**L 929 Cells**

The spontaneous release in the control cultures was 5.9 ± 0.6 percent after two hours and 8.5 ± 0.5 percent in the four hour experiments. Using the stock solution, the amount of $^{51}$Cr-release was approximately 17 percentage points above the control after two hours of evaluation. After four hours of exposure, the radiochromium release increased 41 percentage points over the control value. For the remaining concentrations, the $^{51}$Cr-release was similar to the control (Table II).

**Cell Recovery**

**PDL Fibroblasts**

Cell growth continued when the test solution was replaced with culture medium. A confluent monolayer of cells developed in two days (Fig. 35A). The cell morphology was normal and appeared elongated or spindle-shaped (Fig. 35B).
Cell recovery was stronger after the two hour exposure to materials than for the cultures which had been exposed for four hours to the toxic substance.

L 929 Cells

Cell growth continued after both exposure time when the test solution was replaced with culture medium. Substantial cell growth could be observed after two days of incubation (Fig. 36A). The cells were spindle-shaped and mitotic figures were seen (Fig. 36A, 36B).

Cell recovery was more advanced after two hours exposure to the toxic test material than for the cultures that had been exposed for four hours (Fig. 36B).
DISCUSSION

The culture condition was suitable for the periodontal ligament fibroblasts to grow and the morphology of the cells was similar to animal periodontal ligament fibroblast cultures reported by other investigators (Arnold & Baram 1972, Brunette et al. 1976).

The use of the fourth subculture of the PDL fibroblasts proved to be practical and relatively free of tissue remnants. At this time, minimal alteration of morphology and function due to cultivation will have occurred.

The presence of microvilli covering the outer surfaces of the unlabeled PDL fibroblasts was believed to be important in providing sufficient mechanical stability that maintained the structural integrity of the fibroblasts in vitro (Vasiliev & Gelfand 1977).

The frequency of Golgi complexes observed was lower in cultured L 929 cells than PDL fibroblasts. The Golgi complexes in the former, in fact, were sometimes difficult to identify. The Golgi complex plays an important part in the secretory process which is required for the final stages in the processing and transportation of protein to specific sites in the cell (Sheldon & Kimball 1962). The absence of Golgi complexes in L 929 cells may explain the absence of extracellular collagen.

Of the 75 uC of $^{51}$Cr used to label a 75 cm$^2$ culture flask, about 5 percent of the isotopes were usually taken up by the cells.
during an overnight labeling. Consequently, when the culture medium was poured off at the end of the labeling period, 95 percent of the radioactivity was discarded. This resulted in less than 0.15 uC per ml of radiation in the cell suspension used for the experiments. Thus the amount of sodium chromate remaining in the culture after completed labeling was approximately $4 \times 10^{-7}$ mg/ml. This was very low and nontoxic to the cells as observed by Eadie & Brown (1955), Gibson & Scheitlin (1955), and Spangberg (1973). It has been established that approximately 1 mg/ml of sodium chromate can be tolerated in culture before toxic responses are observed (Spangberg & Al-Nazhan 1987).

The labeled L 929 cells of the control cultures spread faster than the labeled PDL cells after two hours or four hours incubation. They exhibit a spindle-like elongated shape, whereas the PDL cells were roughly elongated and had areas of lamellar cytoplasm (the lamelloplasm) surrounding the cell structure (the endoplasm). According to Vasiliev & Gelfand (1977), the lamelloplasm contains no particulate organelles and the endoplasm contains the nucleus and the remaining organelles.

Both cell types were covered with microvilli which was important for their attachment to the substrate to provide good spreading. The presence of the microvilli was a characteristic feature of the spreading of L 929 cells (Vasiliev & Gelfand 1977).

As the cells begin to spread, the microvilli shorten and decrease in number (Erickson & Trinkaus 1976). The degree of spreading varies with time, and therefore the shape of the spreading cells varied from one cell to another. After a two hour exposure to the stock solution, changes in the general cell morphology were seen.
Both cell types become round in shape as a result of cytoplasmic shrinkage sometimes causing the cells to detach from the substrate. Small to large vacuoles of the cytoplasm were present in both cell types. The extent of vacuolization was directly proportional to the concentration of the test solution and the exposure time (Belkin et al. 1962, Yang et al. 1965). Using phase contrast microscopy, Lettre (1954) and Berliner et al. (1967) studied the action of some drugs on fibroblasts and reported that as the cells round up, cell processes shorten up, vacuoles appear, and bubbling occurs at the cell surface. Observations made in this study confirmed these data.

Total cell lysis of individual cells was observed only in the cultures of L 929 cells and only when the exposure time was increased to four hours at high concentration of test solution (Fig. 30A). All of the structural organelles of L 929 cells were seriously damaged to a degree beyond identification. Such a severe degree of damage inhibits cell division and metabolism.

The presence of blebs on the surfaces of injured cells may be due to cytoplasmic shrinkage. Breaks of the cell membranes occurred in these blebs resulting in excretion of cell content including organelles (Fig. 26).

When the labeled cells were exposed to the higher concentration of the acrylic test solution, an increase of radiochromium release was observed. Twenty percent was recorded for the PDL cells and 50 percent for the L 929 cells. This amount of chromium found in the culture medium reflects the degree of cell damage caused by the test material. This result indicated that the L 929 cells were more sensitive to this toxic material than the PDL cells.
When the PDL fibroblasts were exposed to low concentrations of the test solution, the chromium release value was similar to the control. The radiochromium release pattern was different and did not follow a clear dose-response pattern. With increased concentration of test solution, the release of radiochromium first decreased below the control values before dramatically increasing at full strength concentration (Table II). Such decrease of release has been observed earlier (Spangberg 1973) and is specific for L 929 cells. The decrease was an indication of early cell injury (Fig. 24A).

The amount of chromium release is dependent upon the evaluated substance or material, cell type, the cell-material contact, and the exposure time (Kokubu & Pollak 1962, Spangberg 1973).

Spangberg (1973) suggested that 70 percent chromium release was an indicator of total cell lysis. In this study, the $^{51}$Cr-release was always below the seventy percent level indicating that total cell lysis may not have occurred. This was also confirmed by transmission electron microscopy.

The lower degree of cell injury for the PDL cells compared to the L 929 cells, as recorded by radiochromium release, was also observed microscopically.

When the test solutions were replaced by fresh culture medium and incubated for one to two days, a substantial recovery was observed. The recovery was better for the PDL cells compared to the L 929 cells.

This higher release of radiochromium, more morphological damage, and less recovery for the L 929 cells compared to the PDL cells indicates a greater sensitivity by the L 929 cells to this specific
toxic material. Such a difference may be due to several factors. Thus, the culture may consist of several subpopulations with various degrees of sensitivity to this toxic agent or the agent may selectively affect one specific cell function. The waiting period between cell divisions is significantly shorter for the L 929 cell compared to the PDL cell. Thus, if the toxic substance causes specific injury to the cell during its cell dividing cell cycle, it is more likely that the more rapidly dividing cell will be more exposed to a specific metabolic injury than a cell with a longer waiting period.

The results also indicate that the recorded release of radiocromium does not reflect injury of similar degree to all cells in the population. The observation shows that some cells were severely damaged when other cells seem totally unaltered. Thus, the release seems to reflect the relative frequency of severely damaged cells. The resulting release of the label during an experiment seems to be the combined effect of a low level of spontaneous release at a level of approximately one to two percent per hour of experimental time (Spangberg 1973) and the release from irreversibly altered cells. This observation has not been reported earlier.

Spangberg (1973) described the degree of cell damage as uniform when performing experiments with phenol at various concentrations. Phenol is a strong cytotoxic agent which coagulates cellular proteins. Such toxic injury to cells may very well be equal throughout the cell population as the protein coagulation process is unrelated to the various metabolic stages of the cell cycle. The results of the present study using extracted toxic component of a freshly prepared polymethacrylate material indicate that the toxic effect of this
material on cells is more specific and may leave certain cells unaffected. Such a conclusion leads to the need for further studies of the toxic mechanisms when using cytotoxicity assays in vitro.

Hank et al. (1981) compared the response of human PDL fibroblasts and the 3T3 cell line to a number of dental cements. They found that PDL fibroblasts were less severely affected than 3T3 cells. They relate that to altered cell membrane properties of 3T3 cells.

Guess et al. (1965), using the agar overlay method, found that chick embryo fibroblasts were less sensitive to toxic materials than L 929 cells.

Meryon & Riches (1982), using the Millipore filter method, found that mouse macrophages were more sensitive than syrian hamster kidney fibroblasts.

Koskinen et al. (1981), using the $^{51}$Cr-release method, found that human lymphocytes were more sensitive than human skin fibroblasts. They related their findings to the strong adherence of the fibroblasts to the substrate and to the differences in morphology. These discussions, relative to what cell line was more or less sensitive to cytotoxic agents, must be reevaluated after the findings presented in these experiments. The confusion in the literature related to sensitivity is most likely caused by the great variations of the test materials used in such tests. Depending upon cytotoxic mechanisms, various cells will be affected with different severity depending upon the target mechanism for the cytotoxic action.
CONCLUSIONS

Based on observations from this study, it would appear that:

1. There are no differences between the basic ultrastructure of the periodontal ligament fibroblasts and the L 929 cells in culture. The only difference is in the frequency of Golgi complex. It is lower in cultured L 929 cells than in PDL fibroblasts. The shape of the outer surface of L 929 cells is covered with blebs, where the PDL fibroblast is covered with microvilli.

2. The PDL fibroblasts are less sensitive cells compared to the L 929 cells when used to evaluate cytotoxicity of an acrylic test solution.

3. The chromium labeling has no detectable effect on the PDL or L 929 cell morphology or ultrastructure.

4. The labeled L 929 cells of the control culture spread faster at two and four hours incubation than the PDL fibroblasts. They are spindle-shaped, where the PDL fibroblasts are roughly spindle-shaped and the cell body surrounded with lamellar cytoplasm.

5. There is a clear relationship between the $^{51}$Cr-release and the degree of cell damage.

6. Surviving cells could recover. Such recovery of PDL fibroblasts and L 929 cells took place in one to two days after replacing the acrylic test solution with fresh culture medium.
Figure 1.

A. Phase contrast of confluent monolayer of elongated periodontal ligament fibroblasts grown in tissue culture for 5 days. Note the parallel orientation of the fibroblasts. (Mag. 20 X 10).

B. A spindle-shaped PDL fibroblasts with oval-shaped nuclei and two nucleoli. (32 X 10).

C. Scanning electron micrograph of PDL fibroblasts grown in tissue culture for 5 days. The cells are elongated in shape and covered with microvilli. Notice the parallel arrangement of the cells. (2 X $10^3$).
Figure 2.

Electron micrographs of normal PDL fibroblast grown in tissue culture for 5 days. Nucleus with nucleoli, mitochondria (m), endoplasmic reticulum (er), Golgi complex (G) and lysosomes (L) are well developed. (10 X 10³).
Figure 3.

A. Phase contrast micrograph of monolayer culture of L 929 cells grown in control culture for 5 days. The cells are spindle, fusiform in shape. Large number of cell mitosis (arrow) are seen. (32 X 10).

B. Scanning electron micrograph of L 929 cells grown in tissue culture for 5 days. Cells have a spindle-shape with blebs covering the cell body. (5 X 10^3).

C. Cell mitosis covered with microvilli for cell-substrate attachment. (2 X 10^3).
Figure 4.

Electron micrograph of normal L 929 cells grown in tissue culture for 5 days. Nucleus has double membrane (arrow). Well-developed and round shaped mitochondria (m) and endoplasmic reticulum (er) are seen. Golgi complex (G) and vacuoles are occasionally seen. (16 X 10^3).
Figure 5.

A. Phase contrast micrograph of nontreated PDL fibroblasts labeled with $^{51}$Cr after 2 hrs of dispensation. The cells spread nicely and started to form the normal spindle-shape morphology. Round cells of mitosis are seen. (32 X 10).

B. Scanning electron micrograph of PDL fibroblast labeled with $^{51}$Cr after 2 hrs of dispensation. The cell started to form the spindle-shape morphology. Numerous microvilli are seen at the left side of the cell body. Lamellar cytoplasm is seen at the peripheries of the right side of the cell. (1 X $10^3$).
Figure 6.

Electron micrograph of 2 hr control of nontreated PDL fibroblasts labeled with $^{51}$Cr.

A. The nucleus has double membrane (arrow). Microchondria, Golgi complex, endoplasmic reticulum and lysosome are well developed. (16 X $10^3$).
B. Nucleus of irregular-shape. More than one nucleoli are seen. (10 X 10^3).
Figure 7.

A. Phase contrast micrograph of 4 hr control of nontreated PDL fibroblasts labeled with $^{51}$Cr. The spindle, fusiform-shape of the cells and cell mitosis are seen (arrow). (32 X 10).

B. Scanning electron micrograph of 4 hr control of nontreated PDL fibroblast labeled with $^{51}$Cr. Cells are elongated or fusiform-shaped. Lamellipodia surrounding the cell body. (500).

C. Spindle-shape cell. Numerous microvilli covered the cell body and Lamellipodia surrounding the cell body. (1 X 10$^3$).
Figure 8.

Electron micrograph of 4 hr control of nontreated PDL fibroblast labeled with $^{51}$Cr. All of the intrastructural organelles are well developed. ($8 \times 10^3$).
Figure 9.

Scanning micrograph of 2 hr control of nontreated L 929 cells labeled with $^{51}$Cr.

A. Cell is spindle, stellate or fusiform-shaped. Microvilli covered the cell body. Note the ruffles near the tapering ends of the cell. ($2 \times 10^3$).

B. Stellate-shape and round cells are seen. ($1 \times 10^3$).
Figure 10.

Electron micrograph of 2 hr control of nontreated L 929 cells labeled with $^{51}$Cr. Nucleus and well-developed Golgi complex (G) are seen. (6.3 X $10^3$).
Figure 11.

A. Phase contrast microscopy of 4 hr control of nontreated L 929 cells labeled with $^{51}$Cr. Cells have normal morphology of spindle-shape and mitoses are seen (arrow). (20 X 10).

B. Scanning electron micrograph of 4 hr control of nontreated L 929 cells labeled with $^{51}$Cr. Cells are spindle-shaped, covered with microvilli for attachment. (1 X $10^3$).

C. Note ruffles at the end of cell edge at the upper right corner and at the other cells. (2 X $10^3$).
Figure 12.

Electron micrograph of 4 hr control of nontreated L 929 cell labeled with $^{51}$Cr. All the intrastructural organelles are seen. ($8 \times 10^3$).
Figure 13.

A. Phase contrast microscopy exposed for 2 hr to the stock solution. A large number of floating round cells detaching from the substrate. Cells with the normal spindle-shape morphology are seen. (Mag. 32 X 10).

B. Scanning micrograph of PDL fibroblast exposed for 2 hrs to the stock solution. Fried egg-like shaped cell with lamellipodia and ruffles around the cell body. (1 X 10³).
Figure 14.

PDL fibroblasts exposed for 2 hr to the stock solution. Myelin figure (mf), large vacuoles and autophagic vacuoles (Au) are seen. Numerous profiles of endoplasmic reticulum scattered through the cytoplasm. (8 X 10^3).
Figure 15.

A. Phase contrast microscopy of PDL fibroblasts exposed for 2 hr to 1:1/4 concentration of the test solution. Number of floating round-shaped and spreading cells of spindle-shape are seen. (32 X 10).

B. Scanning micrograph of PDL fibroblasts exposed for 2 hr to 1:1/4 concentration of the test solution. The cells are covered with blebs. Note division of the cell body into peripheral lamelloplasm and central endoplasm. Microvilli are seen at the peripheries of the endoplasm. (1 X 10^3).

C. Ruffles at the cell edges. (1 X 10^3).
Figure 16.

Electron micrograph of PDL fibroblasts exposed for 2 hr to 1:1/4 concentration of test solution. Nucleus with double membrane (arrow), mitochondria, Golgi complex, endoplasmic reticulum, large number of lysosomes and small vacuoles are seen. (8 X 10³).
Figure 17.

A. Phase contrast micrograph of PDL fibroblasts exposed for 2 hr to 1:1/512 concentration of the test solution. Cells spread nicely and exhibit the normal spindle-shape morphology. (32 X 10).

B. Scanning micrograph of PDL fibroblast exposed for 2 hrs to 1:1/512 concentration of test solution. Cells have smooth surface, and are flattened with lamellipodia around the cell body. (500).
Figure 18.

A. Phase contrast microscopy of PDL cells exposed for 4 hrs to the stock solution of the test solution. Round floating cells are seen. (32 X 10).

B. Scanning micrograph of PDL fibroblasts exposed for 4 hrs to the stock solution. Cells are round-shaped and the cell body is covered with blebs at the peripheries. (1 X 10^3).
Figure 19.

Electron micrograph of PDL fibroblasts exposed for 4 hrs to the stock solution.

A. Nucleus has double membrane (arrow). Numerous large vacuoles are seen. Few number of endoplasmic reticulum and mitochondria are present. (12.6 X 10³).
B. Number of autophagic vacuoles (Au). \((16 \times 10^3)\).
Figure 20.

A. Phase contrast microscopy of PDL cells exposed for 4 hrs to 1:1/4 concentration of the test solution. Number of round cells and normal spindle-shaped cells are seen. (32 X 10).

Scanning micrograph of PDL fibroblast exposed for 4 hrs to 1:1/4 concentration of test solution.

B. Cell covered with blebs at the cell edge. Microvilli are seen at the edge of the endoplasm. (500).

C. Cell is fan-shaped covered with ruffles around the cell edge. (1 X 10^3).
Figure 21.

PDL fibroblast exposed for 4 hrs to 1:1/4 concentration of test solution.

A. Nucleus with double membrane (arrow). Myelin figure in lysosome (L), vacuoles and endoplasmic reticulum are seen. (16 X 10^3).
B. Swollen endoplasmic reticulum (ER) is seen. Cell border is covered with microfilaments (f). (5 X 10^3).
Figure 22.

A. Phase contrast micrograph of PDL cells exposed for 4 hrs to 1:1/512 concentration of test solution. The cells exhibited the normal spindle, fusiform-shape. Few round cells (mitosis) are seen. (32 X 10).

B. Scanning micrograph of PDL fibroblasts exposed at 4 hrs to 1:1/512 concentration of test solution. Cells are completely spread on the substrate. Cell body is covered with numerous microvilli. (2 X 10³).
Figure 23.

A. Phase contrast micrograph of L 929 cell exposed for 2 hrs to the stock solution. The cells are round-shaped due to cytoplasmic shrinkage. (32 X 10).

Scanning micrograph illustrated the effect of the stock solution on L 929 cells at 2 hr exposure.

B. Cells are round-shaped and the cell body covered with blebs. (5 X 10^3).

C. Round-shaped cells covered with microvilli. (5 X 10^3).
Figure 24.

Effect of the stock solution on L 929 cells at 2 hr exposure. Cells are round in outline covered with blebs.

A. Nucleus is irregular in shape. Endoplasmic reticulum disappeared. Mitochondria (m) is rough in shape, partially destroyed and the cristae disappeared. (16 X 10^3).
B. Nucleus with double membrane (arrow). Mitochondria (m) is rough in shape, partially destroyed and the cristae disappeared. (12.6 X 10).
Figure 25.

A. L 929 cells exposed for 2 hrs with 1:1/4 concentration of the test solution. Cells are round-shaped. (32 X 10).

Scanning micrographs illustrate the effect of 1:1/4 concentration of test solution on L 929 cells at 2 hr exposure.

B. Cells are round-shaped and covered with blebs. Note ruffles around the cell border. (2 X 10^3).

C. Round cells are covered with microvilli. (2 X 10^3).
Figure 26.

Electron micrograph of L 929 cells exposed at 2 hrs to 1:1/4 concentration of test solution. Large number of mitochondria is seen. Cell is roughly round-shaped, covered with blebs and part of the cell membrane is damaged (arrow). (8 X 10^3).
Figure 27.

Scanning micrograph of L 929 cells exposed for 2 hr to 1:1/32 concentration. Round and irregular spindle-shaped cells are covered with microvilli. (1 X 10\(^3\)).

Figure 28.

Effect of 1:1/512 concentration of test solution on L 929 cells at 2 hr exposure. Cell is spindle-shaped and covered with microvilli. Cell attached by filopodial processes. (2 X 10\(^3\)).
A. Phase contrast micrograph illustrates the effect of the stock solution of test solution on L 929 cells at 4 hr exposure. Cells are round in shape showing degeneration. (32 X 10).

Scanning micrograph of L 929 cells exposed at 4 hrs to stock solution. Cells are roughly round-shaped.

B. Note the crack at the cell surface. Microvilli could be seen. (5 X 10^3).

C. Large blebs covering the cell surface. (5 X 10^3).
Figure 30.

Electron micrograph of L 929 cells exposed at 4 hr to the stock solution.

A. Complete destruction of the cell membrane. Large vacuole at the center of the cell could be interpreted to mean that the nucleus is lost. The other structural organelles are difficult to identify. (6.3 X 10³).
B. The nuclear membrane is destroyed, swollen endoplasmic reticulum (ER) and the shape of the mitochondria (M) is roughly round and the cristae are completely disappeared. (16 X 10^3).
C. The damage is less. Cell is round-shaped and the nucleus is roughly round-shaped. The cristae of mitochondria (m) are difficult to identify and vacuoles are seen. (8 X 10^3).
A. Phase contrast micrograph illustrates the effect of 1:1/4 concentration of test solution on L 929 cells at 4 hr exposure. Cells are round-shaped. (32 X 10).

B. Scanning micrograph illustrates the effect of 1:1/4 concentration of the test solution on L 929 cells at 4 hr exposure. Cell is round-shaped and covered with blebs. (2 X 10^3).
Figure 32.

Electron micrograph of L 929 cells exposed to 1:1/4 concentration of test solution for 4 hrs. Blebs (B) covered the cell border. Mitochondria (m) has no cristae. (8 X 10^3).
Figure 33.

Scanning micrograph illustrates the effect of 1:1/32 concentration of test solution on L 929 cells at 4 hrs. Spindle and round-shaped cells covered with microvilli and/or blebs. (500).

Figure 34.

Scanning micrograph of L 929 cells exposed for 4 hrs to 1:1/512 concentration of test solution. Round and spindle-shaped cells are seen. They are covered with microvilli. (1 X 10^3).
Figure 35.

Phase contrast micrograph illustrated PDL cell recovery after 48 hrs of replacing the test solution with culture medium.

A. The cells formed a confluent layer. (20 X 10).

B. PDL cell with spindle-shaped morphology. (32 X 10).
Figure 36.

Phase contrast micrograph of L 929 cells after 48 hrs of replacing the test solution with the culture medium.

A. A substantial cell growth and cell mitosis are present (arrow). (32 X 10).

B. L 929 cells with spindle-shaped morphology. (32 X 10).
### TABLE I

EFFECT OF DIFFERENT CONCENTRATIONS OF ACRYLIC TEST SOLUTION ON PERIODONTAL LIGAMENT (PDL) FIBROBLASTS

<table>
<thead>
<tr>
<th>Conc. in Test Solution</th>
<th>Cell-Material Contact Time</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2 Hours</td>
</tr>
<tr>
<td></td>
<td>( \bar{X} \pm SE )</td>
</tr>
<tr>
<td>Control</td>
<td>3.6 ± 0.6</td>
</tr>
<tr>
<td>Stock 1:1</td>
<td>18.8 ± 1.1</td>
</tr>
<tr>
<td>1:1/2</td>
<td>4.0 ± 0.4</td>
</tr>
<tr>
<td>1:1/4</td>
<td>3.5 ± 0.1</td>
</tr>
<tr>
<td>1:1/8</td>
<td>3.3 ± 0.2</td>
</tr>
<tr>
<td>1:1/16</td>
<td>3.5 ± 0.6</td>
</tr>
<tr>
<td>1:1/32</td>
<td>3.6 ± 0.4</td>
</tr>
<tr>
<td>1:1/64</td>
<td>3.9 ± 0.4</td>
</tr>
<tr>
<td>1:1/128</td>
<td>3.5 ± 0.3</td>
</tr>
<tr>
<td>1:1/256</td>
<td>3.5 ± 0.1</td>
</tr>
<tr>
<td>1:1/512</td>
<td>3.3 ± 0.3</td>
</tr>
</tbody>
</table>

Release of \(^{51}\text{Cr}\) in percent.

(n) = Number of experiment.
### TABLE II

**EFFECT OF DIFFERENT CONCENTRATIONS OF ACRYLIC TEST SOLUTION ON L 929 CELLS**

<table>
<thead>
<tr>
<th>Conc. in Test Solution</th>
<th>2 Hours</th>
<th>4 Hours</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\bar{X} \pm SE$</td>
<td>(n)</td>
</tr>
<tr>
<td>Control</td>
<td>5.9 ± 0.6</td>
<td>(4)</td>
</tr>
<tr>
<td>Stock 1:1</td>
<td>32.7 ± 4.7</td>
<td>(2)</td>
</tr>
<tr>
<td>1:1/2</td>
<td>4.4 ± 0.1</td>
<td>(2)</td>
</tr>
<tr>
<td>1:1/4</td>
<td>4.7 ± 0.8</td>
<td>(2)</td>
</tr>
<tr>
<td>1:1/8</td>
<td>5.2 ± 1.1</td>
<td>(2)</td>
</tr>
<tr>
<td>1:1/16</td>
<td>5.3 ± 0.8</td>
<td>(2)</td>
</tr>
<tr>
<td>1:1/32</td>
<td>5.7 ± 1.8</td>
<td>(2)</td>
</tr>
<tr>
<td>1:1/64</td>
<td>5.3 ± 0.1</td>
<td>(2)</td>
</tr>
<tr>
<td>1:1/128</td>
<td>5.4 ± 0.6</td>
<td>(2)</td>
</tr>
<tr>
<td>1:1/256</td>
<td>5.9 ± 0.7</td>
<td>(2)</td>
</tr>
<tr>
<td>1:1/512</td>
<td>5.2 ± 0.8</td>
<td>(2)</td>
</tr>
</tbody>
</table>

Release of $^{51}$Cr in percent.

(n) = Number of experiment.
BIBLIOGRAPHY


Kawahara, H., Imanishi, Y. and Oshima, H.: Biological evaluation of 
Kellner, G. and Keresztesi, L.: Die Wirkung metallischer Wurzelfull-
1965.
Kokubu, T. and Pollak, O.: Influence of nicotine on cells of rabbits 
aorta and myocardium in tissue cultures. Exp. and Molec. Path. 
Kollar, E. and Baird, G.: The influence of the dental papilla on the 
development of tooth shape in embryonic mouse tooth germs. J. 
Kollar, E. and Baird, G.: Tissue interactions in embryonic mouse 
tooth germs. II. The inductive role of the dental papilla. J. 
Koskinen, K., Rahkamo, A. and Tuompo, H.: Cytotoxicity of some solu-
tions used for root canal treatment assessed with human fibro-
Kroeger, D., Gonzales, F. and Krivoy, W.: Transmembrane potentials of 
Leirskar, J. and Helgeland, K.: A methodological study of the effect 
of dental materials on growth and adhesion of animal cells in 


