JOURNAL OF MATERIALS SCIENCE: MATERIALS IN MEDICINE 5 (1994) 43–51

New developments in the filter test system for cytotoxicity testing

G. SCHMALZ, K. -A. HILLER, F. DÖRTER-ASLAN
University of Regensburg, Clinic of Operative Dentistry and Periodontics, 93042 Regensburg, Germany

The objective of this study was (1) to improve the succinate dehydrogenase (SDH) staining procedure of the filter test system, and (2) to study the suitability of hydrolases as markers for cell vitality by means of fluorescein diacetate, instead of SDH. The test materials included zinc phosphate cements, conventional and light-cured glass ionomer cements, a composite resin, and methylmethacrylate monomer. Four series of experiments were performed using L-929 mouse fibroblasts: (1) original method, (2) 24 h incubation time with procedural modifications; (3) use of FDA as marker for cell vitality; and (4) the agar overlay method. The staining intensity of the cells in the first series was insufficient. In the second series filter staining was good and showed distinct zones of damaged cells. In the third series cell staining was very distinct and easier to handle than with SDH. The results obtained in the second, third, and fourth series were in agreement with results from other cell culture tests. To compare results from different series an evaluation system based on the area of damaged cells was introduced. Correlations were high between series 2 and 3 as well as between 3 and 4. Our results indicate that the modifications of the filter test improve the method.

1. Introduction

The filter method for cytotoxicity testing of dental materials as it was first described by Tronstad *et al.* [1] is today a well known standard toxicity test system [2–9]. With this test system the influence of a toxicant upon the succinate dehydrogenase (SDH) enzyme activity of monolayer cells is measured. A zone of damaged and therefore SDH-negative cells may be produced under or around a test sample, the extent of this zone being related to the toxicity of the material. A scoring system is used to describe the extent of the zone. The results obtained with the filter method are reported [1–6] to be meaningful and relevant.

Meryon *et al.* [10] modified the filter method with respect to the preparation of the specimens and with respect to the evaluation system, taking into account that in certain cases demonstration of SDH activity was not very distinct in the original method. The same method was used by Meryon and Browne [11] testing glass ionomer cements. These authors increased the exposure time from 4 h, as described by Tronstad and Wennberg [1–5], to 24 h, and showed that the method became more sensitive [11]. This was confirmed in a further study by Meryon [12]. However, SDH activity as a marker for cell vitality still remains one drawback of the filter test method. The original method is time consuming and sometimes does not yield a clear distinction between damaged and undamaged cells [10].

The agar overlay method is technically easier to perform and is one of the most widely used and recommended cytotoxicity test systems [7–9, 13–15]. This test system measures the influence of a toxicant upon the membranes of monolayer cells (lysosomes), which in the presence of Neutral Red at a neutral pH exhibit a red colour [16]. The extent of the zone of decolouration around a test sample, which appears after the destruction of the membranes by a toxic material, is related to the degree of cytotoxicity of the test sample [13, 15, 17]. However, besides the extent of the "zone of decolouration" the degree of cell-lysis ("lysis-index") within the zone of decolouration is estimated, since some materials may lead to decolouration but are not toxic [15, 17]. The determination of the degree of cell lysis is subject to individual interpretation which is considered to be one of the decisive drawbacks of this method when used as a standard technique. With the filter test method mentioned above no estimate of the degree of cell-lysis is necessary as in the agar overlay test.

The aim of the present study was to change the evaluation procedure of the filter test system in order to improve the test method as such and to make it technically as easy to be performed as the agar overlay test. Four series of experiments were performed. In the first series, the original filter test method was used. Two modifications of the test procedure were chosen. The first modification (series 2) involved technical changes in the test procedure. The second involved a more basic change (series 3): instead of using SDH-activity as a marker for cell vitality, fluorescein diacetate (FDA) was used for that purpose. FDA was introduced in the *in vitro* cytotoxicity testing of dental materials [18–21]. Through the transformation of

0957–4530 © *1994 Chapman & Hall*

non-fluorescent FDA into flourescing fluorescein by non-specific hydrolases, metabolic activity of a cell can easily be visualized [19]. As a standard reference test system the agar overlay method was used (series 4).

The suitability of the proposed modifications was evaluated by testing dental materials at different ageing times and storing conditions with the original filter procedure, the two modified filter techniques, and the agar overlay method.

2. Materials and methods

2.1. Materials

The test materials presented in Table I were mixed according to the manufacturers' instructions, filled into glass rings (5 mm internal diameter, 3 mm height) and stored for 1 h, 24 h and 7 days, either at 0% or at 100% relative humidity, and at 37 °C. Methylmethacrylate monomer was used as delivered from the manufacturer without further purification. 10 µl were pipetted onto a paper filter disk (15 mm diameter) which was placed on the millipore filter.

TABLE I Test materials

Test Material	Manufacturer	Batch-Number
series 1 to 3a		
Zincphosphate cement (Harvard, normal setting)	Richter	Fluid 067/Powder 396
Glass ionomer cement (Ketac-Fil)	Espe	R 032/T 304
Glass ionomer cement (Aqua-Cem)	DeTrey/Dentsply	891108
Composite resin (Brilliant Lux)	Coltene	21
Methylmethacrylate Monomer	Merk-Schuchardt	Art. 800590
series 3b and 4		
Zincphosphate cement (Tenet)	Vivadent	217030/646901
Glass ionomer cement (Ketac-Fil)	Espe	R 032/T 304
Glass ionomer cement (Ketac-Silver)	Espe	V 119
Glass ionomer cement (Ketac-Bond; 340 mg powder/100 µl liquid)	Espe	T 354/T 346/1
Glass ionomer cement (Ketac-Endo)	Espe	0004
Glass ionomer cement (Chemfil II; 680 mg powder/100 µl liquid)	DeTrey/Dentsply	990114
Glass ionomer cement (Base-Line)	DeTrey/Dentsply	900409
Glass ionomer cement (Vitrebond)	3M	91101
Glass ionomer cement (XR-Ionomer)	Kerr	20700/92160
Glass ionomer cement (Photac-Bond)	Espe	W 105
Zinc oxide	Mainland	L545935
Eugenol	Caelo-Caesar	902650281

2.2. Methods

2.2.1. Experimental procedure

In the first series of experiments the original filter method was performed as described in ISO-TR 7405 [9], using 0.45 µm pore size filter discs (Millipore, HAWG 047S3) and L-929 mouse fibroblasts grown in BME-medium (GIBCO, 073-01300) with 5% fetal calf serum (GIBCO, 023-06010). The cytotoxic reaction was recorded according to the original scoring system and, additionally, the area of the zone showing no enzyme reactivity was measured by means of an image analysing system (Videoplan, Kontron/Zeiss).

In the second series of experiments the original method was changed in the following ways: (1) the incubation time of the test material with the cell loaded filter was prolonged from 4 h (original method) to 24 h; (2) the filters were secured with glass rings (4.5 cm internal diameter) to prevent floating in the medium; and (3) the filters were incubated with the staining solution for at least 3 h in the dark in a CO_2-free atmosphere. The results were recorded as described above.

In the third series of experiments the original method was followed for the growth of a cell monolayer on the filter discs. The test samples were placed on the filter and the cultures were incubated for 24 h at 37 °C and 100% relative humidity in a 5% CO_2-containing air atmosphere. Then the samples were removed, the filter was placed cell side up and a solution of 20 µl FDA in 10 ml PBS applied for 15 min at 4 °C. Then the filter was placed either under an inverted microscope with UV stimulation (490 nm) and 12 filter (Leitz MPV Compact) or under a UV-lamp (combination of 312 nm, 365 nm and 254 nm, Bioblock Scientific, 67403 Illkirch, France) and the zone of cell damage was evaluated by measuring the diameter of the unstained zone. Standardized colour diapositives were taken and the area of cell damage (lack of fluorescence) was recorded by means of an image analysing system (Videoplan, Kontron/Zeiss). Some of the test materials (series 3a) were tested with this modification in order to compare the results with the original filter method. A larger amount of test materials was then tested (series 3b) in order to compare the results obtained with those derived from the agar overlay test (series 4).

In the fourth series of experiments the agar overlay test [7–9, 13, 15, 17] was performed according to the original method by grading the zone of decolouration and the degree of cell lysis within this zone.

Eight replicate experiments were performed for each method and each test material.

2.2.2. Mathematical and statistical procedures

For the fourth series of experiments the area of the zone of decolouration was calculated on the basis of the results of the grading. For each zone grade we assigned a particular diameter for the calculation of the area of the circular zone, as indicated below:

Zone (grade)	0	1	2	3	4	5
Diameter (mm)	0	2.5	10	20	60	95

The area calculation based on these assigned diameters is designated area without lysis.

In order to take the grading of cell lysis into account, this area without lysis was multiplied by a weighting factor, $f(d)$, related to the degree, d, of cell lysis:

$$f(d) = 0.2*d \quad (\text{for all } d = 0, 1, \ldots, 5)$$

The result of the area calculation based on the assigned diameters and the degree of cell lysis is designated area with lysis.

In order to compare the data the area values were transformed. Each individual area was divided by the area of the Millipore filter for the filter test and the area of the Petri dish for the agar overlay test and then multiplied by 100. This transformation yielded relative data, expressed as a percentage.

The means of both the areas and relative areas, the corresponding standard deviations, the medians, together with the corresponding 25% and 75% quantiles (= 1st and 3rd quartiles) were calculated for all of the samples. Statistical analysis for differences between the materials was performed using the non-parametric Mann-Whitney Test [22] for area data. The correlation coefficients of the medians for area data and relative data were calculated for the different methods and the correlation coefficients based on rank assignment were calculated according to the SPSS-programme [22].

3. Results

Fig. 1 shows the results of the original method (series 1) for zinc phosphate cement. Only for this test material stored at 0% relative humidity a zone of SDH inhibition can be observed. The overall staining is insufficient for an unequivocal interpretation of the cell response. The data of series 1 are summarized in Table II. It is obvious that this test procedure only detects very toxic materials.

Fig. 2 shows the results for zinc phosphate cement derived from the modified method using SDH as a demonstration marker for undamaged cells (series 2).

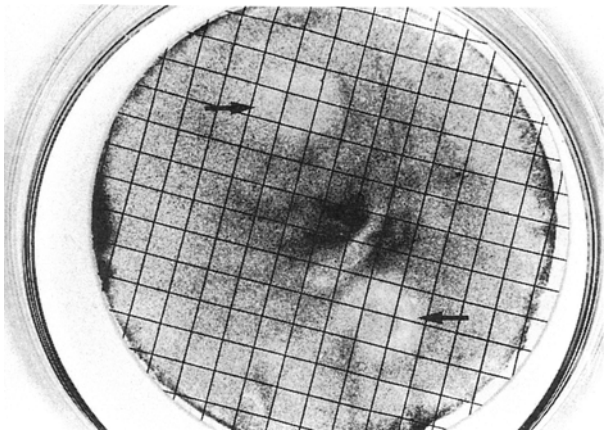

Figure 1 Results of the original filter method using SDH as a marker for cell vitality (series 1) for zinc phosphate cement, 1 h, 0% relative humidity: a zone of reduced staining indicates an area of damaged cells around each of the two test samples (arrows); the distinction between damaged and undamaged cells is insufficient.

In contrast to the first series, the staining is more intense and the distinct zones around the test specimens allow unequivocal interpretation of the cell response. The data of series 2 are summarized in Table III.

Fig. 3 shows the results for zinc phosphate cement derived from the method using FDA-turnover as a marker for vital cells (series 3). Again, distinct zones of cell damage can be observed. The photomicrograph (Fig. 4) shows fibroblasts with normal appearance on the filter outside the reaction zone. The results of series 3a are summarized in Table IV.

Fig. 5 shows the results for the zinc phosphate cement with the agar overlay test; Fig. 6, the corresponding photomicrograph. The zone of decolouration is clearly discernable, and also the change of the morphology of the cells. However, the grading of the cell destruction (grade 3) is subject to individual interpretation. The results of series 3b and 4 are summarized in Table V.

Comparison of the results of series 1 and 2 (Tables II and III) shows that the prolongation of the incubation time from 4 h to 24 h and the technical changes have made the method more sensitive.

Comparison of the results of series 2 and 3a shows that with both methods significant differences can be observed between the different humidity storage conditions for the cements but not with the composite resins. Within each series tests for significant differences were only performed if one of the variables (relative humidity or material) was changed (Table VI). The bar-diagram for the medians (Fig. 7) demonstrates good agreement of the results between the two series. The correlation coefficient with the area data is $r = 0.98$ (means and medians) being significantly different from 0 ($p < 0.001$). The correlation coefficient by assigned ranks is $r = 0.96$ (means) and $r = 0.98$ (medians), being significantly different from 0 ($p < 0.001$).

Comparison of the results of series 3b and 4 shows that for both test systems the effect of relative humidity during the storage of the specimens upon the toxic reaction is evident. The same can be observed for the influence of the setting speed: Ketac Endo, which by intention has a comparatively low setting speed, shows a prolonged toxicity in both test series. The phosphate cement showed initially higher scores of toxicity than the conventional glass ionomer cements in both test systems, but it was less toxic than zinc oxide eugenol.

The visible light cured material Vitrebond was by far the most toxic glass ionomer cement within this series again in both the filter and the agar overlay test. The other visible light cured glass ionomer cements (XR Ionomer and Photac-Bond) showed less toxicity, although at the set state at 100% relative humidity it was slightly more toxic than most of the conventional glass ionomer cements at corresponding storage conditions. In both test systems zinc oxide eugenol was the most toxic material within the whole series. However, in the agar overlay test all cells of the plate (95 mm diameter) were destroyed, whereas in the filter test system only part of the filter showed cell damage.

TABLE II Results of series 1 (original filter method); 8 samples in each category

Test material	% relative humidity	Biological reaction				
		Diameter (mm) Mean ± SD	Area (mm^2)			
			Mean ± SD	Median	Quantiles	
					25%	75%
Harvard	0	8.75 ± 0.96	63.5 ± 12.2	60.0	54.5	76.0
Harvard	100	Ø	Ø	Ø		
Ketac-Fil	0	Ø	Ø	Ø		
Ketac-Fil	100	Ø	Ø	Ø		
Aqua-Cem	0	Ø	Ø	Ø		
Aqua-Cem	100	Ø	Ø	Ø		
Composite	0	Ø	Ø	Ø		
Composite	100	Ø	Ø	Ø		
MMA	–	Ø	Ø	Ø		

Ø Method incapable of evaluation of biological reaction

TABLE III Results of series 2 (modified SDH determination); 8 samples in each category

Test material	% relative humidity	Biological reaction				
		Diameter (mm) Mean ± SD	Area (mm^2)			
			Mean ± SD	Median	Quantiles	
					25%	75%
Harvard	0	23.1 ± 4.7	413.7 ± 146.5	481.0	256.5	538.0
Harvard	100	10.5 ± 1.5	85.9 ± 20.3	90.0	74.5	101.5
Ketac-Fil	0	10.2 ± 2.8	86.5 ± 39.5	83.0	53.0	116.0
Ketac-Fil	100	1.2 ± 2.6	8.4 ± 18.7	0.0	0.0	0.0
Aqua-Cem	0	8.6 ± 1.4	62.8 ± 10.8	61.0	56.0	68.0
Aqua-Cem	100	1.6 ± 2.8	9.9 ± 17.0	0.0	0.0	35.0
Composite	0	7.4 ± 1.5	52.6 ± 12.9	50.0	45.0	57.5
Composite	100	7.0 ± 1.3	48.1 ± 12.4	43.0	40.0	60.0
MMA	–	13.5 ± 1.3	143.6 ± 28.1	159.0	116.5	165.0

TABLE IV Results of series 3a (fluorescein diacetate as marker for cell vitality); 8 samples in each category

Test material	% relative humidity	Biological reaction				
		Diameter (mm) Mean ± SD	Area (mm^2)			
			Mean ± SD	Median	Quantiles	
					25%	75%
Harvard	0	18.6 ± 1.7	296.8 ± 51.3	299.0	249.8	340.8
Harvard	100	10.2 ± 1.7	85.6 ± 27.3	74.5	68.8	99.0
Ketac-Fil	0	6.3 ± 1.6	44.9 ± 18.2	44.0	31.5	54.5
Ketac-Fil	100	1.1 ± 3.3	8.7 ± 26.0	0.0	0.0	0.0
Aqua-Cem	0	7.3 ± 1.5	51.1 ± 18.4	46.0	38.0	66.5
Aqua-Cem	100	0.2 ± 0.6	1.6 ± 4.7	0.0	0.0	0.0
Composite	0	5.7 ± 1.9	36.3 ± 16.0	34.0	27.3	52.3
Composite	100	6.2 ± 1.5	38.1 ± 16.0	32.0	24.3	57.0
MMA	–	12.8 ± 2.9	140.4 ± 43.3	153.0	133.8	157.3

The correlation coefficients between the results of series 3b and 4 (Table VII) with the area data were calculated to be 0.69 (without lysis) and 0.68 (with lysis); the correlation coefficients by assigned ranks, 0.79 (without lysis) and 0.85 (with lysis). Relative area data correlation coefficients are the same as area data correlation coefficients. All correlation coefficients are significantly different from 0 ($p < 0.001$).

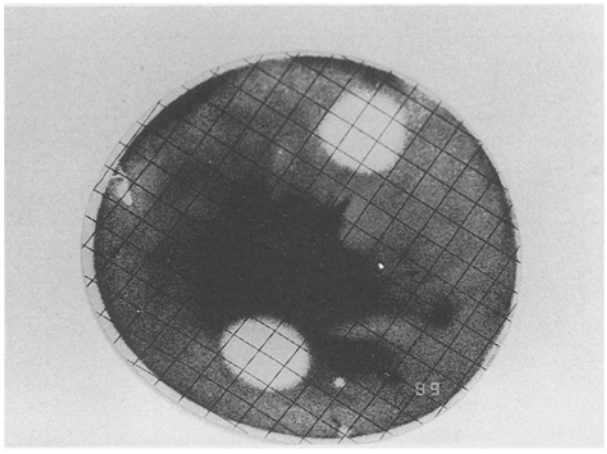

Figure 2 Results of the modified filter method using SDH as a marker for cell vitality (series 2) for zinc phosphate cement, 1 h, 0% relative humidity: an unstained zone indicates an area of damaged cells around each of the two test samples; the distinction between damaged and undamaged cells is sufficient.

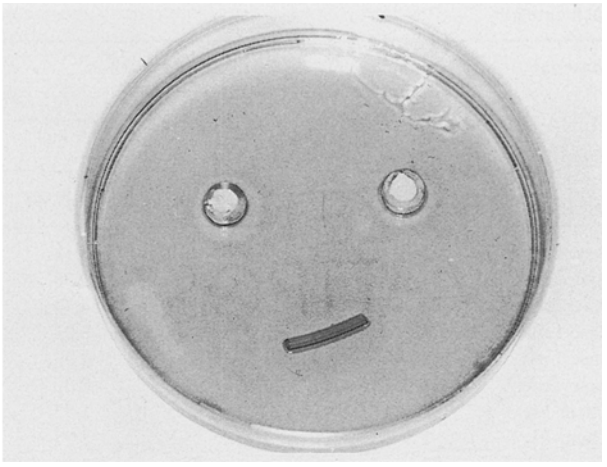

Figure 5 Results of the agar overlay test (series 4) for zinc phosphate cement, 1 h, 0% relative humidity: a zone of decolouration around each of the two test samples indicates an area of damaged cells (strip = control); the distinction between damaged and undamaged cells is sufficient.

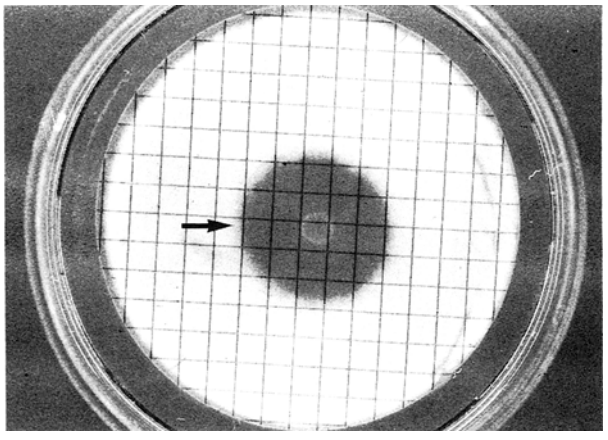

Figure 3 Results of the modified filter method using FDA as a marker for cell vitality (series 3) for zinc phosphate cement, 1 h, 0% relative humidity: an unstained zone indicates an area of damaged cells around the test sample; the distinction between damaged and undamaged cells is sufficient.

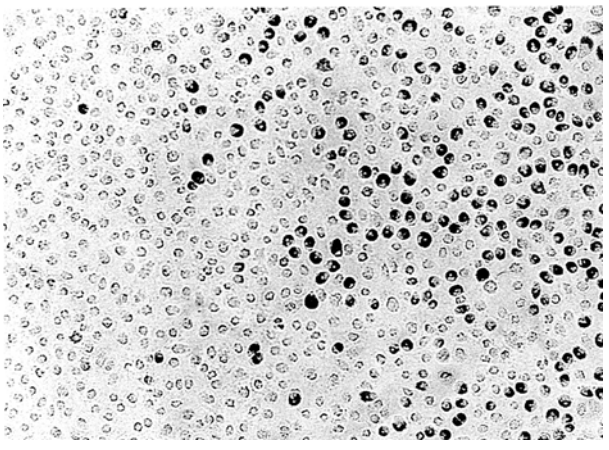

Figure 6 Photomicrograph (100×) of the cell reaction of the agar overlay test (series 4) for zinc phosphate cement, 1 h, 0% relative humidity: the storage of neutral red inside the cell is used as an indicator for undamaged cells by which they can easily be distinguished from dead cells; however, the grading of the cell lysis (grade 3) is subject to individual interpretation.

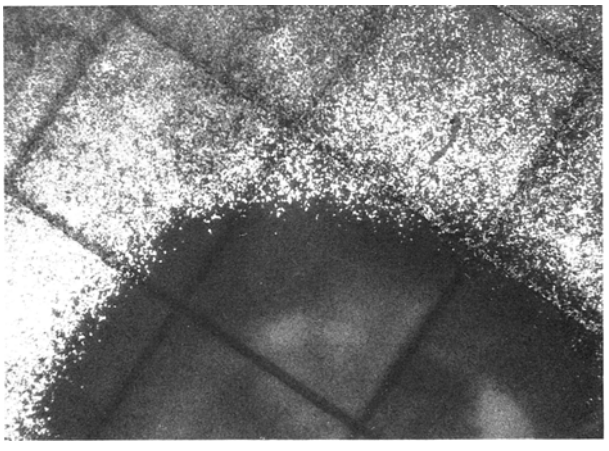

Figure 4 Photomicrograph (50×) of the cell reaction of the modified filter method using FDA as a marker for cell vitality (series 3) for zinc phosphate cement, 1 h, 0% relative humidity: vital cells show a light fluorescence by which they can easily be distinguished from dead cells.

4. Discussion

The influence of the storage conditions on the toxicity of the cements tested is consistent with findings in other cell culture test systems [13, 16, 21] and may be attributed to incomplete setting of the cements when stored at 0% relative humidity. The lack of such an influence with the composite resin, as was demonstrated in this study, was also reported in previous publications using different cell culture systems [13, 16, 21]. Also the observation that zinc phosphate cements are more cytotoxic than glass ionomer cements under corresponding conditions is in agreement with the literature [11], as well as the findings of the comparatively high toxicity for the visible light cured glass ionomer cement Vitrebond [23, 24] and the very high cytotoxicity of zinc oxide eugenol [25, 26]. The high toxicity of Vitrebond may be attributed to the high amount of hydroxyethyl methacrylate (HEMA) in the material which is needed to combine the hydro-

TABLE V Results of series 3b (fluorescein diacetate as marker for cell vitality) and of series 4 (agar overlay test); medians of 8 samples in each category

Material	Storage time (h)	% Rel. humidity	Filter method (series 3b)		Agar overlay test (series 4)				
			Test area		Zone/Lysis (grades)	Area without Lysis		Area with Lysis	
			(mm^2)	(Rel. %)		(mm^2)	(Rel. %)	(mm^2)	(Rel. %)
Base Line	1	0	98.2	6.18	1.0/2.0	4.9	0.07	2.0	0.03
Base Line	1	100	5.8	0.36	0.0/0.0	0.0	0.00	0.0	0.00
Base Line	24	0	75.4	4.74	1.0/1.5	4.9	0.07	1.5	0.02
Base Line	24	100	0.4	0.02	0.0/0.0	0.0	0.00	0.0	0.00
Base Line	168	0	64.0	4.03	2.0/2.0	78.5	1.11	31.4	0.44
Base Line	168	100	0.0	0.00	0.0/0.0	0.0	0.00	0.0	0.00
Chem Fil	1	0	88.5	5.57	2.0/3.0	78.5	1.11	47.1	0.66
Chem Fil	1	100	56.8	3.57	1.0/2.0	4.9	0.07	2.0	0.03
Chem Fil	24	0	78.6	4.94	2.0/2.0	78.5	1.11	31.4	0.44
Chem Fil	24	100	48.7	3.06	1.0/1.0	4.9	0.07	1.0	0.01
Chem Fil	168	0	52.6	3.31	1.0/1.0	4.9	0.07	1.0	0.01
Chem Fil	168	100	1.0	0.07	1.0/1.0	4.9	0.07	1.0	0.01
Ketac Bond	1	0	157.8	9.92	1.0/2.0	4.9	0.07	2.0	0.03
Ketac Bond	1	100	60.1	3.78	1.0/1.0	4.9	0.07	1.0	0.01
Ketac Bond	24	0	103.3	6.50	1.0/2.0	4.9	0.07	2.0	0.03
Ketac Bond	24	100	35.0	2.20	0.0/0.0	0.0	0.00	0.0	0.00
Ketac Bond	168	0	61.0	3.83	1.0/1.0	4.9	0.07	1.0	0.01
Ketac Bond	168	100	0.0	0.00	0.0/0.0	0.0	0.00	0.0	0.00
Ketac Endo	1	0	355.3	22.35	3.0/4.0	314.1	4.43	251.3	3.55
Ketac Endo	1	100	262.7	16.52	2.0/2.0	78.5	1.11	31.4	0.44
Ketac Endo	24	0	243.5	15.31	3.0/4.0	314.1	4.43	251.3	3.55
Ketac Endo	24	100	121.8	7.66	1.0/2.0	4.9	0.07	2.0	0.03
Ketac Endo	168	0	111.8	7.03	2.0/2.0	78.5	1.11	31.4	0.44
Ketac Endo	168	100	11.7	0.74	1.0/1.0	4.9	0.07	1.0	0.01
Ketac Fil	1	0	59.5	3.74	1.0/1.0	4.9	0.07	1.0	0.01
Ketac Fil	1	100	29.2	1.84	0.0/0.0	0.0	0.00	0.0	0.00
Ketac Fil	24	0	62.6	3.94	1.0/2.0	4.9	0.07	2.0	0.03
Ketac Fil	24	100	0.0	0.00	0.0/0.0	0.0	0.00	0.0	0.00
Ketac Fil	168	0	91.7	5.77	2.0/3.0	78.5	1.11	47.1	0.66
Ketac Fil	168	100	0.0	0.00	0.0/0.0	0.0	0.00	0.0	0.00
Ketac Silver	1	0	140.8	8.84	2.0/4.0	78.5	1.11	62.8	0.89
Ketac Silver	1	100	14.1	0.89	1.0/1.0	4.9	0.07	1.0	0.01
Ketac Silver	24	0	102.2	6.42	2.0/3.0	78.5	1.11	47.1	0.66
Ketac Silver	24	100	0.0	0.00	0.0/0.0	0.0	0.00	0.0	0.00
Ketac Silver	168	0	99.2	6.24	2.0/3.0	78.5	1.11	47.1	0.66
Ketac Silver	168	100	0.0	0.00	0.0/0.0	0.0	0.00	0.0	0.00
Tenet	1	0	278.5	17.51	2.0/4.0	78.5	1.11	62.8	0.89
Tenet	1	100	128.4	8.07	1.0/3.0	4.9	0.07	2.9	0.04
Tenet	24	0	196.7	12.37	2.0/3.0	78.5	1.11	47.1	0.66
Tenet	24	100	113.8	7.16	1.0/1.0	4.9	0.07	1.0	0.01
Tenet	168	0	227.8	14.32	1.0/3.0	4.9	0.07	2.9	0.04
Tenet	168	100	0.0	0.00	0.0/0.0	0.0	0.00	0.0	0.00
Vitre Bond	1	0	492.0	30.94	4.0/4.0	2826.9	39.90	261.5	31.92
Vitre Bond	1	100	319.1	20.07	3.0/3.0	314.1	4.43	188.5	2.66
Vitre Bond	24	0	381.4	23.99	3.0/4.0	314.1	4.43	251.3	3.55
Vitre Bond	24	100	248.2	15.61	2.0/3.0	78.5	1.11	47.1	0.66
Vitre Bond	168	0	256.1	16.10	3.0/3.0	314.1	4.43	188.5	2.66
Vitre Bond	168	100	233.8	14.70	1.0/3.0	4.9	0.07	2.9	0.04
XR-Ionomer	1	0	157.8	9.92	2.0/3.0	78.5	1.11	47.1	0.66
XR-Ionomer	1	100	69.9	4.40	1.0/2.0	4.9	0.07	2.0	0.03
XR-Ionomer	24	0	108.9	6.85	2.0/2.0	78.5	1.11	31.4	0.44
XR-Ionomer	24	100	41.7	2.62	1.0/1.0	4.9	0.07	1.0	0.01
XR-Ionomer	168	0	82.4	5.18	2.0/2.0	78.5	1.11	31.4	0.44
XR-Ionomer	168	100	31.1	1.96	1.0/1.0	4.9	0.07	1.0	0.01
Photac-Bond	1	0	28.0	1.76	2.0/1.5	78.5	1.11	23.5	0.33
Photac-Bond	1	100	24.5	1.54	1.0/1.0	4.9	0.07	1.0	0.01
Photac-Bond	24	0	31.0	1.95	2.0/3.0	78.5	1.11	47.1	0.66
Photac-Bond	24	100	22.0	1.39	2.0/2.0	78.5	1.11	31.4	0.44
Photac-Bond	168	0	35.0	2.20	2.0/3.0	78.5	1.11	47.1	0.66
Photac-Bond	168	100	14.5	0.91	1.0/1.0	4.9	0.07	1.0	0.01
ZnO-Eugenol	1	0	531.4	33.42	5.0/5.0	7086.9	100.00	7086.9	100.00
ZnO-Eugenol	1	100	419.0	26.35	4.0/5.0	2826.9	39.90	2826.9	39.90
ZnO-Eugenol	24	0	399.0	25.09	5.0/5.0	7086.9	100.00	7086.9	100.00
ZnO-Eugenol	24	100	309.2	19.44	4.0/5.0	2826.9	39.90	2826.9	39.90
ZnO-Eugenol	168	0	387.2	24.35	4.0/5.0	2826.9	39.90	2826.9	39.90
ZnO-Eugenol	168	100	235.4	14.81	4.0/4.0	2826.9	39.90	2261.5	31.92

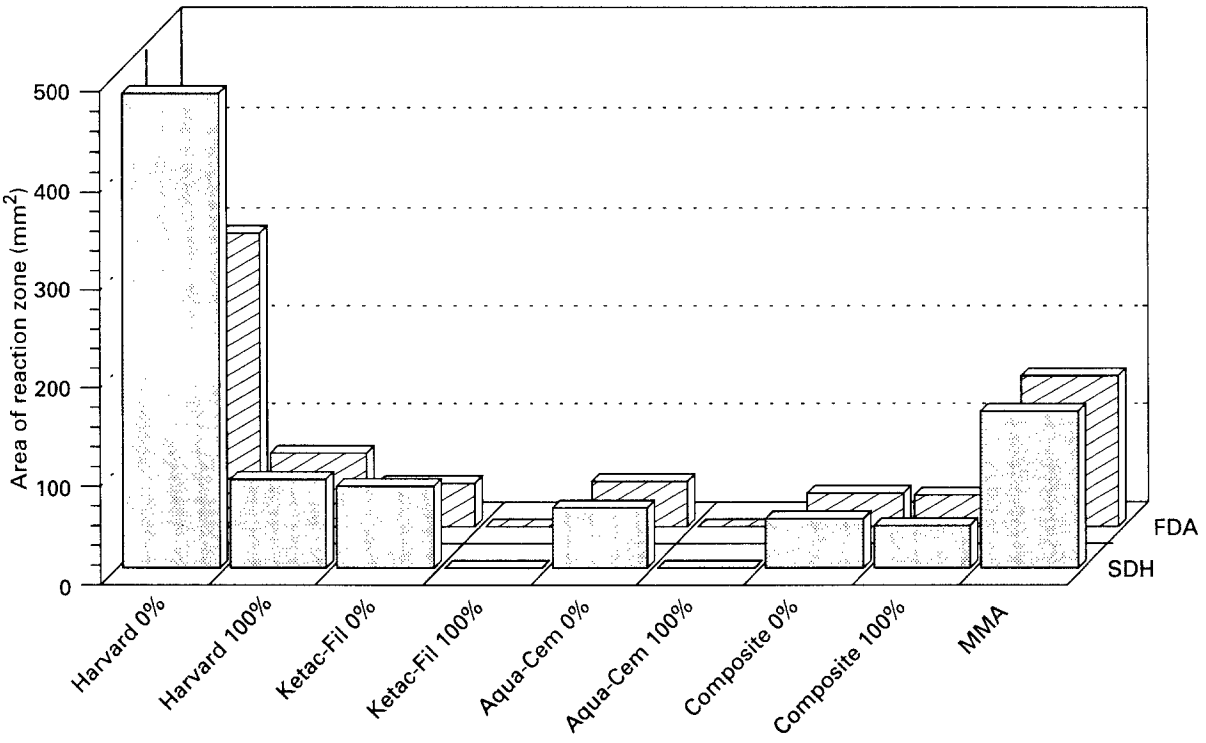

Figure 7 Comparison of the results (medians) of series 2 and of series 3a: a high degree of correlation of the results for the different test materials between the two test methods can be demonstrated.

TABLE VI Statistical analysis (Mann–Whitney test) for the different materials in each of the test systems; top and right: series 2; bottom and left: series 3a

	Harv 0%	Harv 100%	KeFi 0%	KeFi 100%	AquC 0%	AquC 100%	Comp. 0%	Comp. 100%	MMA
Harv 0%		++	++	n.t.	++	n.t.	++	n.t.	++
Harv 100%	++		n.t.	++	n.t.	++	n.t.	++	++
KeFi 0%	++	n.t.		++	n.s.	n.t.	++	n.t.	++
KeFi 100%	n.t.	++	++		n.t.	n.s.	n.t.	++	++
AquC 0%	++	n.t.	n.s.	n.t.		++	+	n.t.	++
AquC 100%	n.t.	++	n.t.	n.s.	++		n.t.	++	++
Comp. 0%	++	n.t.	n.s.	n.t.	n.s.	n.t.		n.s.	++
Comp. 100%	n.t.	++	n.t.	+	n.t.	++	n.s.		n.t.
MMA	++	+	++	++	++	++	++	n.t.	

+ $p < 0.05$
++ $p < 0.01$
n.s. no significant difference ($p < 0.05$)
n.t. not tested statistically

phobic monomer-system to the hydrophilic acrylic acid system or to the specific catalyst. HEMA is reported to be very cytotoxic [27]. Therefore, it can be concluded that the data generated with the modified SDH test and with the FDA method are consistent with literature reports.

The extension of the exposure time from 4 h (series 1, ISO-TR 7504) to 24 h (series 2) makes the original method more sensitive, which is in accordance with the results of Meryon and Browne [11]. The technical changes that were introduced are taken from other biochemical techniques [28]. As a consequence the staining of the filters is more intense and the interpretation of the cell response is unequivocal.

The use of FDA, instead of the rather complicated and time consuming SDH, yields results as unequivo-

TABLE VII Correlation coefficient results of statistical analysis between the median data of the filter method (series 3b) and the agar overlay test (series 4)

Correlation parameters	Area data		Relative area data	
	Usual corr. coef.	Corr. coef. by assigned rank	Usual corr. coef.	Corr. coef. by assigned rank
Filter method versus agar overlay without lysis	0.6927	0.7946	0.6927	0.7937
Filter method versus agar overlay with lysis	0.6766	0.8537	0.6767	0.8528

All coefficients are significantly different from 0 ($p < 0.001$).

cal as the modified SDH technique and is technically as easy and as fast to perform as the agar overlay method.

The use of the area of cell damage as a measure of the toxicity, instead of the diameter (or scores), has the advantage that irregular shaped zones can be evaluated. Furthermore, the area of cell damage seems from a theoretical point of view better related to the amount of toxicants eluted from a material than the diameter of the zone, or a scoring system related to the diameter of the zone. Modern means of computerized methods for measuring areas make this way of evaluation easy and fast to perform.

Comparison of the area data between the filter test and the agar overlay test (series 3b and 4) shows generally good agreement as can be demonstrated by the high correlation coefficients. This is in contrast to findings by Hensten-Pettersen and Helgeland [29] who reported little agreement between different cell culture methods. However, these authors used completely different test systems with different cell lines and different elution conditions. In the present study the cell lines were identical as well as the elution conditions of the test specimens: they were placed on top of a culture, one side being in contact with the test system (filter/agar) the other side having contact with air. Only the zinc oxide eugenol showed some discrepancy in both test systems: it destroyed all the cells in the agar overlay test, but damaged only part of the monolayer on the filter. This demonstrates that although these test systems are very closely related, the results cannot be used interchangeably. Probably the different conditions for diffusion through the agar on the one side and through the filter on the other side may account for these differences. Therefore, cytotoxicity data should only be interpreted from a relative toxicity analysis, using one strictly defined test system and a series of control materials. The higher values of the correlation coefficients by assigned ranks in comparison with those calculated from the area data underline this conclusion, which is in agreement with other reports [30]. However, in the new ISO-specification No. 10993 part V [31], which covers cytotoxicity tests for the whole field of medical devices, no specific method for cytotoxicity testing is mentioned, but only guidelines on how to perform such tests. Therefore we recommend that defined methods be included in the corresponding dental standard (ISO TR 7405, under revision), in order to make the results of different investigators comparable.

Concern has been expressed over the evaluation system of the agar diffusion test since the grading of the lysis index is subject to individual interpretation. However, the present results show a higher correlation coefficient by assigned rank when the lysis index is used, compared with when it is omitted. This is in agreement with former findings in our laboratory [30] and demonstrates the importance of the lysis index when used by an experienced investigator.

From the present study it can be concluded that the filter method as described in ISO-TR 7405 should be revised in the following ways:

1. If SDH is used as a marker for cell vitality, the modified method should be used.
2. As an alternative, the use of FDA as a marker for vitality should be adopted.
3. The area of cell damage should be used as a measure of material toxicity.
4. In addition of ISO 19993, part V, a precise description of cell culture tests should be included in the dental standard ISO-TR 7405, which is under revision.

Acknowledgement

The authors wish to thank Dr L. J. Nunez for valuable discussions.

References

1. L. TRONSTAD, G. HASSELGREN and A. WENNBERG, *J. Dent. Res. A* **56** (1977) 119.
2. L. TRONSTAD, A. WENNBERG and G. HASSELGREN, *J. Endodont.* **4** (1978) 304.
3. A. WENNBERG, G. HASSELGREN and A. TRONSTAD, *J. Biomed. Mater. Res.* **13** (1979) 109.
4. L. TRONSTAD and A. WENNBERG, *Int. Endodont. J.* **13** (1980) 13.
5. A. WENNBERG, *J. Endodont.* **6** (1980) 784.
6. C. YESILSOY and R. J. FEIGAL, *ibid.* **11** (1985) 401.
7. DIN-NORMENAUSSCHUß DENTAL, DIN-V 13930 (Westliche 56, 7350 Pforzheim, FRG).
8. ASSOCIATION FRANCAISE DE NORMALISATION, "Evaluation biologique des produits dentaire", Document No. 85058 (1985) 91.
9. INTERNATIONAL ORGANISATION FOR STANDARDIZATION "Biological evaluation of dental materials", Technical Report 7405.
10. S. D. MERYON, P. G. STEPHENS and R. BROWNE, *Int. Endodont. J.* **15** (1982) 197.
11. S. D. MERYON and R. M. BROWNE, *Cell Biochem. Func.* **2** (1984) 43.
12. S. D. MERYON, *Biomater.* **5** (1984) 293.

13. G. SCHMALZ, *Int. J. Endodont.* **21** (1988) 59.
14. AMERICAN DENTAL ASSOCIATION, *J. Amer. Dent. Assoc.* **99** (1979) 637.
15. W. L. GUESS, S. A. ROSENBLUTH, B. SCHMIDT and J. AUTIAN, *J. Pharm. Sci.* **54** (1965) 1545.
16. G. SCHMALZ and M. SHARAF, *Z. Zahnärztl. Implantol.* **4** (1988) 240.
17. G. SCHMALZ, "Die Gewebeverträglichkeit Zahnärztlicher Materialien-Möglichkeiten der Prüfung in der Zellkultur" (Thieme, Stuttgart New York, 1980).
18. G. SCHMALZ and L. NETUSCHIL, *J. Dent. Res.* **62** (1983) 451.
19. *Idem, J. Biomed. Mater. Res.* **19** (1985) 653.
20. G. SCHMALZ and H. KÜBLER, *J. Dent. Res.* **66** (1987) 352.
21. G. SCHMALZ and H. SCHWEIKL, *J. Mater. Sci.: Mater. Med.* **1** (1990) 228.
22. SPSS/PC +, SPSS Inc., Chicago, IL, USA.
23. T. KANAOKA, E. YOSHII and K. HIROTA, *J. Dent. Res.* **70** (1991) 798.
24. J. P. SALOMON, G. DeSOUSA, M. ROCHE, M. GOLDBERG and R. RAHMANI, *ibid.* **70** (1991) 740.
25. W. R. HUME, *Int. Dent. J.* **21** (1988) 85.
26. G. SCHMALZ and J. ROTGANS, *Ned. Tijdschrift Tandheelk* **86** (1979) 85.
27. W. MAILE, T. LINDL and D. G. WEISS, *Mol. Toxicol.* **1** (1987) 427.
28. T. P. SINGER, E. B. KEARNEY and W. C. KENNEY, *Adv. Enzymol.* **37** (1973) 189.
29. A. HENSTEN-PETTERSEN and K. HELGELAND, *Scand. J. Dent. Res.* **85** (1977) 291.
30. G. SCHMALZ, *J. Dent. Res.* **61** (1984) 577.
31. INTERNATIONAL ORGANIZATION FOR STANDARDIZATION, "Biological evaluation of medical devices" ISO 10993-5 (DJS)

Received 4 January
and accepted 19 May 1993