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Methylcellulose cell culture as a new cytotoxicity test system for biomaterials

M. J. A. VAN LUYN, P. B. VAN WACHEM, P. NIEUWENHUIS
Department of Histology and Cell Biology, University of Groningen, Oostersingel 69/2, 9713 EZ Groningen, The Netherlands

L. OLDE DAMINK, H. TEN HOOPEN, J. FEIJEN
Department of Chemical Technology, University of Twente, P.O. Box 217, 7500 AE Enschede, The Netherlands

The cytotoxicity of biomaterials can be tested *in vitro* using various culture systems. Liquid culture systems may detect cytotoxicity of a material either by culture of cells with extracts or with the material itself. In the latter instance, renewing the medium will remove possible released cytotoxic products. The agar-overlay test is a short term semi-solid culture system in which the possible cytotoxicity of biomaterials is identified only by the presence of cell free zones. The aim of this study was to develop a more sensitive cytotoxicity test system for biomaterials, using methylcellulose as a culture gel, mixed with human fibroblasts. The main advantage of the test system is the possibility of evaluating cytotoxicity for a period of up to seven days without renewal of the culture gel. Furthermore it is possible to both quantitatively evaluate by counting absolute cell numbers and to qualitatively evaluate by studying cell morphology with light- and/or electron microscopy. Processed dermal sheep collagen was selected as test material, since contradictory results concerning the cytotoxicity of its extracts have been reported by others [2, 15, 18, 19]. Using our test system, both primary and secondary cytotoxic effects were found. Primary cytotoxicity is due to direct leakage of products from the material, detected by testing, extracts of the collagen or the collagen itself. Secondary cytotoxicity is due to release of cytotoxic products resulting from cell–biomaterial interactions. We conclude that our test system is extremely useful to test materials which are suspected of primary and/or secondary cytotoxicity, either with slow release of cytotoxic products or release of products with late cytotoxic effects.

Introduction

The aim of this study was to develop a sensitive *in vitro* cytotoxicity test system for biomaterials. When using liquid culture test systems there are two possibilities. Cells may be cultured in the presence of extracts of biomaterial [1, 2], or a biomaterial is put into the medium in (in-)direct contact with the cells [3–8]. When the latter system is used in long term testing, a disadvantage is the necessary changing of the medium, with removal of possible released cytotoxic products as a consequence.

The so-called solidified agar-overlay test system, such as described by NIH (NIH 77-1294), is widely used and often required for biomaterial testing [6, 9–11]. In this system the material is put on top of the agar, separating it from a layer of cells. Usually the cultures are examined after one or two days and a possible cytotoxic effect is only identified by a cell free zone under the test sample [6, 9–11]. In our opinion, disadvantages of the agar-overlay test system are the rather ill-defined way of cytotoxicity scoring, using the relatively insensitive model of a stable cell layer as compared to a growing cell culture and the short observation period.

In order to overcome the disadvantages of both liquid culture and agar-overlay test systems described above, we developed a semi-solid test system in which we used the rather water soluble methylcellulose (MC). As a culture gel, MC is widely used for culturing haemopoietic colonies [12–14]. In our semi-solid test system we mixed MC culture gel with human skin fibroblasts. Cells are allowed to grow in the presence of (or extracts of) biomaterials for a period of up to seven days without refreshing the culture gel. Scoring of possible cytotoxicity is easily performed by cell counting. Furthermore, cell morphology may be examined by phase contrast light microscopy and/or by transmission electron microscopy.

In this study we tested the biocompatibility of hexamethylenediisocyanate (HMDIC)-tanned dermal sheep collagen (HDSC). Previously, Metselaar [15] observed that fibroblasts exposed to extracts of HDSC did not show inhibition of DNA synthesis within 24 h, as indicated by ^{3}H–thymidine incorporation. However, HDSC may be suspected of cytotoxic effects, because of the tanning agent used. HMDIC as tanning agent has been used by others for other types of collagen [2, 16–19]. Although HMDIC was reported

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to be a more adequate tanning agent than glutaraldehyde with respect to cytotoxicity [2, 15, 18, 19], Ulreich *et al.* [2] observed 22% inhibition in ^{3}H–thymidine incorporation, when testing an extract of a collagen sponge tanned with HMDIC. Cytotoxic effects of diisocyanates have been repeatedly reported [20–23], and 1,6–diaminohexane, which is a cytotoxic hydrolysis product of HMDIC [24–26], may be released from collagens tanned with HMDIC. Thus we considered HDSC as a proper test material in the cytotoxicity test system presented in this paper.

2. Materials and methods

2.1. Materials

2.1.1. Culture conditions

Human skin fibroblasts (HF, from the established cell line PK 84) were routinely cultured in RPMI 1640 medium (Gibco Biocult Co, Paisley, UK), supplemented with 10% fetal calf serum (FCS), 2 mM ml^{-1} glutamine (Glut) (Merck, Darmstadt, FRG), penicillin (Pen) and streptomycin (Strep), both 100 U ml^{-1} (Gibco). The cells were incubated at 37 °C in air containing 5% CO_2.

A stock gel of methylcellulose (MC), using Methocel® high viscosity (3000–5000 mPa) from Fluka, Bio Chemica, Buchs, Switzerland, was prepared according to Iscoves and Schreier [12], with Iscoves modification of Dulbecco's medium (IMDM) (Flow Lab, Irvine, UK) in a final concentration of 2.25% (w/v).

2.1.2. Materials and extracts tested

Hexamethylenediisocyanate-tanned dermal sheep collagen (HDSC), was obtained from the Zuid Nederlandse Zeemlederfabriek, Oosterhout, The Netherlands. Since glutaraldehyde(G)-tanned collagens are widely used as bioprostheses, a G-tanned dermal sheep collagen (GDSC), also obtained from the above mentioned company, was used as a reference material. Discs with a diameter of 8 mm were punched from both HDSC and GDSC and the individual weight of each disc was established. These discs were sterilized by gamma irradiation, 2.5 Mrad (Gammaster, Ede, The Netherlands). Low density polyethylene (PE) sheets (Talas, Ommen, The Netherlands) with a thickness of 0.05 mm were also punched into discs of 8 mm. PE discs were gas-sterilized with ethylene oxide and used as a control material for the O_2/CO_2-exchange in the culture systems.

A 10 day extract (E1) of HDSC was made by shaking 1 g of HDSC per 20 ml of IMDM (supplemented with Pen and Strep) for 10 days at 37 °C. This ratio corresponded to the ratio of the total weight of two HDSC discs to the volume of the culture gel in each well (see later). The extracted HDSC, after preparation of E1, was punched under sterile conditions into discs of 8 mm. In another experiment the extracted HDSC was washed twice with IMDM and for a second time incubated with fresh IMDM for another 10 days at 37 °C, resulting in a second 10 day extract (E2). The twice extracted HDSC, left after preparation of E2, was also punched into discs of 8 mm. Furthermore, control IMDM was made by shaking IMDM for 20 days at 37 °C to test quality maintenance of culture medium while preparing extract E1 or E2.

2.2. Methods

2.2.1. Human fibroblasts (HF)

After washing twice with phosphate buffered saline (PBS) (NPBI, Emmer-Compascuum, The Netherlands), HF were harvested from routine culture using 0.25% (w/v) trypsin in Ca\Mg free Hank's Salt Solution (Gibco). Thereafter cells were centrifuged and resuspended in fresh IMDM.

2.2.2. HF/gel mixtures

An ultimate HF/IMDM suspension of 4.17 $\times 10^4$ HF ml^{-1} was prepared. HF/gel mixtures were made by gently but thoroughly mixing 30% (v/v) HF/IMDM with 50% (v/v) MC stock gel and 20% (v/v) FCS. Volumes of Pen, Strep and Glut, added to IMDM, has been adjusted in order to obtain the same final concentration in the culture gel as described for routine culture medium.

2.2.3. HF/gel extract mixtures

Cultures of HF containing extract E1, E2 or control IMDM were made by adding HF in a minimal volume to a mixture of 50% (v/v) of MC, 20% (v/v) of FCS and 30% (v/v) of E1, E2 or control IMDM to give a final concentration of 1.25×10^4 HF ml^{-1} culture gel (cultures C, D and H respectively, see later).

For all cultures, a final volume of 4.0 ml culture gel containing 5×10^4 HF was placed into each well of 6 wells Tissue Culture Plates (Greiner B. V., Alphen a/d Rijn, The Netherlands) using a plastic syringe. Since each well has a surface area of 10 cm^2 the final seeding density was 5×10^3 HF cm^{-2}. All cell cultures were set up in triplicate. Cultures were incubated at 37 °C in air containing 5% CO_2. After 24 h, two discs of (extracted) HDSC (cultures B, E, F, see later), GDSC (culture G) or PE (culture I), were put on top of the HF/gel mixture in each well and the discs immersed just below the gel surface (Fig. 1).

Cultures
- A: Control HF/gel culture.
- B: HF/gel culture with discs of HDSC with a total weight of approximately 30 mg per well.
- C: HF/gel culture with 30% of extract E1.
- D: HF/gel culture with 30% of extract E2.
- E: HF/gel culture with discs of once extracted HDSC remaining from preparation of E1.
- F: HF/gel culture with discs of twice extracted HDSC remaining from preparation of E2.
- G: HF/gel culture with discs of GDSC with a total weight of approximately 30 mg per well.
- H: HF/gel culture with PE discs.
- I: HF/gel culture with 30% control IMDM.

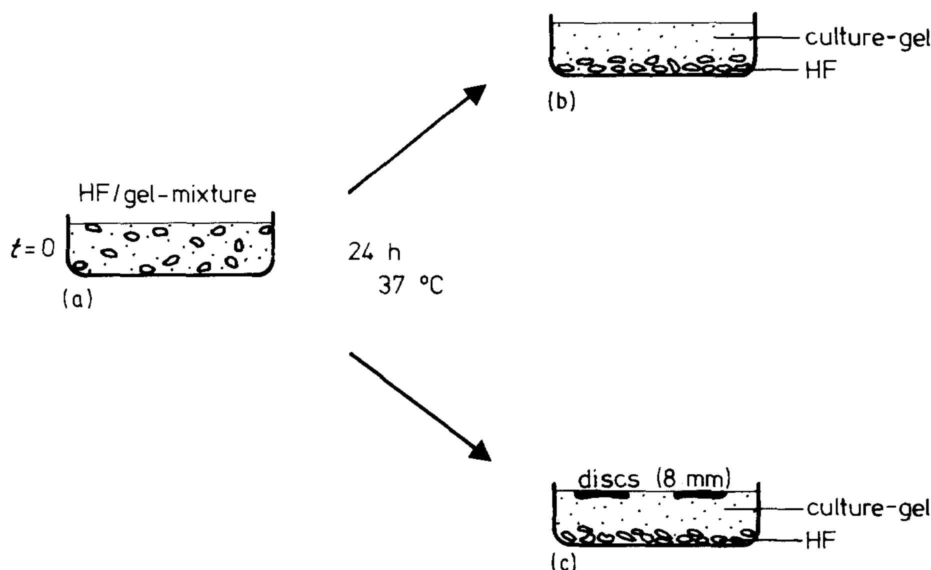


Figure 1 (a) At $t = 0$ h 4.0 ml of culture gel containing 5×10^4 HF was placed in each well of 6 wells tissue culture plates. (b) Thereafter HF migrated towards the bottom of the wells and adhered. (c) After 24 h two discs of (extracted) HDSC (cultures B, E, F), two discs of GDSC (culture G) or two discs of PE (culture H) were placed on top of the gel.

2.3. Cell counts

At 3, 5 or 7 days after starting the cultures, the gel was removed without damaging the cells using a syringe and, in the case of cultures B, E, F and H, after removal of the discs. The cell layers remaining on the bottom of the wells were extensively washed (3 or 4 times) with 4 ml of PBS in order to completely remove the gel. The cells were trypsinized, re-suspended and counted in a Bürker counting chamber. Cell numbers ($\times 10^3$ cm^{-2} $\pm$ standard deviation) represent the mean of counts of cell numbers in triplicate wells. From these numbers the inhibition of cell proliferation, expressed as a percentage of the cell number in culture A, was calculated each time point, using the following relationship:

$$\text{inhibition (\%)} = \frac{\text{A} - \text{(e.g.) B}}{\text{A}} \times 100\%,$$

in which A and B represent the number of cells of respectively culture A and culture B.

2.4. Microscopy

Light microscopic evaluation of the cells is easily performed without removing culture gel and discs. *In situ* photography with the phase contrast inverted light microscope was done after washing, when the cells were covered with PBS. For transmission electron microscopy, trypsinized cells, pooled from the three wells, were washed with PBS and centrifuged. The resulting pellets were fixed with 2% glutaraldehyde in PBS and cut into 1 mm^3 blocks. Postfixation was done by 1% osmium, thereafter cells were dehydrated with increasing concentrations of ethanol and embedded in Epon 812. Ultrathin sections were stained with uranylacetate, dissolved in methanol and examined with a Philips 201 transmission electron microscope.

3. Results

3.1. Cell counts

Human fibroblasts (HF) were seeded in culture gel to a final density of 5×10^3 HF cm^{-2} and incubated at 37 °C. Within 24 h HF migrated towards the bottom of the wells where they adhered (Fig. 1). Subsequently cells started to proliferate.

Cell numbers in control culture A increased from 16.8×10^3 HF cm^{-2} on day 3 to 128.3×10^3 HF cm^{-2} on day 7 (Fig. 2). In culture B, with discs of HDSC on top of the culture gel. HF numbers increased from 13.5×10^3 cm^{-2} on day 3 to 59.0×10^3 cm^{-2} on day 7 (Fig. 2). From these data it was calculated that the presence of the HDSC results in an inhibition of cell proliferation of 54.0% on day 7 (Table I).

To test whether this inhibition of cell proliferation is due to leakage of products from HDSC, HDSC was extracted during a period of 10 days (extract E1) and during a second period of 10 days (extract E2) in IMDM at 37 °C. When using extract E1 in the culture

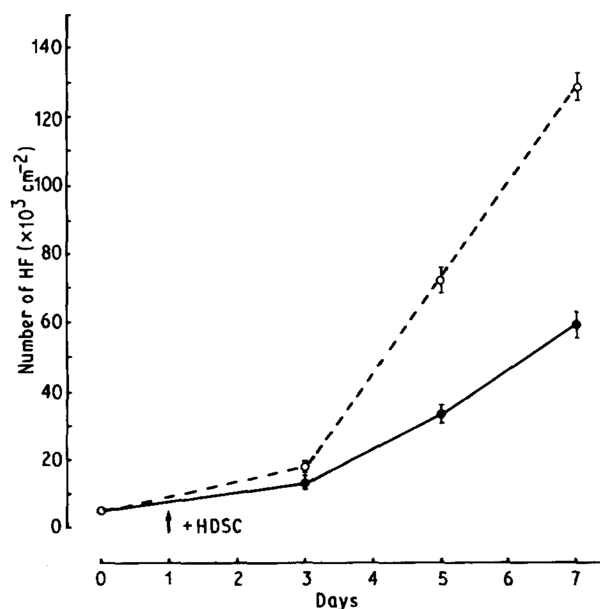


Figure 2 Numbers of HF ($\times 10^3$ cm^{-2} $\pm$ s.d.) in: (---) control culture A and (——) culture B as measured on days 3, 5 and 7. In culture B two discs of HDSC were placed on top of the gel after 24 h.

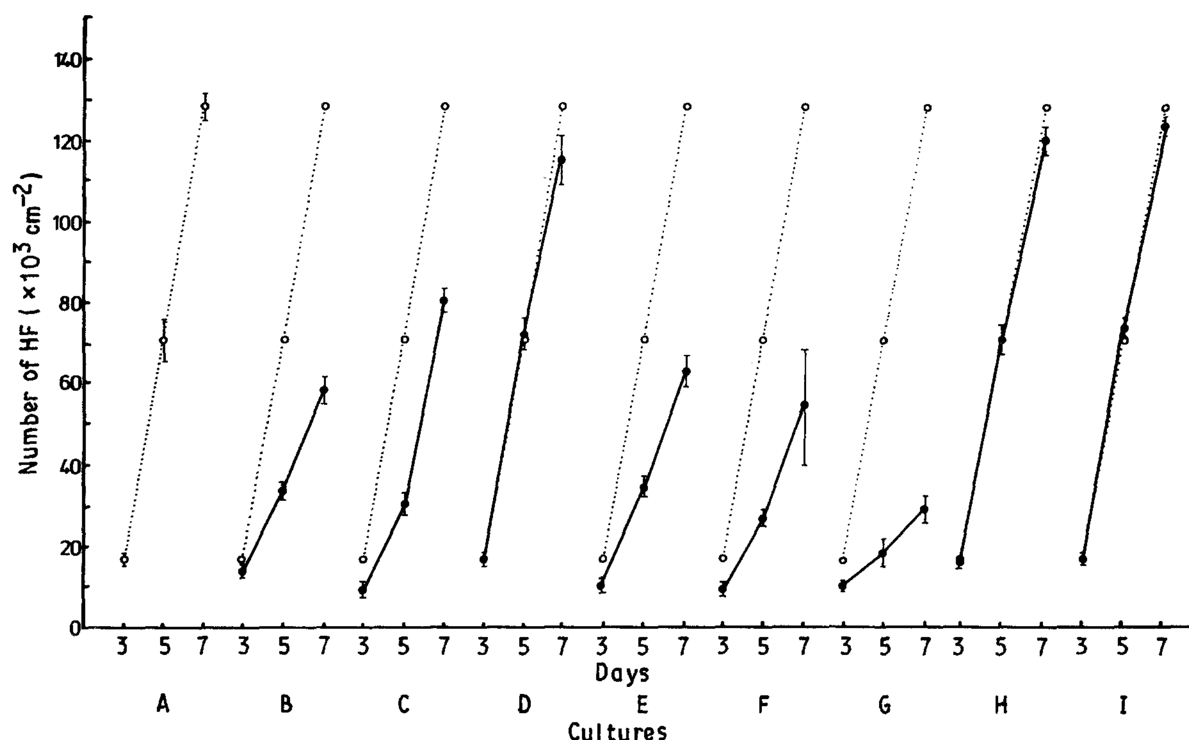


Figure 3 Numbers of HF ($\times$ 10^3 cm^{-2} $\pm$ s.d.) in cultures: (---) A and (——) B–I as measured on days 3, 5 and 7. For explanation of the different cultures, see Materials and Methods.

gel (culture C), lower cell numbers as compared to the control culture were found (Fig. 3) and on day 7 an inhibition of cell proliferation of approximately 40% was found (Table I). However, when extract E2 (culture D) was used an inhibition of only 2.8% was obtained (Table I).

In cultures E and F, with discs of HDSC, remaining from preparation of extract E1 and extract E2 respectively, an inhibition of cell proliferation of approximately 50% in both cultures on days 3, 5 and 7 as calculated (Table I). These inhibitions differed strikingly from the 2.8% inhibition obtained with extract E2 (Table I). The standard deviation on day 7 in culture F was relatively high (Fig. 3). As a reference, GDSC (culture G) was tested. This resulted in an inhibition of approximately 77% on day 7.

Cell numbers in culture H, in which PE discs were used, did not differ from cell numbers in culture A (Fig. 3 and Table I). This result excludes the possibility of decrease in cell numbers due to poor culture conditions, i.e., poor O_2/CO_2-exchange because of the mere physical presence of the discs. No differences in cell numbers were found when control IMDM was used (culture I) as compared to culture A in which fresh IMDM was used (Fig. 3 and Table I). This result excludes the possibility of a decrease in cell proliferation in cultures C and D by using low quality IMDM due to prolonged shaking at 37 °C during preparation of the extracts.

TABLE I Inhibitions (%) of proliferation of HF on days 3, 5 and 7 in cultures B–I as compared to proliferation in culture A. For explanation of the different cultures, see Materials and Methods.

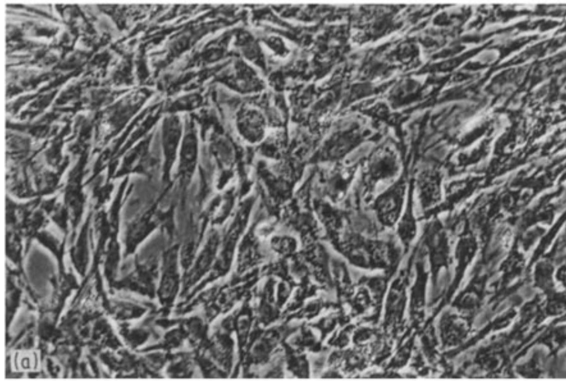
Cultures	day 3	day 5	day 7
B	28.2	53.9	54.0
C	46.5	58.0	37.7
D	0.0	0.0	2.8
E	43.9	51.3	47.6
F	37.4	42.0	58.0
G	53.5	73.8	76.7
H	0.0	0.0	6.7
I	0.0	0.0	2.0

3.2. Microscopy

In control culture A, a multilayer of completely spread HF was observed on day 7 with the light microscope. Some illuminated particles were present (Fig. 4a). Cells of cultures D, H and I had morphologies similar to those described for culture A.

In cultures with DSC discs (B, E, F, and G) cell free zones were macroscopically observed upon disc removal on days 5 and 7. Light microscopically, cells cultured in the presence of HDSC (culture B, Fig. 4b), extract E1 (culture C), extracted HDSC (cultures E and F) or GDSC (culture G) had decreased numbers with proportionally larger cells and poorly adhering cells with elongated or spiderlike structures and illuminated edges. Furthermore, increased numbers of illuminated particles were observed in their cytoplasm (Fig. 4b).

Transmission electron microscopic examination of trypsinized cells confirmed and extended the differences outlined above in cell morphology, in cultures B, C, E, F and G as compared to control culture A (Fig. 5a and b). The illuminated particles, as observed with the light microscope, represent lipid droplets, which were found in high quantities on days 5 and 7



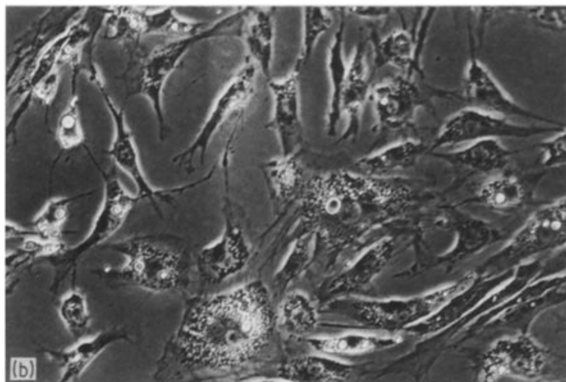
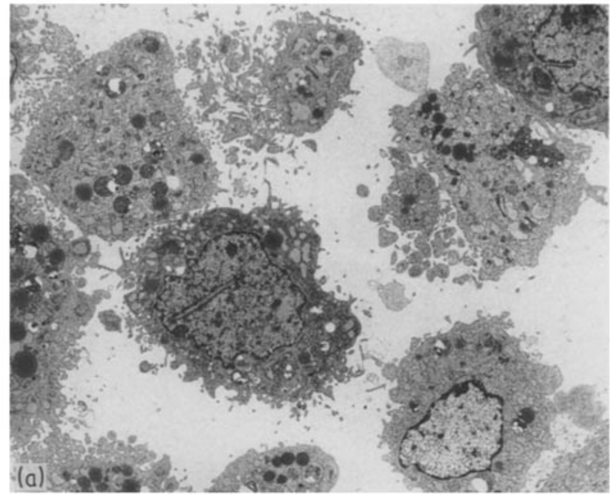


Figure 4 (a) Light microscopical micrographs of HF in culture A and (b) culture B on day 7. In culture A, a multilayer of completely spread cells is observed. Decreased numbers with proportionally larger and poorly adhering cells with many illuminated particles are present in culture B. Magnification, 110 ×.



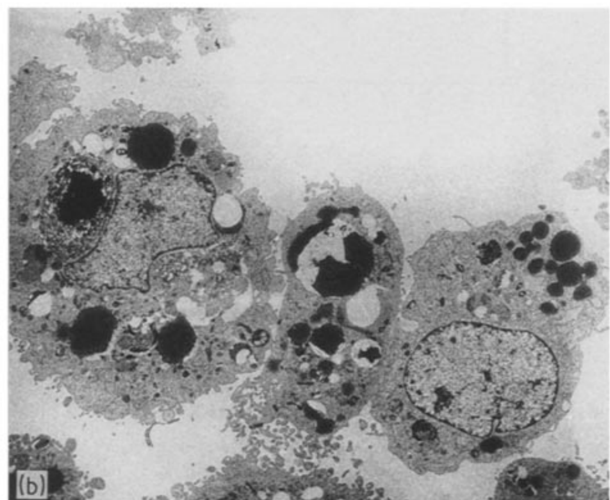


Figure 5 Transmission electron micrographs of trypsinized HF from: (a) culture A and (b) culture B on day 7. In culture A, cells contain small lipid droplets (L) and well developed endoplasmatic reticulum (ER). Many cytoplasmic processes are present. In culture B, cells are sometimes larger. Rounded cell membranes, high quantities of lipid droplets (L), decreased presence of ER and cytoplasmic inclusions with remnants of dead cells (I) are observed. Magnification, 4685 × .

and were sometimes observed as huge unilocular lipid accumulations (shown for culture B in Fig. 5b). A reduction in the amount and dilatation of endoplasmatic reticulum (ER) in contrast to the ER in control culture A was observed. Strikingly, many cytoplasmic inclusions with remnants of dead cells were found in cells of cultures B, C, E, F and G. Furthermore, cells of these cultures had, in general, rather rounded membranes in contrast with many *pseudopodia* of control cells. In the case of GDSC (culture G) morphology differed extremely from control cells, especially with regard to the huge accumulations of lipid, whereas cells cultured with extract E1 (C) had the least differences. Cells of cultures F, H and I had similar morphologies as compared to the morphology of cells in control culture A.

4. Discussion

In this paper we describe a new cytotoxicity test system for biomaterials in which we used methylcellulose culture gel and human fibroblasts. The main advantage of the test system is the possibility of evaluating cytotoxicity for a period of up to 7 days without renewing the culture gel, thus allowing the accumulation of (possibly delayed) released cytotoxic products within the test system. Furthermore, late cytotoxic influences of released products may be detected. Cell proliferation at the bottom of the wells was easily followed by light microscopy and by cell counting after trypsinization. Cell morphology could be adequately examined by both light- and transmission electronmicroscopy. Processed dermal sheep collagen (HDSC) tanned with hexamethylenediisocyanate (HMDIC) was selected as the test material, since contradictory results concerning the cytotoxicity of its extracts have been reported by others [2, 15, 18, 19]. Using this sensitive test system, both primary and secondary cytotoxic effects of HDSC were found.

Primary cytotoxicity is due to direct leakage of products from HDSC. This was detected by testing a 10 day extract of HDSC (E1) which resulted in an approximate 40% inhibition of cell proliferation. When HDSC itself was tested, primary as well as secondary, cytotoxic effects may occur. Secondary cytotoxic effects may be due to release of cytotoxic products as a consequence of cell–biomaterial interactions. Proof for the occurrence of secondary cytotoxicity was found when the second 10 day extract E2 and once and twice extracted HDSC were tested.

Extract E2 did not result in inhibition of cell proliferation or deviant morphology, indicating that no further direct leakage of cytotoxic products, at least by preparing the extract in this way, had occurred. The measured inhibitions of approximately 50% and deviant morphology with once and twice extracted HDSC therefore must be due to cell–biomaterial interactions. The relatively high standard deviation (s.d.) in cell number as observed on day 7 with twice extracted HDSC appears to represent differences in the amount of released cytotoxic products from different discs.

Our results are at variance with the results obtained by Metselaar [15], who did not find a decrease in cell numbers with extracts of HDSC. However, their test system was less sensitive, since they used a liquid culture system, a 3 day extract and a test period of only 24 h. The observed cytotoxicity of HDSC, on the other hand is in agreement with the 22% inhibition in ^{3}H–thymidine incorporation as reported by Ulreich and Chvapil [2], when they tested an extract of a collagen sponge tanned with HMDIC.

Since HMDIC is the tanning agent and cytotoxic effects of both diisocyanates [20–23] and the hydrolysis product 1,6–diaminohexane (DAH) [24–26] have been repeatedly reported, HMDIC and/or DAH may be responsible for primary cytotoxicity. Furthermore, other products may be responsible since many steps were involved in the processing procedure of HDSC [27].

Products responsible for secondary cytotoxicity may vary. We hypothesize that the fibroblasts interact with HDSC by production and release of, e.g., proteolytic enzymes (such as collagenase) [28–31] into the culture gel, reaching HDSC by diffusion, resulting in its degradation. The molecular structure of (cytotoxic) degradation products may depend on the available binding sites on the collagen for proteolytic enzymes.

The described deviant cell morphology, i.e., changes in ER, huge accumulations of lipid and increased inclusions of cell remnants, suggest a lipid degenerative-like influence [32, 33] of HMDIC, DAH and/or other products. However, this does not give a clue to the biochemical processes involved. Lipid-degeneration of cells, a result of cytotoxic effects, was also and most extremely induced by glutaraldehyde(G)-tanned dermal sheep collagen (GDSC). GDSC was used as a reference since G-tanned prostheses, such as porcine heart valves [34–36] or human umbilical cord vein based vascular grafts [37], are routinely used for clinical implantations. The strong cytotoxic effects of GDSC is not surprising since the cytotoxicity of G-tanned materials/prostheses has been reported before [3, 15, 38–40]. The consequences for preferable use of HMDIC-tanning versus G-tanning of collagens for clinical implantations remain to be seen.

In conclusion our test system offers many advantages over liquid culture systems [1–8] and the agar-overlay test [6, 9–11]. While testing HDSC proof of primary release of cytotoxic products as well as secondary release of cytotoxic products due to cellular interactions was found.

Therefore we recommend the use of this test system in those cases, when materials are suspected of: (1) Slow release of cytotoxic products; (2) release of products with late cytotoxic influence; (3) release of cytotoxic products due to cellular interactions with the material. In principle it is possible to prolong the 7 day test period to a 14 day test period by using lower cell seedling densities, larger test surface areas and higher volumes of culture gel. We feel that our test system thus mimics the *in vivo* situation to a high degree as it also allows the detection of secondary released products due to cell–biomaterial interactions.

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