JOURNAL OF MATERIALS SCIENCE: MATERIALS IN MEDICINE 1 (1990) 228–232

A semi-automated cell culture evaluation system for cytotoxicity testing of dental materials

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A rapid and quantitative determination of viable cells in the cytotoxicity testing of dental materials is desirable to evaluate large numbers of samples in a short time. For this purpose a new and semi-automated cell culture evaluation system was developed using the fluorogenic dye fluorescein diacetate (FDA) for vital staining and microtiter plates for culture vessels. Our methodological experiments showed that in this system the fluorescence intensity was linearily related to the cell number from 500 to 20 000 cells per culture^{-1} and that fluorescence recording was stable for between at least 1 to 2 h using a quencher solution for absorbing extracellular fluorescence. The results from toxicity testing of different dental materials (two glass ionomer cements, a phosphate cement, a composite resin and monomeric methylmethacrylate) corresponded to those derived from other, standard test methods. Because of the ease of performance, the quantitative, rapid evaluation system and the small culture vessels requiring only few cells per culture, the test method presented may be an interesting alternative to other cell culture techniques for cytotoxicity testing.

1. Introduction

Cell culture techniques for cytotoxicity testing of dental materials have many advantages compared with animal experimentation and are therefore included in standard biological testing protocols [1–4]. However, the evaluation systems of some of these methods are time-consuming (e.g. protein determination) or require expensive equipment (e.g. ^{51}Cr-release determination) [5, 6]. In other, simpler tests (e.g. the agar overlay method or the filter method) the evaluation is only semiquantitative [7, 8]. Furthermore, for these methods large numbers of cells are needed, which may be a problem when primary cells (target cells) are to be used in the experiment.

Ideally, a bioassay detecting cell viability should be sufficiently rapid to process large numbers of samples and it should provide quantitative results in detecting viable cells in a mixture of dead and living cells. Besides a number of different methods for quantifying cell viability by means of vital staining, flourescent dyes, which can be detected in very small quantities, are an attractive alternative. Fluorescein diacetate (FDA), a non-fluorescent, non-polar fatty acid ester, quickly diffuses into the cells, when its cleavage by intracellular esterases results in the fluorescent, charged fluorescein [9, 10].

In a recent study FDA proved to be appropriate for vital staining of cells in a modification of the agar overlay test [11]. The objectives of this study were (1) to establish a semi-automated evaluation system of cell growth in microtiter plates, where cells were stained with FDA (methodological experiments) and (2) to evaluate the suitability of this approach for cytotoxicity testing of dental materials.

2. Materials and methods

2.1. Methodological experiments

Preliminary experiments had established that fluorescence measurements were independent of the microtiter plate well position. Inner wells showed no difference from outer wells. For determining the time-dependence of fluorescence intensity, 5000 cells (L-929 mouse fibroblasts) in a total volume of 200 μl BME cell culture medium supplemented with 5% foetal calf serum (GIBCO-Biocult Laboratories, Glasgow, UK) were seeded into each well of a 96-well flat-bottomed microtiter plate (Tecnomara, Fernwald, FRG). The cells were incubated for 3 h in an air atmosphere with 5% CO_2 at 37° C and 95% relative humidity. After incubation the plates were centrifuged at 1200 r.p.m. for 7 min (Rotixa/P, Hettich, Tuttlingen, FRG), supernatants were decanted, and of 200 μl different concentrations of FDA (4 to 108 μM) (Sigma, Deisenhofen, FRG), diluted in phosphate buffered saline (PBS; pH 7.4), were added to the cell cultures. In parallel experiments cell cultures were stained with FDA dissolved in a special quencher solution containing Colanyl-black (Leitz, Wetzlar, FRG), diluted 200-fold in PBS. The function of this quencher solution was to absorb extracellular fluorescence. Eight replicate cultures were stained with each FDA concentration and the fluorescence intensity was recorded continuously up to 2.5 h.

The intracellularly produced fluorescein tends to

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leak from viable cells [12]. In order to discover if this occurred in our experimental set-up and what influence it would have on the evaluation system, cell cultures were supplied with quencher solution and incubated with 24 μM FDA followed by an incubation without FDA.

To investigate the relationship between the fluorescence intensity and the cell number, different numbers of cells (500 to 20 000) were seeded in each well of the microtiter plates, eight replicate cultures for each cell number. After incubation (3 h), centrifugation and decanting (s.a.) 24 μM FDA in the quencher was added and fluorescence was recorded after 60 min.

2.2. Cytotoxicity testing of dental materials

The dental materials used for cytotoxicity testing (Table I) were mixed according to the manufacturers' instructions, filled in glass rings (height 3 mm, diameter 5 mm) and stored for 24 h at 37° C and at 0% or at 100% relative humidity, respectively. From one to six samples were introduced into 20 ml BME cell culture medium supplemented with 5% foetal calf serum (pH 7.4) and incubated at 37° C without shaking for 24 h. To avoid fluorescence measurements arising from dispersed particles, the eluates were filtered using 0.2 μm membrane filters (Minisart, Sartorius, Göttingen, FRG). The methylmethacrylate monomer was used without purification and diluted in BME cell culture medium supplemented with 5% foetal calf serum for concentrations ranging from 0.1 to 2% (v/v). For toxicity testing, 200 μl of each eluate were used per well.

For the cytotoxicity testing experiments the 96 wells of a microtiter plate were divided into three groups: the experimental cultures (56 wells, containing cells and eluate), the 100% cell growth controls (24 wells, containing cells and culture medium) and the 0% cell growth controls (16 wells, containing culture medium). In each well of the experimental cultures and of the 100% cell growth controls, 5000 cells were seeded. To the 0% and 100% cell growth control wells, 200 μl cell culture medium were added. Into the experimental wells, 200 μl of different eluates, representing different materials and different numbers of samples of each material being eluated, were pipetted, eight replicas for each eluate. After incubation for 48 h at 37° C in an air atmosphere containing 5% CO_2 the cultures were treated as described above and the fluorescence intensity was recorded 45 to 60 min after application of 24 μM FDA diluted in quencher solution.

TABLE I Test materials

Material	Manufacturer	Batch no.
Phosphate cement (Harvard cement)	Richter & Hoffmann, Berlin, FRG	Powder 3891 Liquid 867
Glass ionomer cement (AquaCem)	De Trey, Konstanz, FRG	HD44 88/04
Glass ionomer cement (KetaFil)	ESPE, Seefeld, FRG	R125
Composite resin (Brilliant Lux)	Coltene, Konstanz, FRG	34
Methylmethacrylate monomer	Merck, Darmstadt, FRG	800590

2.3. Automated recording of the fluorescence intensity

A system composed of a microfluorometer and an inverted microscope (MPV compact MT, Leitz, Wetzlar, FRG) equipped with a scanning table was used. The microtiter plate was placed on the scanning table and was viewed by eye through the microscope to estimate the well with the highest fluorescence signal. This signal was used to calibrate the microfluorometer, and thus original readings of fluorescence from the other wells were obtained as "relative fluorescent units". By means of a computer-controlled stepping motor, each well of the microtiter plates was centred under the microscope, fluorescence was recorded and the next well was centred. A xenon XBO 75 lamp served as a light source. It was mounted outside the microscope box and fed from a stabilized power supply. The excitation filter was a 450 to 490 nm bandpass filter (Leitz Pleomopak, I2) and the emissions filter was a 530 to 560 nm bandpass filter (Leitz Pleomopak, N2).

2.4. Data processing

Original fluorescence intensity data were stored in a personal computer and printed out in a 96-well format corresponding to the original position on the plate. For the cytotoxicity experiments the averaged values from eight replicates of the experimental cultures were further transformed into percentage of relative growth by relating them to the values derived from the 0% and 100% cell growth controls. Mean values (and standard deviations) of percentage of relative growth together with the doses (number of specimens per 20 ml culture medium) were plotted as dose–response curves. In order to compare these dose–response curves with each other, the area A under each curve was calculated and related to the area T under the curve for the 100% cell growth control. The resulting area coefficient C (in %) was calculated by

$$C = (A/T) \times 100$$

3. Results

3.1. Time-dependence of the fluorescence intensity

When cell cultures were incubated with increasing FDA concentrations (dissolved in PBS) a rapid increase of the fluorescence intensity was observed during the first minutes of incubation (Fig. 1). The relationship between the fluorescence intensity and the incubation time was not linear for any FDA concentration tested. However, after 2.5 h the fluorescence intensity arising from the lowest FDA concentration seemed to level off at a maximum value, indicating that nearly all of the FDA available to cleavage was transformed into fluorescein and diacetate.

When cell cultures were incubated with FDA dissolved in quencher solution, the fluorescence signals from the cultures were less intense (Fig. 2). Similar curves were obtained for all FDA concentrations,

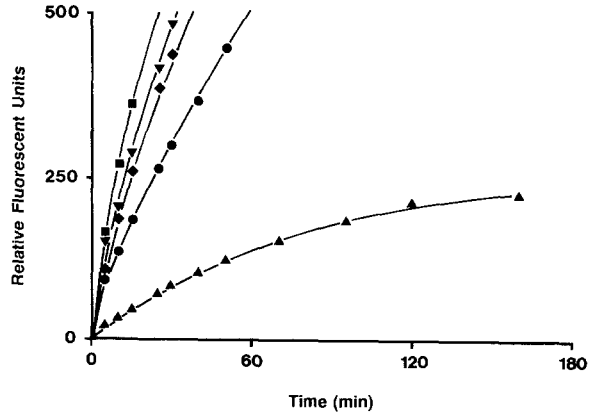

Figure 1 Fluorescence intensity as a function of the incubation time and different FDA concentrations in PBS. Cell cultures were incubated with (▲) 4, (●) 12, (◆) 24, (▼) 36 and (■) 108 μM FDA.

reaching a maximum fluorescence intensity after approximately 45 to 60 min, which remained constant up to 2.5 h.

3.2. Leakage of fluorescein from viable cells

In the corresponding experiments quencher solution was supplied to the cultures with and without FDA (Fig. 3). The fluorescence intensity increased rapidly after a short time of incubation in the presence of FDA and reached a constant level after about 60 min. When FDA was now removed from the cell cultures with the quencher still present, the fluorescence intensity immediately decreased, indicating a loss of intracellular fluorescein.

Using FDA (24 μM) in quencher solution, a linear proportionality between the fluorescence intensity and the number of viable cells was observed over the range 500 to 20 000 cells per well^{-1} (Fig. 4).

3.3. Cytotoxicity testing of dental materials

The dose–response curves for the cements (Figs 5 to 7) show a clear dependence of relative growth upon the storage conditions of the samples. Those samples stored at 100% relative humidity were less cytotoxic than those stored at 0%. This effect was more pronounced with the phosphate cements than with the glass ionomer cements. No such dependence could be demonstrated with the composite resin (Fig. 8). The dose–response curves also show that the phosphate

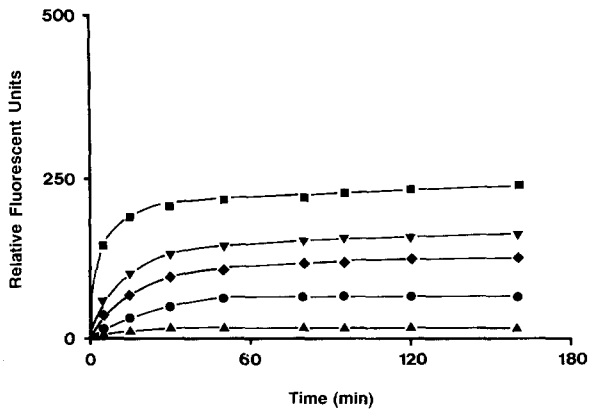

Figure 2 Fluorescence intensity as a function of the incubation time and different FDA concentrations in quencher. Key as for Fig. 1.

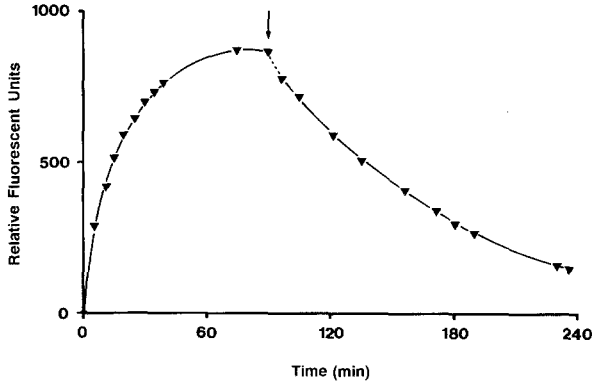

Figure 3 Fluorescence intensity with and without FDA present in quencher. The arrow indicates the removal of FDA (24 μM) from cell cultures.

cement was more toxic than the glass ionomer cements, especially if the samples were stored at 0% relative humidity. The results derived from the dose–response curves can be substantiated by the area coefficients (Table II).

A 50% growth inhibition by methylmethacrylate monomer (Fig. 9) was found for a 1% solution.

4. Discussion

We have described an assay that may be useful for cytotoxicity testing of dental materials. The cleavage of FDA by living, metabolically active cells is directly proportional to the cell number over a wide range, and the fluorescence intensity derived from various FDA concentrations is independent of time if a quencher solution is used (Fig. 2). A serious problem in cell viability staining with FDA is spontaneous leakage of fluorescein from viable cells [12]. For this reason, in recent cytotoxicity studies, fluorescein was replaced by more-polar and more-impermeable derivates [13, 14]. Similarly, in our investigation the drop in the fluorescence intensity after removing FDA from cell cultures is evidently caused by spontaneous leakage of fluorescein from the cells (Fig. 3), whereas the fluorescence remains constant with FDA still present (Fig. 2). Therefore, it is evident that the quencher solution is effective in absorbing extracellular fluorescence, leaving the intracellular fluorescein for recording. The fluorescence intensity remains constant at least over a 1 to 2 h period, presumably because fluorescein leakage from cells is balanced by the amount of newly intracellularly produced fluorescein. The FDA concentration that we used for cytotoxicity

TABLE II Cytotoxicity of dental materials expressed as area coefficients

Material	Area coefficient	
	0% relative humidity	100% relative humidity
Glass ionomer cement (Ketac Fil)	72	86
Glass ionomer cement (Aqua Cem)	71	82
Phosphate cement	11	69
Composite resin	64	57

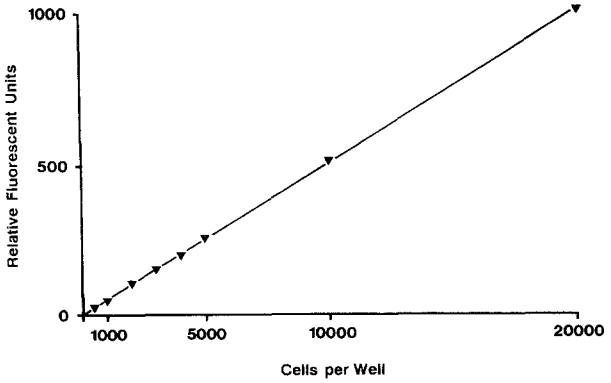

Figure 4 Fluorescence intensity as a function of the cell number. Cell cultures were incubated with 24 μM FDA dissolved in quencher solution.

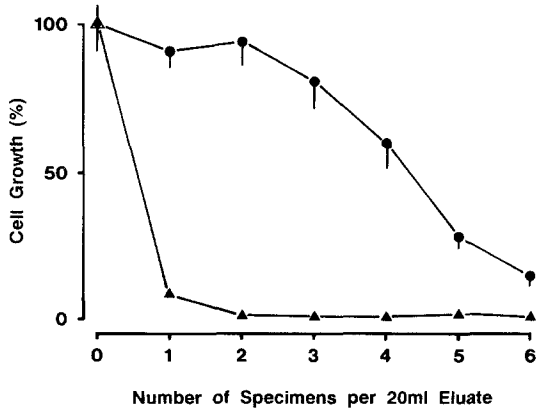

Figure 7 Cytotoxic effect of a phosphate cement stored at (●) 100% and (▲) 0% relative humidity.

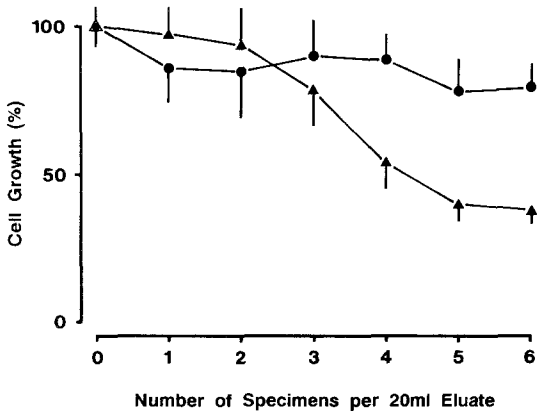

Figure 5 Cytotoxic effects of a glass ionomer cement (KetacFil) stored at (●) 100% and (▲) 0% relative humidity.

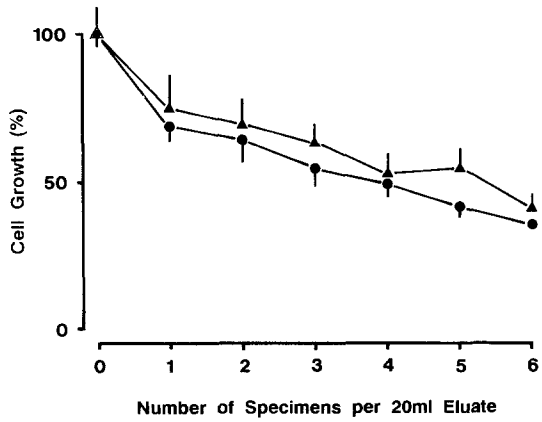

Figure 8 Cytotoxic effect of a composite resin stored at (●) 100% and (▲) 0% relative humidity.

testing of dental materials is appropriate for our purposes. As was shown in former experiments [11], the dye itself was not toxic to the cell cultures at this concentration. Although loss of sensitivity results from the use of a quencher, it provides a measurement of viable cells over a wide range of cell numbers and over a sufficiently long period.

The data obtained from cytotoxicity tests showing the influence of storage conditions for cements on growth inhibition are in accordance with other reports in the literature [6, 15]. The same is true for the cytotoxicity relationships of the glass ionomer cements and the phosphate cement [16]. The high toxicity of the cement samples stored at 0% relative humidity may be attributed to incomplete setting of these materials. Hydration is essential for the setting process, which may be impeded by storage at 0% relative humidity.

The calculation of an area coefficient may be useful in quantifying dose-related toxic effects of dental materials. It especially helps ranking the test materials from the least toxic to the most-toxic substance. However, it should be kept in mind that the area coefficient is a mean value for the whole dose–response curve and does not reflect special characteristics of the individual dose–response curve for a given material.

The 50% inhibition of cell growth by a 1% monomeric methylmethacrylate solution is in accordance

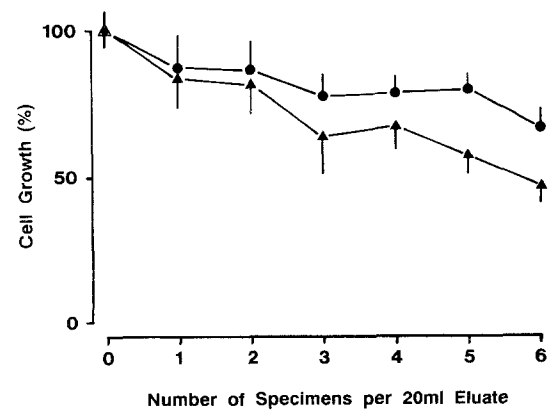

Figure 6 Cytotoxic effect of a glass ionomer cement (AquaCem) stored at (●) 100% and (▲) 0% relative humidity.

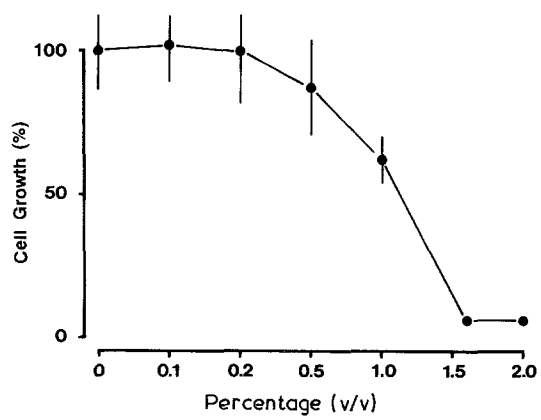

Figure 9 Cytotoxic effect of different concentrations of monomeric methylmethacrylate.

with data from growth inhibition tests by means of protein determination and shows that the test presented here has a sensitivity similar to other standard cell culture methods [5, 6, 17].

Our results indicate that the test described in this study may be suitable for cytotoxicity testing of dental materials. The technical requirements for a standard cytotoxicity testing system as decribed in [18] are fulfilled and the results correspond well to other standard assays. Furthermore, the use of radiochemicals which is required for the ^{51}Cr-releasing test is avoided. The moderate costs of equipment should be acceptable, considering the advantages of this test system. Through the miniaturization of the single-cell culture using microtiter plates and through the use of an automated reading system, a large number of tests requiring only a few cells per culture can be performed in a short time.

Acknowledgement

The authors acknowledge the help of Mr K.-A. Hiller for the mathematical and statistical analysis of the results.

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Received 22 March
and accepted 31 May 1990