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Morphological Cell Changes due to Chemical Toxicity of a Dental Material: An Electron Microscopic Study on Human Periodontal Ligament Fibroblasts and L929 Cells

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New endodontic materials with polymer bases may be more difficult to evaluate in cell cultures in vitro than conventional zinc oxide-eugenol cements. In order to study the morphological changes taking place in cells exposed to such materials, L929 cells and human periodontal fibroblasts were observed using scanning electron microscopic and transmission electron microscopic techniques. The morphological changes of the cells were correlated to the quantitative results observed simultaneously in cytotoxicity studies using the radiochromium release method. Results showed there was a relationship between the chromium release and the degree of individual cell damage. The periodontal ligament fibroblasts were more resistant to this kind of chemical injury than the L929 cells. Consequently, it may be proper to use periodontally derived cells for the study of cytotoxic mechanisms of polymer endodontic filling materials.

Endodontic filling materials have been the objects of many biological assessments in regard to their biocompatability. A number of methods utilizing in vitro cell culture techniques have been proposed (1–4). Similar methods have been included in national (ADA 156) (5) and international (FDI) (6) standards for biomaterial evaluation. Endodontic materials are difficult to study with tissue culture methodology due to the many variations in chemical compositions. Most of the original endodontic materials were slightly modified zinc oxide-eugenol cements. Such materials are easy to assess through in vitro methodologies as the eugenol's protein coagulating effect results in general cell injury and death.

Newer endodontic materials tend to be polymers with very different compositions. The potential toxic components of these materials will most likely not influence cells as general toxins but rather be targeted at certain metabolic pathways. Little is known about the cellular changes occurring during cytotoxicity testing. None of the commonly used methods (1–4) required detailed observation of the target cells and little attention has been paid to the morphological alteration of the test cells. The most commonly reported change, as a result of cell injury during cytotoxicity evaluation, is cell detachment (7, 8). Cell shape is also altered. Thus, the spindle shape of fibroblasts changes to a round appearance as a result of cytoplasmic shrinkage. Such morphological changes have been observed using phase contrast microscopy (9–11). The ultrastructure changes of cells in culture, as a result of chemical injury, have not yet been reported.

The purpose of the present investigation was to study, in vitro, at an electron microscopic level, the morphological changes occurring in the target cells as a result of chemical toxicity induced by a polymer material. These changes will be related to the quantitative results observed simultaneously with the radiochromium release method (2). Results obtained in cultures of human periodontal ligament fibroblasts and L929 cells will be compared.

MATERIALS AND METHODS

Cells

Three- to 5-day-old cultures of L929 mouse fibroblasts and human periodontal ligament fibroblasts (PDL) were used. The PDL cells were obtained from a maxillary premolar tooth extracted for orthodontic therapy from a 14-yr-old boy. 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.

Cuture Medium

Eagle's minimal essential medium with Earl's balanced salt solution (Flow Laboratories, Mclean, VA) supplemented with 10% (vol/vol) fetal calf serum, 2 mM L-glutamine, and 2.2 mg of sodium bicarbonate per ml was used. In addition, 100 IU/ml penicillin and 50 μg/ml streptomycin were added to the culture medium (2).

Cell Labeling

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

Test Material

Cold-curing acrylic resin (batch 11137; Coldpac, Chicago, IL) was mixed in a powder to liquid ratio of 2:1 according to the manufacturer's directions. Three grams of this mixture were spread over the whole bottom surface of 55-cm^2 culture dishes. Twenty milliliters of culture medium were added to each dish, which was stored refrigerated for 24 h. Subsequently, the culture medium containing the leaked toxic component of the acrylic resin was filtered and stored in glass bottles at +4°C. This solution was called stock solution. The stock solution and several dilutions made in culture medium were used as test solutions for the experiment. These dilutions were made to provide decreasing concentrations of toxins.

Experimental Procedures

The labeled cells were harvested with 0.02% trypsin in calcium and magnesium-free phosphate-buffered saline solution before being suspended in culture medium. The cell suspension was washed four times in culture medium. The cells were suspended in culture medium at a density of 5×10^5 cells per ml. The experiments were performed in plastic tissue culture clusters (Costar, Cambridge, MA) containing 24 wells, each with an inner diameter of 16 mm. One milliliter of cell suspension was mixed with 1 ml of the test solution in each culture well and incubated at 37°C and 100% humidity. At the same time 1 ml of the cell suspension and 1 ml of the culture medium were mixed in culture wells to serve as a negative control of spontaneous release.

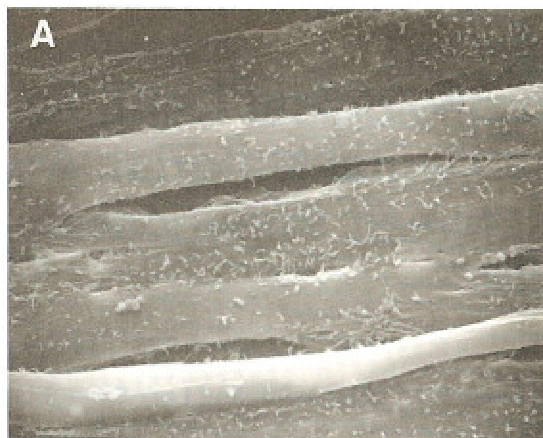
The cultures were incubated for 2 and 4 h. At the end of the incubation period, 1 ml of culture medium was withdrawn from each culture well and transferred to test tubes. These tubes were centrifuged for 10 min at $500 \times g$, after which 0.5 ml of the supernatant in each test tube was withdrawn and counted for 1 min 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 ^{51}Cr release in the experiments. The percentage of ^{51}Cr release was calculated on the basis of the total amount incorporated in the target cells using the following formula:

$$\text{Release } \% = \frac{2 \times {}^{51}\text{Cr in } t \text{ samples}}{{}^{51}\text{Cr in } r \text{ samples}} \times 100$$

RESULTS

Normal Morphology

Figures 1 and 2 illustrate the normal morphological features of the PDL fibroblasts and L929 cells in vitro.



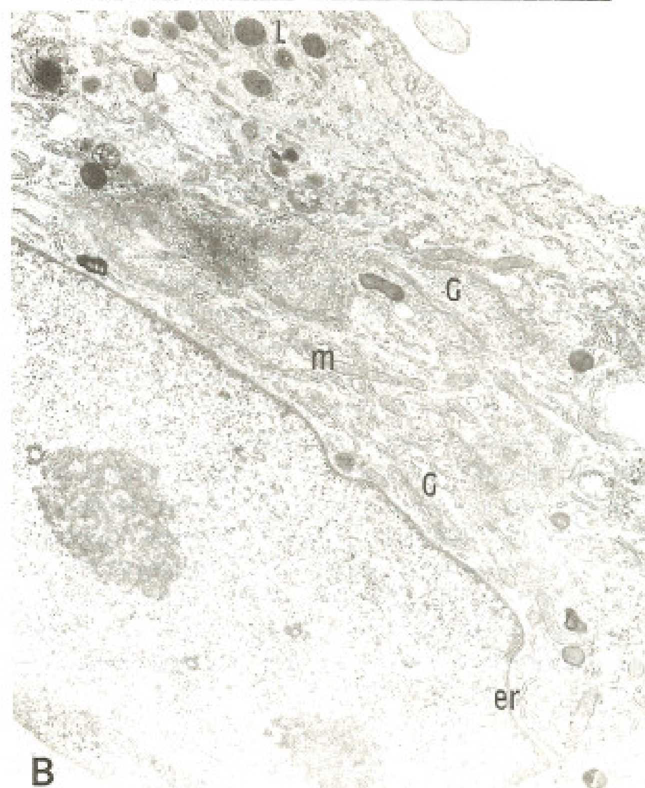


Fig 1. *A*, Scanning electron micrograph of PDL fibroblasts grown in tissue culture. The cells are elongated in shape and covered with microvilli. Notice the parallel arrangement of the cells (2×10^3). *B*, Electron micrograph of normal PDL fibroblast grown in tissue culture. Nucleus with nucleoli, mitochondria (*m*), endoplasmic reticulum (*er*), Golgi complex (*G*), and lysosomes (*L*) are well developed (20×10^3).

Effect of the Test Solutions on Cell Morphology

TWO-H EXPOSURE

The 2-h exposure of the PDL fibroblasts and L929 cells to the acrylic test solution caused the cells to round up and sometimes detach from the substratum. After 2-h exposure to the stock solution, the PDL fibroblasts had a "fried egg-like" appearance with a smooth surface and ruffles covering the cell border (Fig. 3*A*). Myelin figures, numerous vacuoles, and autophagic vacuoles were observed (Fig. 3*B*). The Golgi complex was difficult to identify. Mitochondria and lysosomes

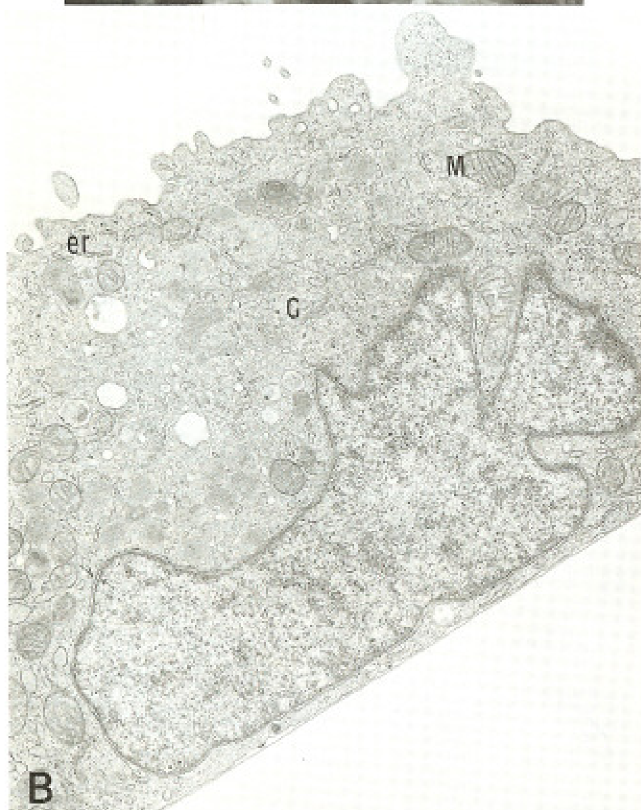
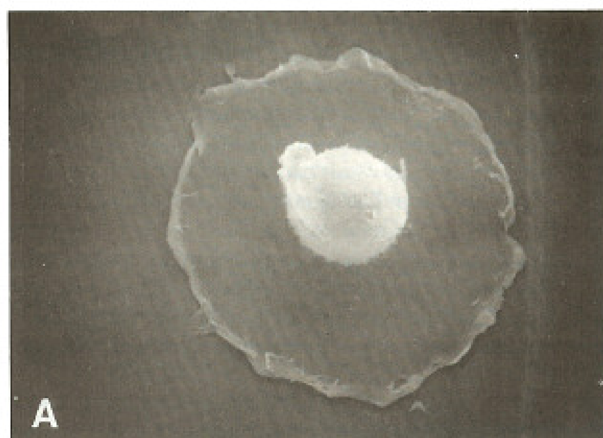


FIG 2. *A*, Scanning electron micrograph of L929 cell grown in tissue culture. Cell has a spindle shape with blebs covering the cell body (5 $\times$ 10^3). *B*, Electron micrograph of normal L929 cell grown in tissue culture. Nucleus has double membrane (*arrows*). Well-developed and round mitochondria (*m*) and endoplamic reticulur (*er*) are seen. Golgi complex (*G*) and vacuoles are occasionally seen (32 $\times$ 10^3).

were occasionally seen. The nuclei and endoplasmic reticulum appeared to be normal.

With decreasing concentrations of acrylic diffusion products, the PDL cells appeared to be more normal. The general morphology of the PDL cells when exposed to a four-time dilution was round and covered with microvilli or blebs. Many lysosomes, vacuoles, and Golgi complexes were present.

After 2-h exposure to stock solution, the L929 cells became round and the cell surfaces were covered with blebs. A number of small and large vacuoles and autophagic vacuoles were seen. The cytoplasm appeared to be less dense. Golgi complexes, mitochondria, and endoplasmic reticulum were difficult to identify. When the test solution was diluted, the damage to the L929 cells was less noticeable. The cells were round and covered with blebs. Few cells had microvilli. Large numbers of mitochondria were seen. Normal cells were randomly seen in both types of cell cultures at the highest concentrations.



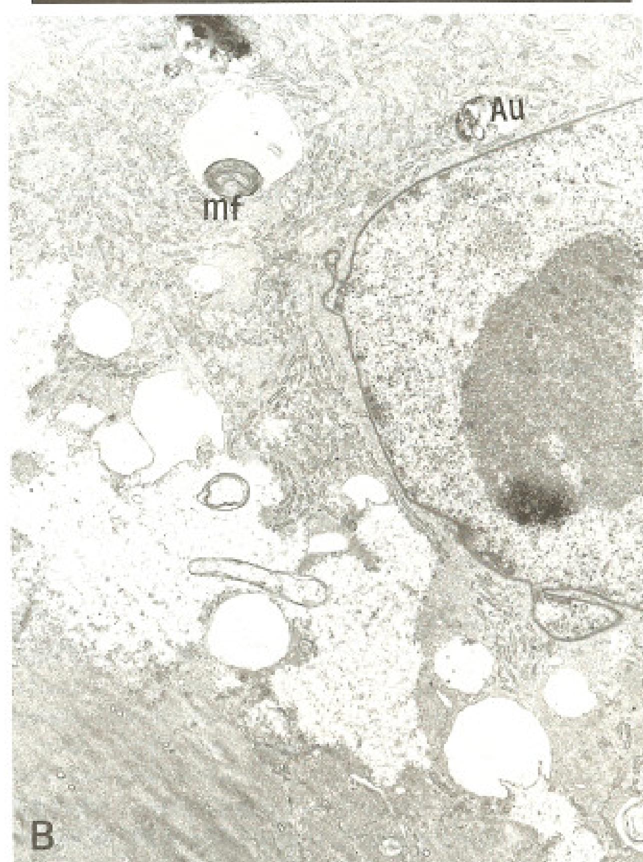


FIG 3. *A*, Scanning micrograph of PDL fibroblast exposed for 2 h to the stock solution. Fried egg-like shaped cell with lamellipodia and ruffles around the cell body (1 $\times$ 10^3). *B*, PDL fibroblast exposed for 2 h to the stock solution. Myelin figure (*mf*), large vacuoles, and autophagic vacuoles (*Au*) are seen. Numerous profiles of endoplasmic reticulum scattered through the cytoplasm (16 $\times$ 10^3).

FOUR-H EXPOSURE

When the experimental time was increased to 4 h, the injury to the cells became more severe. PDL fibroblasts were

roughly round with blebs covering the cell border. 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. Lysosomes, myelin figures, and autophagic vacuoles were observed. Mitochondria were occasionally seen. The nuclei appeared to be normal. Cell alterations decreased with increased dilution.

The general morphology of the PDL cells, when exposed to a four-time dilution, was mostly round but sometimes spindle shaped. Cells were covered with blebs, microvilli, or ruffles. Myelin figures in lysosomes, vacuoles, and endoplasmic reticulum were seen.

Four-hour exposure of the L929 cells to the acrylic solution caused greater disturbance of the cell content and cell nuclei. Mitochondria were completely disorganized. The Golgi complex and cell membrane had disappeared (Fig. 4*B*). The endoplasmic reticulum was swollen. The outer surface of the cells was covered with blebs (Fig. 4*A*). Very few cells were unaffected at this exposure time. When exposed to four-time dilution, the L929 cells were round and covered with blebs. With lower concentrations, more mitochondria and vacuoles were observed.

Chromium Release

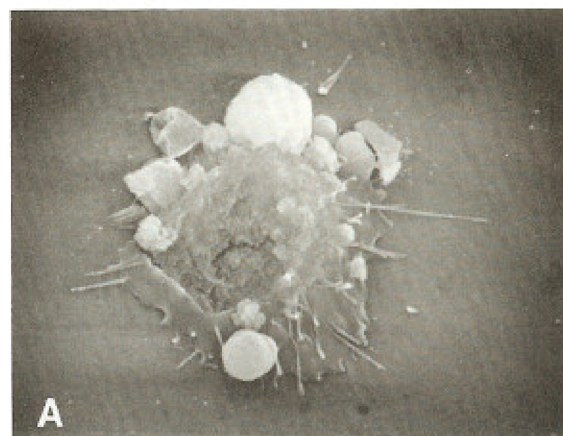
The results are summarized in Tables 1 and 2.

PDL Fibroblasts

The spontaneous release in the control cultures of the PDL cells was 3.6 ± 0.6% after 2-h exposure and 6.6 ± 0.3% in the 4-h experiments. The amount of ^{51}Cr release at 2 and 4 h 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 not different to the control (Table 1).

L929 Cells

The spontaneous release in the control cultures of the L929 cells was 5.9 ± 0.6% after 2 h and 8.5 ± 0.5% in the 4-h experiments. Using the stock solution, the amount of ^{51}Cr release was approximately 17 percentage points above the control after 2 h of evaluation. After 4 h 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 values (Table 2).



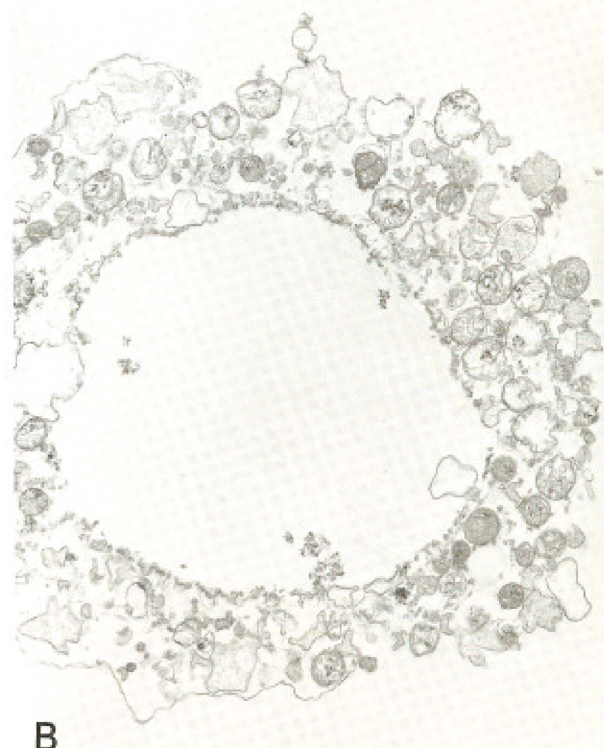


Fig 4. *A*, Scanning micrograph of L929 cell exposed at 4 h to stock solution. Cell is roughly round. Note the crack at the cell surface. Microvilli can be seen (5 × 10^3). *B*, Electron micrograph of L929 cell exposed at 4 h to the stock solution. Complete destruction of the cell membrane. Large vacuole at the center of the cell could be interpreted to mean that the nucleus is lost. The other structural organelles are difficult to identify (32 × 10^3).

DISCUSSION

After a 2-h exposure to the stock solution, changes in cell morphology were observed. Both cell types became round 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 proportional to the concentration of the test solution and the exposure time (13, 14). Using phase contrast microscopy, Lettre (9) and Berliner et al. (15) studied the action of some drugs on fibroblasts in culture 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 findings when studying chemical toxicity of a dental material. The frequency of Golgi complexes observed was lower in cultured L929 cells than in PDL cells. The Golgi complexes were, in fact, sometimes difficult to identify in L929 cells. The Golgi complex plays an important role in the secretory process which is required for the final stages in the processing and transportation of protein to specific sites in the cell (12). The absence of Golgi complexes in L929 cells may explain the absence of extracellular

TABLE 1. Effect of different concentrations of acrylic test solution on PDL fibroblasts*

Concentration in Test Solution	Cell-Material Contact Time 2 h	Cell-Material Contact Time 4 h	n†
	$\bar{X} \pm SE$	$\bar{X} \pm SE$	
Control	3.6 ± 0.6	6.6 ± 0.3	4
Stock			
1:1	18.8 ± 1.1	22.6 ± 3.3	4
1:½	4.0 ± 0.4	6.0 ± 0.1	4
1:¼	3.5 ± 0.1	6.5 ± 0.1	4

* Release of ^{51}Cr in percent.
† Number of experiments.

TABLE 2. Effect of different concentrations of acrylic test solution on L929 cells*

Concentration in Test Solution	Cell-Material Contact Time 2 h	Cell-Material Contact Time 4 h	n†
	$\bar{X} \pm SE$	$\bar{X} \pm SE$	
Control	5.9 ± 0.6	8.5 ± 0.5	4
Stock			
1:1	32.7 ± 4.7	48.9 ± 1.4	4
1:½	4.4 ± 0.1	6.8 ± 0.2	4
1:¼	4.7 ± 0.8	6.6 ± 0.3	4

* Release of ^{51}Cr in percent.
† Number of experiments.

collagen found in cultures of these cells. Cell lysis of individual cells is observed only in the cultures of L929 cells and only when the exposure time is increased to 4 h at high concentrations of test solution (Fig. 4). Under these conditions, the structural organelles of L929 cells are seriously damaged to a degree beyond identification. Such a severe degree of damage inhibits cell function.

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.

When the labeled cells were exposed to the higher concentration of the acrylic test solution, an increase of radiochromium release was observed. Approximately 20% was recorded for the PDL cells and 50% for the L929 cells. This amount of chromium found in the culture medium reflects the degree of cell damage caused by the test material. Both the morphological observations and the radiochromium release results indicate that the L929 cells are more sensitive to this toxic material than the PDL cells.

The amount of chromium release is dependent upon the evaluated substance or material, cell type, the cell-material contact, and the exposure time (2, 16). Spangberg (2) suggested that 70% chromium release is an indicator of total cell lysis. In the present study, the ^{51}Cr release is always below the 70% level, indicating that total cell lysis may not have occurred. This is also confirmed by transmission electron microscopy. Despite the observation of severely injured cells, there are always cells present which have more structure preserved. The lower degree of cell injury for the PDL cells compared with the L929 cells as recorded by radiochromium release was also observed microscopically. This higher release of radiochromium and more morphological damage of the L929 cells compared with the PDL cells indicates a greater sensitivity by the L929 cells to this specific toxic material.

Similar results have been reported with different materials (1, 17–19). 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 division was significantly shorter for the L929 cell than for the PDL cell. Thus, if the toxic substance causes specific injury to the cell during its dividing cycle, it is more likely that the rapidly dividing cell will be more exposed to a specific metabolic injury than a cell with a longer waiting period.

The confusion in the literature related to sensitivity is most likely caused by the great variations of 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. This tends to indicate that when performing cytotoxicity assays of nontraditional endodontic materials, it may be proper to use periodontally derived cells for the study of cytotoxic mechanisms.

The results also indicate that the recorded release of radiochromium does not reflect injury of similar degree to all cells in the population. The observation shows that some cells were severly damaged when other cells seemed totally unaltered. Thus, *the ^{51}Cr release most likely reflects the relative frequency of severly damaged cells.*

The release of the label seems to be due to the combined effect of a low level of spontaneous release at a level of approximately 1 to 2% per h of experimental time (2) and the release from irreversible altered cells. This observation has not been reported earlier. Spangberg (2) 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 will be equal throughout the cell population as the protein coagulation process is unrelated to the various metabolic stages in the cell cycle. The results of the present study using extracted toxic component of a freshly prepared polymer material indicate that the toxic effect of this material on cells is more specific than normally expected when evaluating traditional endodontic filling materials. New compositions of endodontic materials and the increased use of polymers in such materials will most likely require different or modified approaches to the study of biocompatability of such materials. Such a conclusion leads to the need for further studies of the toxic mechanisms when using cytotoxicity assays in vitro for the study of biocompatability of endodontic filling materials.

CONCLUSIONS

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

1. There are some differences between the basic ultrastructure of the periodontal ligament fibroblast and the L929 cell in culture. These differences are not major. The most striking difference is in the frequency of Golgi complex. It is lower in cultured L929 cells than in PDL fibroblasts. The shape of the outer surface of L929 cells is covered with blebs where the PDL fibroblasts are covered with microvilli.

2. There is a clear relationship between the ^{51}Cr release and

the degree of individual cell damage. The ^{51}Cr release reflects the relative frequency of severly damaged cells.

3. The release of the label during an experiment is due to the combined effect of a low level of spontaneous release at a level of approximately 1 to 2% per h of experimental time and the release from irreversible altered cells.

4. The PDL fibroblasts are less sensitive cells compared with the L929 cells when used to evaluate cytotoxicity of an acrylic test solution.

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