Cells and Materials

Volume 5 | Number 1 Article 1

1995

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Warocquier-Clerout, R.; Hachom-Nitcheu, G. C.; and Sigot-Luizard, M. F. (1995) "Reliability of Human Fresh and Frozen Gingiva Explant Culture in Assessing Dental Materials Cytocompatibility," *Cells and Materials*: Vol. 5 : No. 1 , Article 1.
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Cells and Materials, Vol. 5, No. 1, 1995 (Pages 1-14) 1051-6794/95$5.00+.25
Scanning Microscopy International, Chicago (AMF O'Hare), IL 60666 USA

# RELIABILITY OF HUMAN FRESH AND FROZEN GINGIVA EXPLANT CULTURE IN ASSESSING DENTAL MATERIALS CYTOCOMPATIBILITY

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(Received for publication July 9, 1994 and in revised form February 15, 1995)

## Abstract

An explant culture of human gingival epithelium has been set up in order to provide a valuable test for evaluating the cytocompatibility of dental materials. In an attempt to supply a bank of gingiva explants, frozen and freshly excised specimens were cultured in parallel. Optical and scanning electron microscopy showed an early release of cuboidal cells forming a dense layer around the explants. Afterwards, cultures evolved differently. Spread cells grew and migrated more rapidly in fresh than in frozen explant cultures but their adhesion to substratum increased earlier in frozen ones. Epithelial phenotype of cells had been immunologically characterized by using a battery of monoclonal antibodies to cytokeratins (CKs). We found a time increasing expression of CKs 5, 6, 13, 14/15, 16 and 17, whereas amounts of CKs 1, 2, 10 and 11, specific for terminal differentiation, remained constant. The freezing procedure decreased the yield of CKs but did not modify the electrophoretic pattern. These results suggested that the differentiation of epithelial cells might proceed as *in vivo*. As an application, the cytocompatibility of precious (Au, Pd, Ag) and non-precious (Ni-Cr) alloys was assessed, the reference metals being Ti, which was chosen for its cytocompatibility and Cu, which was chosen for its cytotoxicity. Alloys differed by their ability to modulate cell proliferation and migration. Pd and Au exhibited a high migration potential, whereas Au-Pd and Ti allowed efficient cell proliferation but restricted migration. Reduced migration and proliferation attested the low cytocompatibility of Ag. The toxicity of Cu and Ni-Cr prevented cell migration. These results showed the availability of this method for selecting biomaterials.

**Key Words**: Human gingival epithelium, explant culture, cytokeratins, cytocompatibility, dental metals.

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## Introduction

The development and improvement of biomaterials have increased the interest in the biological aspect and has led to a new strategy of biocompatibility assessment. Thus, a second generation of biomaterials appeared which have to satisfy both technical and biological requirements. Biocompatibility studies need the knowledge of cell behaviour at the tissue/material interface and the *in vitro* culture technique, routinely used for analyzing cytotoxicity, must be improved to satisfy this request. However, continuous line cell cultures, relevant to ranking materials as a function of their cytotoxicity (Wataha *et al.*, 1994), were no longer able to resolve the actual problems, mainly the level of tolerance of such biomaterials or their predictive cytocompatibility. A better understanding has been provided by more appropriate culture techniques of differentiated cells related to the use intended for a given biomaterial (Naji and Harmand, 1990). Nevertheless, the *in situ* conditions seemed to be better reproduced in three-dimensional techniques (Gosselin *et al.*, 1990). The concept of *in vitro* organ culture, which reproduced the three-dimensional *in vivo* biological environment and maintained the interaction between the different tissue cells, has evolved from the work of Wolff and Haffen (1952) and Marin (1965). Their technique was modified so that explants incubated in close contact with cell substratum released migrating cells to form a continuous cell tissue, the growth parameters of which depended upon the surface properties of the substratum (Sigot-Luizard *et al.*, 1986, 1988). According to this prerequisite, we proposed an organ culture derived method to grow human gingival epithelium and to investigate the interaction of some dental materials with growth and phenotypic expression of epithelial cells. These cells are characterized by the expression of intermediate filaments of cytoskeleton, the cytokeratin (CK) filaments (Lazarides, 1980; Moll *et al.*, 1982). To date, 19 different CK polypeptides have been identified. They have been classified into two subfamilies: one contained neutral to basic polypeptides (type II keratins numbered 1-8), the other smaller acidic

polypeptides (type I keratins numbered 9-19). The expression of CKs was related to embryonic development and differentiation stage of epithelial tissues (Franke *et al.*, 1982; Sun *et al.*, 1983; Clausen *et al.*, 1986). *In vivo*, pathological states might generate specific changes of CKs pattern. *In vitro*, the environmental biological conditions such as the medium composition, the air-liquid interface or substratum surface properties, might also interfere with their metabolism (Fuchs and Green, 1980; Ouhayoum *et al.*, 1990).

The purpose of this study was to determine the growth characteristics of human gingiva explant cultures and to consider the possibility of frozen specimen storage. The phenotypic expression of the cells released from explants was investigated using the cytokeratins as molecular markers for epithelial differentiation. The immunological procedures involved immunostaining and immunoblotting by monoclonal antibodies to CKs. To further demonstrate the reliability of the technique, we reported the results of cytocompatibility assessment of several dental metals and alloys.

## Materials and Methods

### Materials

Four categories of metals used in dental surgery have been tested:

1. A group of four precious metal alloys with high percentage of gold (Au), palladium (Pd), silver (Ag), and Au plus Pd provided by Metalor (Neucâtel, Switzerland).
2. An alloy made of non-precious metals, namely, nickel and chromium (Ni-Cr), 63.30% and 22.85%, respectively.
3. Titanium (Ti) from Metalor.
4. Copper (Cu) from Metalor.

Material specimens tested had a surface of 1 $cm^2$ and 1 mm thickness. Their density did not exceed 1.5 $g/cm^2$.

### Culture technique

Specimens of human gingival tissue were aseptically removed from the tuberositor pocket during extraction of impacted upper wisdom teeth on subjects between 19 and 25 years old. Biopsies of attached gingiva were stored at 4°C no longer than 24 hours in buffered saline solution added with penicillin (200 UI/ml) and streptomycin (200 $\mu$g/ml) before removing traces of blood and incubated for 12 hours in buffer. A large part of stratum conjonctivum was cut off so as to preserve epithelium basal zones. The epithelium was cut into straight edged fragments of about 1 x 1 mm which were either stored frozen in liquid nitrogen or cultured. The explant culture consisted of layering the fragments onto agar medium, the basal side facing the substratum to be tested as previously described (Sigot-Luizard *et al.*, 1986). Buffered agar medium contained 27% Iscove MEM (minimum essential medium; Boehringer Mannheim, Germany) + 20% colt serum (Institut Pasteur, Paris, France) + 2% Tricin (Merck, Darmstadt, Germany) + 2 mM L-glutamin + penicillin (100 UI/ml) and streptomycin (100 $\mu$g/ml) + 1% Bacto-agar in 50% Gey solution (Difco, Detroit). Cultures were incubated at 37°C in dry atmosphere for 8 to 16 days. Cells started covering Thermanox®, used as reference material, as soon as day 4. The freezing procedure consisted of dispersing 4 explants per ml of medium MEM Iscove containing 10% dimethylsulfoxide, as described for single cells freezing procedure.

### Scanning electron microscopy

Cell layers were fixed in 3% glutaraldehyde in Rembaum buffer (pH 7.4), dehydrated in graded alcohols, critical-point dried from $CO_2$ (Polaron Equipment Ltd., Watford, England), sputter-coated with gold (Polaron Equipment Ltd.) and examined using a JEOL (model JSM 840) scanning electron microscope operated at an accelerating voltage of 15 kV.

### Cytocompatibility assessment

**Cell proliferation and migration** After neutral red staining, the surfaces of the cell layers were measured with a stereo-microscope fitted with a camera lucida and a digitizing tablet connected to a microcomputer. The cells were dissociated in trypsin (0.025%)-EDTA (ethylenediaminetetraacetic acid; 0.020%) and counted with a Multisizer® (Coultronics, Margency, France). The cellular densities were plotted versus cell migration surfaces, each point being the average of 60 explants from 6 donors, each donor providing a mean of 10 explants.

**Adhesion test** The cells were dissociated by trypsin-EDTA treatment for 10 to 75 minutes and summarized in order to establish the curve of percentages of cells released as a function of time (Duval *et al.*, 1988). Then we calculated by integration the area between the curve and the x-axis. The value of the area provided a quantitative measure of the cell adhesion strengths which is inversely proportional to the cell adhesion to the biomaterial. A comparative results diagram with 3 different zones has been drawn: Zone A = area more than 4500 = weak cell adhesion; Zone B = area between 3000 and 4500 = medium cell adhesion and Zone C = area less than 3000 = strong cell adhesion.

**Cell viability** A viability test was performed in two steps: first, cells harvested from reference cultures on Thermanox® were submitted to a trypan blue exclusion test to determine the percentage of viable cells. The cell suspension was counted with a Multisizer® (Coultronics)

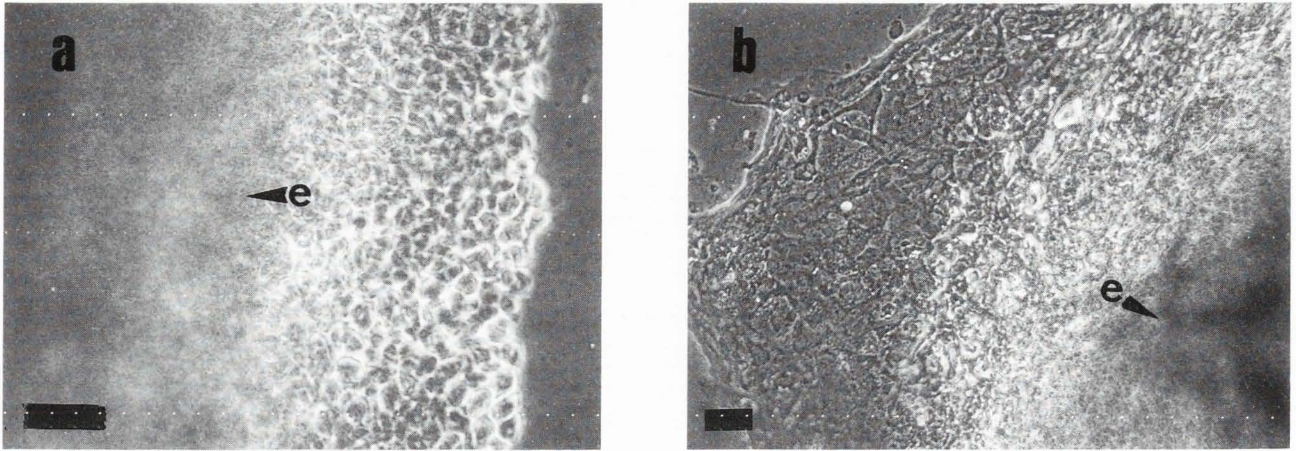


**Figure 1.** Phase contrast micrographs of epithelial cells from gingival explant culture incubated for (**a**) 8 days, (**b**) 10 days. Explants are indicated by the arrow. Bars = 50 $\mu$m.

and the upper and lower levels of the channel were displaced so that the channel was reduced to cover a number of cells equivalent to the number of viable cells. Second, cells harvested from the tested culture were counted successively in channel corresponding to the cell size (y cells) and in channel previously set on the percentage of viable cells in control (x cells). The percentage of viability was calculated as x/y × 100 as previously published (Roux *et al.*, 1992).

**Statistical analysis** The statistical significance between groups was determined by applying Student's t-test. The level of reliability chosen was 95%.

### Characterization of cell phenotype

**Fluorescent immunostaining** Cell layers from frozen and fresh explants incubated for 14 days were first fixed in absolute methanol at -20°C and successively incubated for 1 hour with mouse monoclonal anti-human CK AE1/AE3 (Boehringer Mannheim) and fluorescein conjugated anti-mouse immunoglobulin antibody (Sigma, St. Louis). A washing step with phosphate buffered saline (PBS) + 1% bovine serum albumin (BSA) + 1% Triton X-100 was included between each immunoreaction. The specificity of the immunostainings was controlled in the presence of non-immune serum from mouse. The samples were examined with a Leitz microscope equipped with epifluorecence illumination.

**SDS-PAGE** CKs were extracted from cell layers of explants incubated in contact with Thermanox® according to the technique described by Achtstätter (1986). Cell layers were cleared off explants before homogenization in high-salt buffer (10 mM Tris, 0.15 M NaCl, 1.5 M KCl, 5 mM EDTA, 1% Triton X-100, pH 7.4) for 1 hour at 4°C. The homogenate was centrifuged at 1500 g for 10 minutes and the pellet was resuspended in low-salt buffer (10 mM Tris, 0.07 M NaCl, 5 mM EDTA, 1% Triton X-100, pH 7.4) shaken for 5 minutes at 4°C and centrifuged again. Pellet was then rinsed in PBS and either stored frozen at -70°C or analyzed by SDS acrylamide gel electrophoresis (PAGE) in a discontinuous buffer system of Laemmli's (1970). Then separated proteins were stained with Coomassie blue. Proteins from duplicate unstained gels were transferred in Tris-glycin buffer pH 9.0 added with 20% methanol onto nitrocellulose paper. The papers were stained with Ponceau Red to localize the polypeptide bands and staining was washed off in PBS. Immunoblotting experiments were carried out according to Towbin *et al.* (1979). Briefly, papers were incubated in PBS-Tween 20 (0.05%) for 30 minutes, then in PBS-Tween + 2% BSA to saturate the absorption sites. Papers were incubated overnight with one of the following primary monoclonal antibodies: AE1/AE3 (Boehringer Mannheim); EE21.6 (Biosoft); simple epithelium anti-CKs (Amersham); stratified squamous epithelium anti-CKs (Amersham). They were washed again with PBS-Tween for 30 minutes and the monoclonal antibody was visualized using a second anti-mouse immunoglobulin conjugated to alkaline phosphatase (Bio-Rad). Alkaline phosphatase activity was detected utilizing a kit from Bio-Rad.

## Results

### Cell morphology

Figures 1 and 2 show the ultrastructural morphology of the cell layers grown from fresh and frozen explants incubated for 1-16 days at 37°C. Both cultures exhibited dense area of cuboidal cells around the explants as seen by phase contrast (Fig. 1a) and scanning electron microscopy (Figs. 2a and 2b). With increasing incubation period, cells spread beyond the initial cellular zone and cell organites and nucleus could be distinguished by

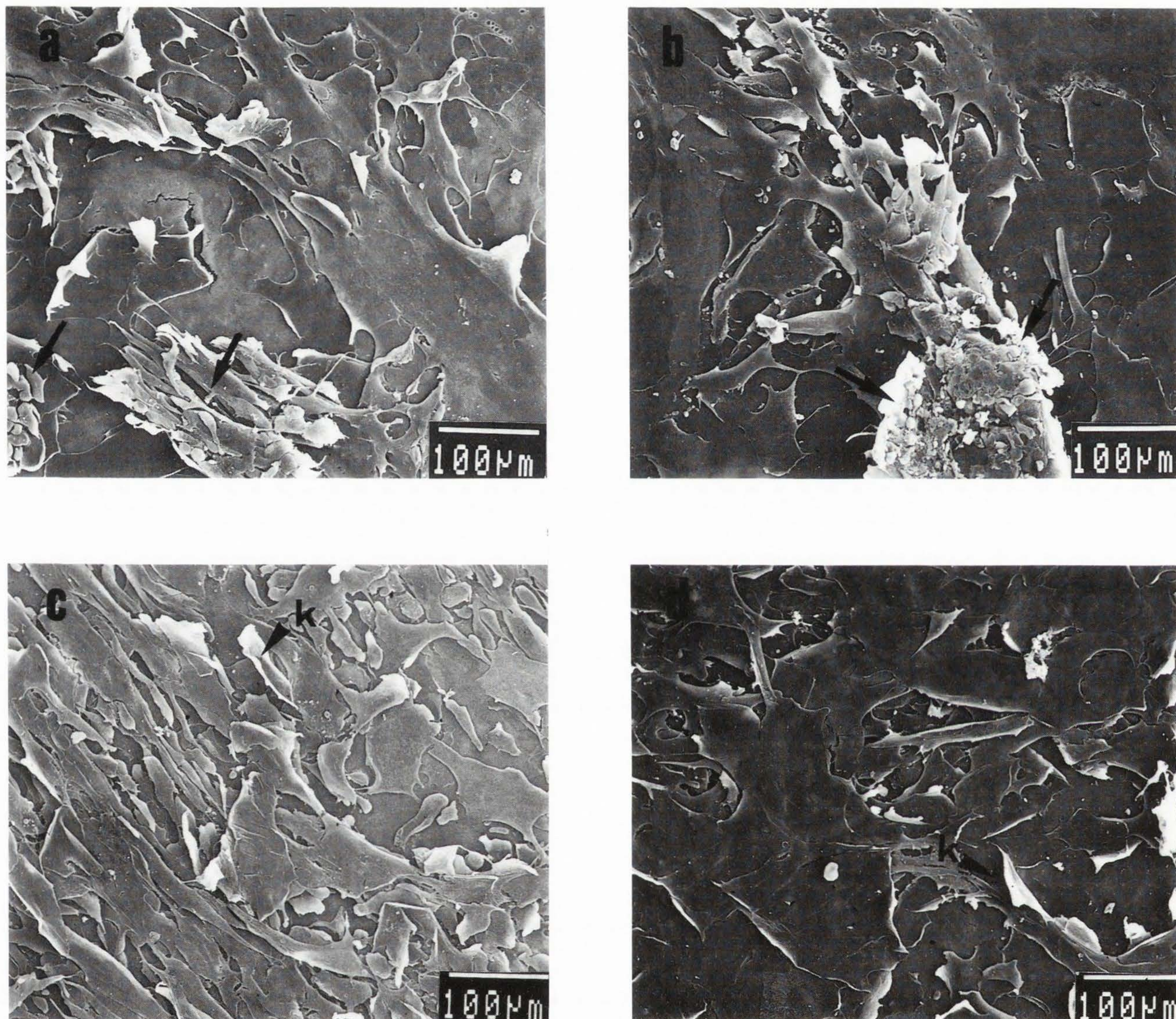


**Figure 2.** Scanning electron micrographs of epithelial cells from (**a**, **c**) fresh and (**b**, **d**) frozen explant cultures incubated for (**a**, **b**) 8 days, (**c**, **d**) 14 days. Arrows indicate the dense cell layer close to the explant. Arrow heads show keratinized cells over the cell layer.

phase contrast microscopy (Fig. 1b). Keratinized cells were observed both at the periphery of the culture by day 12 and scattered over the cell layer (Figs. 2c and 2d). In fresh explant culture, the epithelial cell multilayer culture mostly consisted of high migrating elon-gated cells (Fig. 2c). Prolonged incubation period up to 16 days developed a degenerative process. Early cul-tures from frozen explants displayed significant amount of cell debris (Fig. 2b), which might account for the apparent decrease of the growth rate following the first growth phase. Cell proliferation resumed later and gave rise to a pluristratified layer of keratinized cells, well observed by day 14 (Fig. 2d). Then cells retracted and detached as reported in fresh explants culture.

### Cell growth parameters

Figure 3 deals with the biological parameters of cell growth as migration and proliferation. Kinetics evolved differently in that the area of cell layer which emerged from frozen explant cultures was significantly lower compared with fresh explant cultures ($p \leq 0{,}001$) and the cells migrated less rapidly and to a shorter distance from explant. In both cultures, cell density was as high as 5-6000 cell/mm$^2$ by day 8. Increasing incubation time allowed a burst of cell growth from fresh explants, whereas the cell number surrounding frozen explants decreased by day 10. Cell growth resumed in these cultures by day 12 but the density remained quasi-constant. In both cultures, the migration increased to a high extent

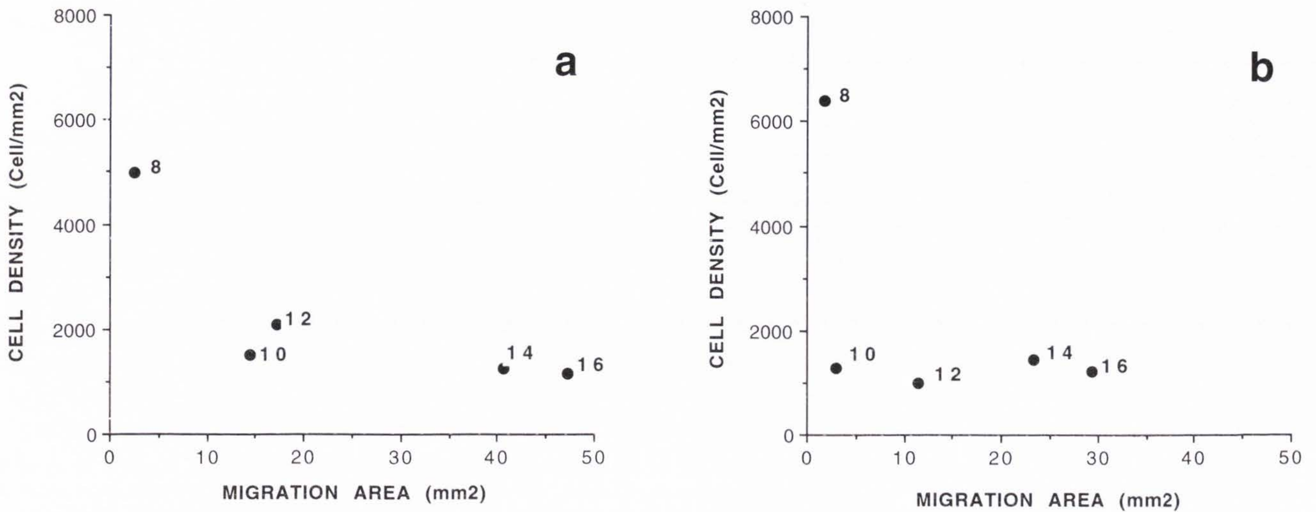


**Figure 3.** Growth and migration of epithelial cells from (a) fresh gingival explants and (b) frozen gingival explants incubated for 8-16 days. Cell densities were plotted versus migration areas.

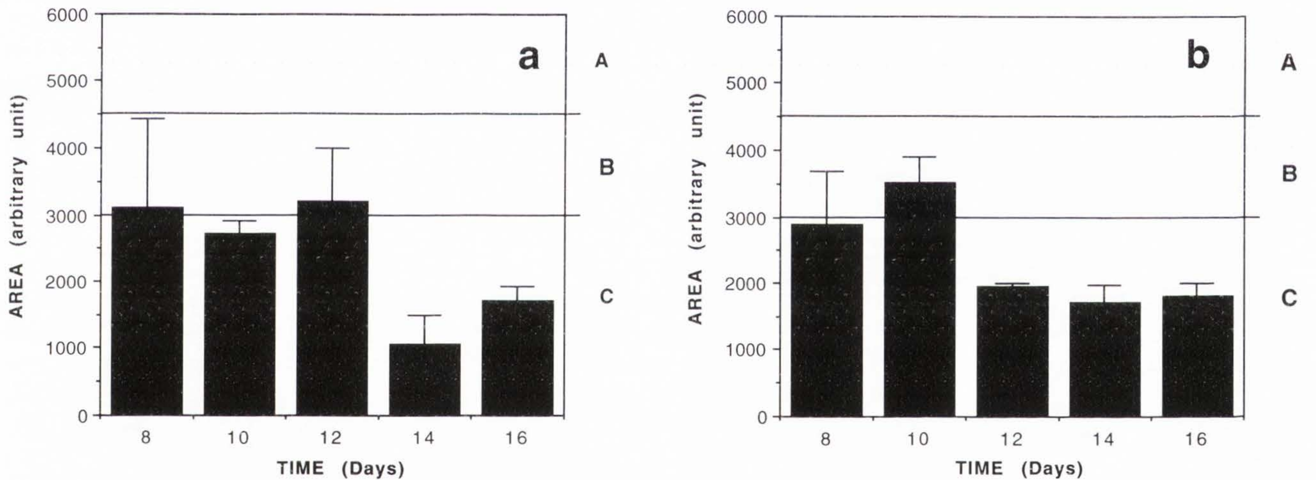


**Figure 4.** Adhesion of epithelial cells grown for 8-16 days from (a) fresh gingival explants and (b) frozen gingival explants on Thermanox®. The areas indicated in the y-axis were previously calculated from the kinetic curves of trypsin cell dissociation, cell percentages being plotted versus incubation time. Areas were calculated at the indicated time. Zone A = weak cell adhesion, Zone B = medium cell adhesion, Zone C = strong cell adhesion.

**Table 1.** Cell viability.

| | Fresh explant culture | | Frozen explant culture | |
|---|---|---|---|---|
| Time days | cells/explant | viable cells (%) | cells/explant | viable cells (%) |
| d + 8 | 12956 ± 5407 | 73 ± 0.8 | 11092 ± 1454 | 64.8 ± 0.8 |
| d + 10 | 22525 ± 16282 | 70.9 ± 4.1 | 3272 ± 419 | 63.8 ± 2 |
| d + 12 | 26009 ± 8440 | 69.1 ± 4.4 | 11488 ± 1855 | 68.7 ± 0.1 |
| d + 14 | 48879 ± 32989 | 70.7 ± 1.2 | 32736 ± 532 | 67.5 ± 1.2 |
| d + 16 | 54278 ± 3473 | 63.75 ± 1.1 | 36730 ± 1259 | 63.4 ± 1.9 |

Each value was mean of 6 x 10 determinations ± standard deviation.

**Figure 5**. Immunofluorescence microscopy of epithelial cells from explant culture stained by monoclonal anti AE1/AE3 antibody. Bar = 10 $\mu$m.

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between day 12 and 14, then migration rate slowed down.

Cell viability assessment expressed as percentages of viable cells harvested by an enzymatic treatment of cultures is summarized in Table 1. Frozen explant cultures exhibited significant lower viability than fresh explant ones incubated for 8-10 days. Then, percentages of viable cells remained quasi-constant in fresh explant cultures, whereas they slightly increased in others. By day 16, decrease of percentages in both cultures indicated a degenerative process due to culture conditions.

Adhesion strengths of cells to Thermanox® were evaluated from a trypsin treatment of the cultures. Their values were inversely proportional to the area calculated from the kinetic curves of cell dissociation. Figure 4 which summarizes the results, shows a significant increase at late incubation time. In the fresh explant cultures, the shift from medium adhesion (Zone B) to strong adhesion (Zone C) occurred after a marked increase of migration by day 14. In the frozen explant cultures, both migration and proliferation were reduced but the adhesion increased as soon as day 12.

### Cell immunostaining

Indirect immunofluorescence assay has been performed in order to characterize the cell phenotype expressed in both explant cultures grown for 14 days. Microscopic examination showed an uniform labelling of cell layers by monoclonal AE1/AE3 anti acid and basic keratins antibody, no matter which migration distance from the explant. Figure 5 shows highly stained cells dispersed over the culture. They might correspond to the keratinized cells previously observed by scanning electron microscopy.

### SDS-PAGE of CKs

CKs have been extracted from fresh and frozen explant cultures at different times through the incubation period and analyzed under reducing conditions in SDS-PAGE. Successive high- and low- salt extractions were carried out from about 300,000 cells which meant that a variable number of explants was treated depending on the cell growth. Twenty microliters of cytokeratins suspension corresponding to the amount extracted from 6,000 cells were deposited into wells of one-dimensional slab gel. Electrophoretic patterns shown in Figure 6 pointed out a progressive appearance of cytokeratin polypeptides. After 8 day incubation, minor amounts of 7 polypeptides were identified as CK2 (65 kD), CK6 (56 kD), CK10/11 (56.5 kD), CK14/15 (50 kD), CK16 (48 kD) in fresh explant culture extracts (Fig. 6a). The amount of each polypeptide increased with incubation period, except the CK2 amount, which remained unchanged. Additional bands appeared, identified as CK1 (67 kD), CK5 (58 kD), CK13 (54 kD) and CK17 (46 kD) according to the nomenclature established by Sun *et al.* (1983). Main cytokeratin polypeptides could have been detected in smaller amounts from the extract of frozen explant cultures which were incubated for 14 days (Fig. 6b). However, their expression seemed delayed compared to fresh explant cultures and the sensitivity of the technique did not allow CKs detection before 12 days. Evidence that polypeptides belonged to the two families of CKs was obtained by using monoclonal AE1/AE3 and anti-stratified epithelium antibody in electrophoretic transfer blot. All the bands stained by Coomassie blue were immunolabelled by the immunoserum AE1/AE3 (lane 1). Anti-CK 1, 2, 9 and 10/11 antibody labelled terminal differentiation CKs seen in the lane 2. Anti-CK 19 and simple epithelium antibody did not label any polypeptide (not shown).

### Biocompatibility assessment of dental materials according to this gingival explant culture

Fresh gingival explants cultured in the conditions

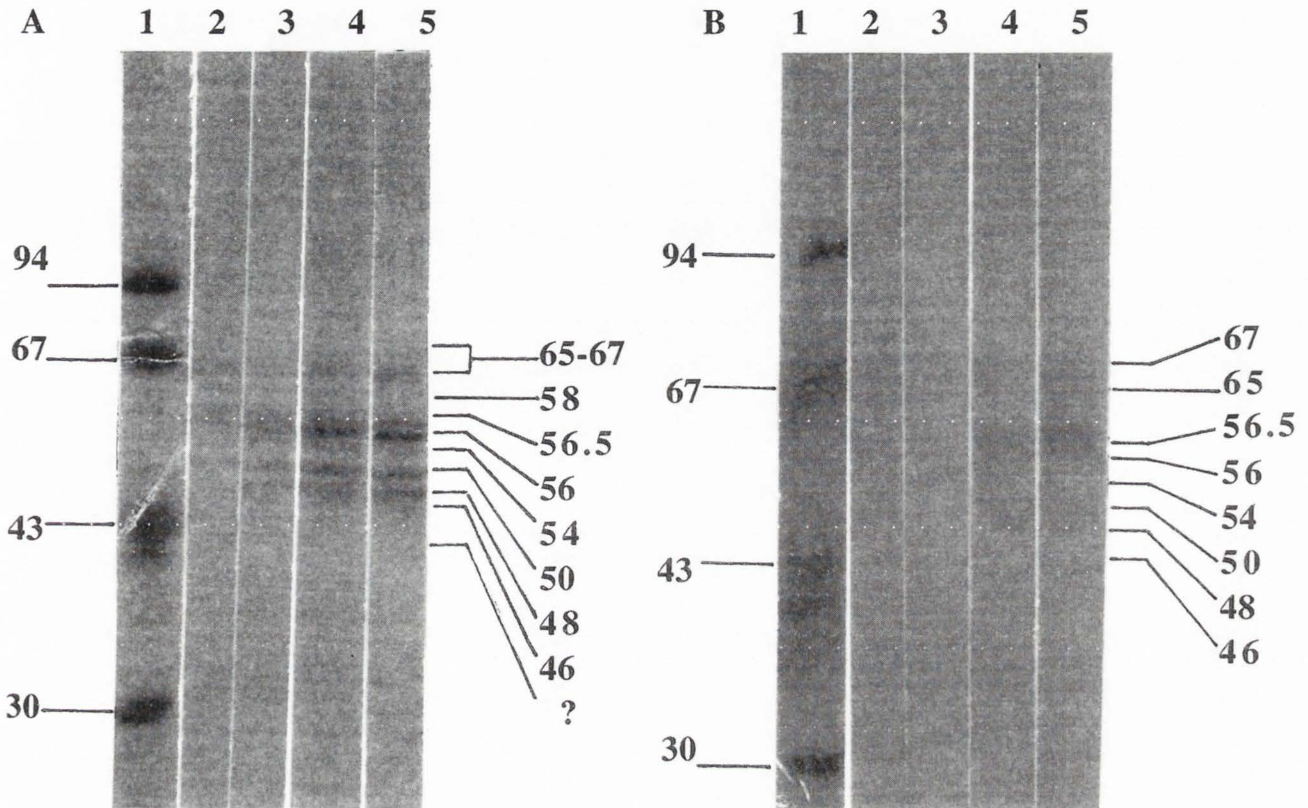


**Figure 6**. SDS-polyacrylamide gel electrophoresis of CKs extracted from similar number of epithelial cells of culture of fresh and frozen gingival explants. Molecular weight markers stained with Coomassie Blue are indicated in lane 1. Electrophoretic patterns of extracts from (**a**) fresh and (**b**) frozen explant cultures incubated for 8, 10, 12 and 14 days are seen in the lanes 2, 3, 4 and 5 respectively. The molecular weights of the CK polypeptides are indicated on the right.

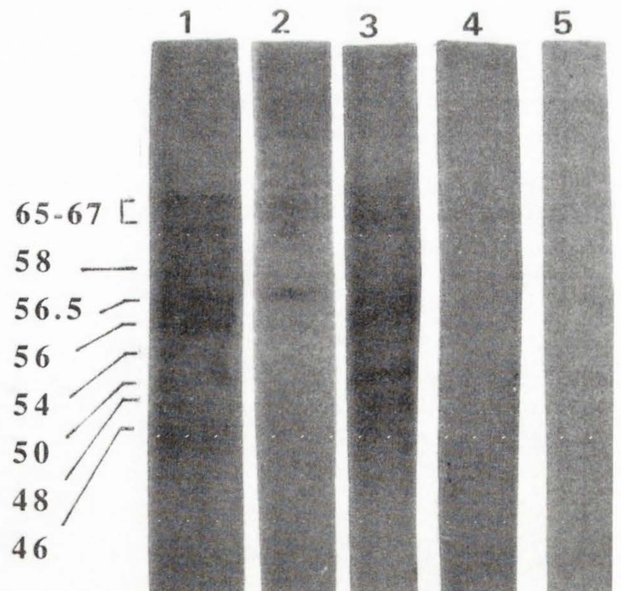


**Figure 7**. Immunoblotting of CK extracted from epithelial cells of fresh explant cultures grown for 14 days. The CKs were electrophoresed on SDS-PAGE then transferred electrophoretically to nitrocellulose paper and stained separately with AE1/AE3 monoclonal antibody (lane 1) and EE21.6 monoclonal antibody (lane 2). The molecular weights of the CK polypeptides revealed by these antibodies after alkaline phosphatase labelling are indicated on the right.

described above were grown in contact with precious and non-precious metal alloys in comparison with Ti, Cu and Ni-Cr. Scanning electron microscopy of the cell layers grown for 14 days displayed various morphological aspects depending on the metal used as substratum (Fig. 8). Pd alloy allowed the synthesis of pluristratified cell layers containing keratinized cells (Fig. 8a). Culture on Au alloy exhibited a continuous layer of well spread cells. Some elongated keratinized cell began overlapping the basal layer (Fig. 8b). Cultures grown on Au-Pd alloy displayed a morphology similar to that on Au alloy but cells migrated to a shorter distance from the explant (not shown). In contrast, the cell morphology was altered on Ag alloy. We observed the presence of rounded cells in mono and multilayers and the absence of keratinized cells (Fig. 8c). Non-precious alloys like titanium and Ni-Cr supported a moderated growth

**Table 2.** Cell viability

| Samples | cells/explant | viable cells (%) |
|---|---|---|
| T(-) | 53667 ± 26691 | 78.1 |
| Au | 29714 ± 11544 | 84 |
| Pd | 51473 ± 6380 | 79 |
| Ag | 7415 ± 770 | 78.7 |
| Au-Pd | 14064 ± 569 | 79.2 |
| Ti | 12089 | 60.2 |
| Ni-Cr | 4182 ± 371 | 60 |
| Cu | 3468 | n.d. |

Each value was mean of 3 x 10 determinations ± standard deviation.

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of cell, the morphology of which resembled that grown on Thermanox®. Cu had a dramatic effect causing cell rounding and presumably death.

Quantitative measurement of cell growth was performed as described above using computerized system. Migration and proliferation parameters are summarized in Figure 9. Materials might be distributed into three groups:

1. assembled materials which favored cell migration, like Pd and Au. Migration areas reached mean values comparable to that on Thermanox®;

2. with Au-Pd, Ag and Ti allowed the synthesis of less extended cell layer than Thermanox® but cell densities of the cultures grown on Ag and Ti were reduced compared to the cultures on Au-Pd and Thermanox®; and

3. included Cu and Ni-Cr responsible for low migrating cell cultures. The number of cells surrounding explant grown on Cu was abnormally high.

Adhesion strengths of cells to metals did not significantly vary from one alloy to another. All of them dis played medium values belonging to Zone B defined above (Au, 4007 ± 693; Ag, 2885 ± 374; Au-Pd, 3204 ± 250; Ni-Cr, 3218 ± 566; Ti, 3370 ± 435). The lower value found on Pd (1602 ± 1419) might signify an increase of the adhesion strength but the significance was not statistically established.

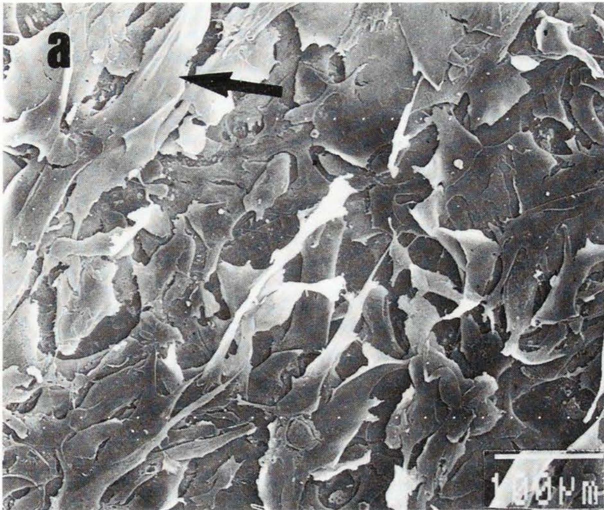
Cell viability measured with the Multisizer as described above was quite comparable in cultures grown on Au, Pd, Ag, Au-Pd alloys. The percentages of viable cells fluctuated between 78-84% (Table 2). Only Ti and Ni-Cr alloys gave percentages as low as 60%. Cells present on Cu were so highly damaged that viable unstained cells could not be clearly identified.

## Discussion

In the present study, we have shown that human gingival epithelium explants incubated on nutrient agar and maintained in close contact with culture substratum enabled outgrowth of epithelial cells. Evidence for the epithelial phenotype was brought by immunostaining with monoclonal anti-CKs antibody which labelled uniformly the cell layer. During the first days following incubation, cuboidal cells surrounded the explants and their density was high. They have the morphological aspect of granular keratinocytes with no visible nucleus and cell organites. Thereafter, the migration process started and a less dense multilayers cell tissue spread over the substratum. It seems that two different cell populations emerged from explants: one, with a cuboidal configuration, might be released from differentiated keratinized cells, possibly located in the spinosum stratum or in the granulosum stratum; another, made up of well-spread cells, might proceed from highly proliferative cell layers belonging to the basal stratum. Cells liberated during the first wave of synthesis could accumulate close to explant. They would have a limited mitotic potential. By cóntrast, cells from the second wave of production were endowed with a high mitotic activity and were responsible for the neoformation of a continuous cell layer which evoluted towards pluristratification and keratinization. This keratinization process could be immunodetected in culture at late incubation time.

Explant culture of both animal and human gingiva was experimented by Bergenholtz (1967) according to an organ culture in liquid medium described by Trowell (1959). Survival of the tissues and maintenance of their specificity appeared highly dependent on the culture medium composition and the biological conditions could retard the appearance of necrosis up to 2 weeks (Pellerin *et al.*, 1988). It was not the purpose of our study to control the viability of the explant tissues. Nevertheless, it could not be excluded that the physiological state of explant might affect the proliferative activity of neoformed cells. In our culture model, the incubation period was limited since nutrient agar medium could not be changed or fed as required for single cell cultures. Beyond 14 days, mitosis and cell viability decreased. Similar observation had been reported by Letort *et al.* (1990) who grew rat gingival explants according to this technique.

The goal of freezing gingival explants came from the wish to plan experiments, independently of surgical acts and from the need of human biological material in sufficient amount to perform ranges of comparative experiments from the same batch of explants. This is a prerequisite to limit the experimental errors due to different donors. However, it has been found that freezing



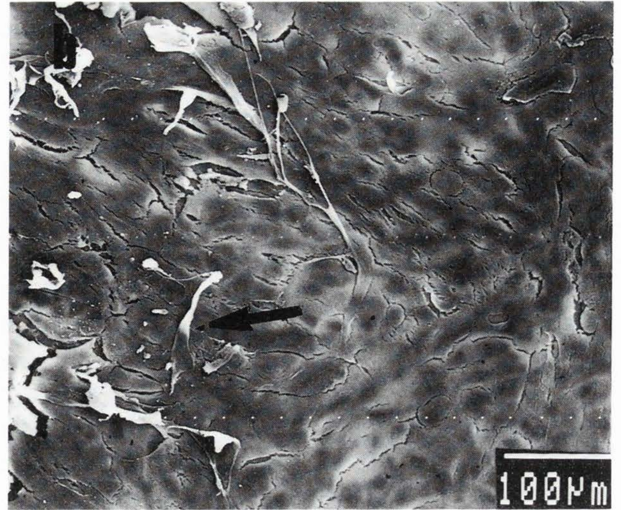
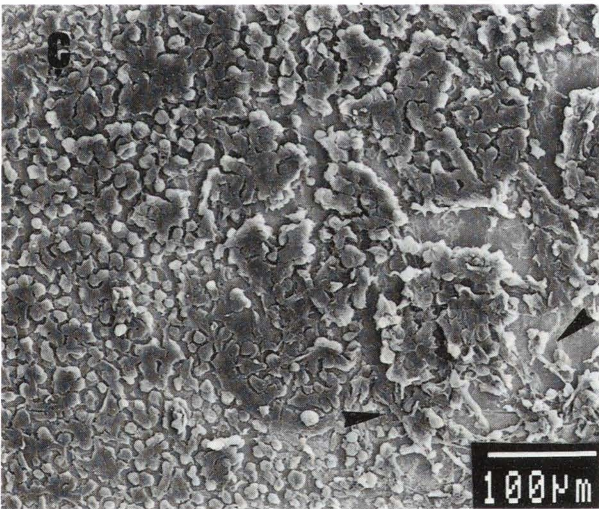




Figure 8. Scanning electron micrographs of epithelial cells from human gingival explants grown for 14 days on: (a) Pd alloy, (b) Au alloy, (c) Ag alloy. Arrows show keratinized cells. Arrow heads indicate multilayer zone.



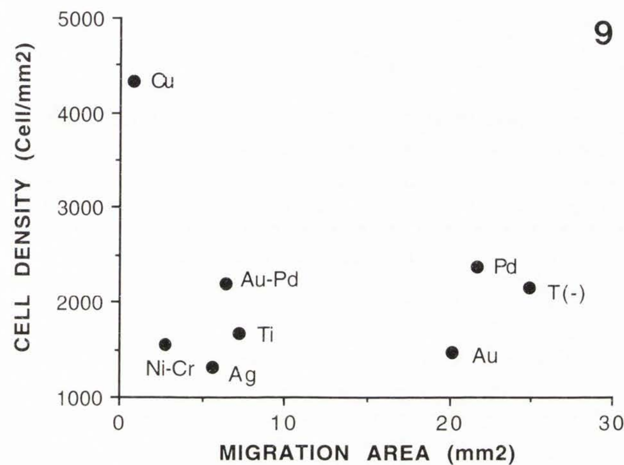




Figure 9 (at bottom of column 1). Growth and migration of epithelial cells grown for 14 days from human gingival explants on alloys and metals. Cell densities were plotted versus migration areas.

and thawing procedure caused a decrease of the cell viability and a delay of the migration process which started a few days later. In contrast, signs of cell degeneration were not retarded, so it turns out that the proliferative phase was shortened and consequently, cell number was reduced. Sublethal cell damages due to low freezing temperature may affect a high percentage of cells. Short incubation period following thawing allowed the action of repair mechanisms as shown by Frim *et al.* (1976), which did not require protein synthesis but resulted from reorganization of plasma lipidic layers (Law *et al.*, 1980).

The SDS-PAGE and immunoblotting analysis with antikeratin monoclonal antibodies demonstrated the presence of CKs 1, 2, 5, 6, 10/11, 13, 14/15, 16, 17 in late fresh explant cultures. Extracts from frozen explant cultures contained lower amounts of CKs. The sensitivity of the Coomassie blue staining method did not allow the detection of CK5. The delay of cell growth in frozen explant culture compared to fresh one may explain this failure. Indeed, CK pattern of day 14 cell extract from frozen explants parallels that of day 10 extract from fresh explants which did not exhibit significant staining of CKs. Expression of CKs 6, 10/11, 14/15 and 16 was

consistent with increasing incubation time, whereas expression of CK2 remained unchanged. CK1, a terminal differentiation specific marker, was faintly detected in late extracts. The cumulative synthesis of CK10/11 throughout the incubation period of explants gave evidence of an *in vitro* keratinization process of the cell sheet which could be observed by scanning electron microscopy. Results for CK13, a specific marker of oesophagus-like keratinized stratified epithelia and CK17, a keratin present in keratinized simple epithelia, poorly expressed in late 12-14 day cultures, parallel the adult gingival cell tissues (Juhl *et al.*, 1989; Ouhayoum *et al.*, 1990). The occurrence of epithelial cell terminal differentiation contrasted with the behavior of other reported epithelial cell cultures which did not express CK1,2 and 10/11, but CK 19, a marker of simple epithelia thought to reflect proliferative potential (Salonen *et al.*, 1989; Oda *et al.*, 1990; Shabana *et al.*, 1991). The reason for this discrepancy may arise from culture physical conditions. Previous studies from Pruniéras *et al.* (1983) and Asselineau *et al.* (1985) reported that epidermal cell culture and corneal layer go through terminal differentiation when they are grown at the air-liquid interface. Normal gingival epithelium even though considered as a humid epithelium since emerging in gingival fluid, undergoes keratinization. In the culture model described, the explant was in contact with a semi-solid phase which enables keratinization.

Differentiation stages of gingival epithelium reconstituted *in vitro* had been investigated following different culture procedures. Human epithelial cells grown on a 3T3 feeder layer or on connective tissue equivalent respectively, expressed the main characteristic CKs 5, 6, 14, 16, 17 except CKs 1, 2 and 10/11 (Gosselin *et al.*, 1990). A similar three-dimensional culture model, made up of collagen lattice populated with fibroblasts, was used in histiotypic culture by Shabana *et al.* (1991). They observed a quasi-constant expression of CKs 5, 6, 14, 16 in each cell culture no matter which epithelium origin. Expression of the others CKs fluctuated as a function of the morphological differentiation of the cell grown.

Application of the explant culture technique to comparative biocompatibility assessments further confirmed previous analysis of short-term cytotoxicity performed in continuous human cell culture, in direct contact with metal ions. The cell viability, reduced by powder of non-precious alloys, was not affected by powder of precious and semi-precious alloys (Hildebrand *et al.*, 1991). However, there were significant differences in the cytotoxicity response depending on the cell line origin and the passage number of the cells (Wataha *et al.*, 1994). Bernstein *et al.* (1992) reported that low noble alloys and non-precious alloys displayed a higher cytotoxic potential in primary fibroblast culture than in permanent cell culture. Naji and Harmond (1990) demonstrated that the assessment of the cytocompatibility of an alloy might decrease as a function of the differentiation state of the cells used. Comparative study of the biocompatibility of titanium, assessed in different cell cultures, allowed the authors to suggest that the increase of the doubling time of human gingival fibroblasts and osteoblasts, compared to continuous line cells, might be responsible for an enhanced sensitivity of the response. Titanium had no effect on Hela and Balb/c 3T3 cell attachment and proliferation, although a late toxic effect has been detected in the presence of human gingival fibroblasts and osteoblasts. In our test model, titanium ranked among a group of materials, different from the negative control, which allowed a fair cell proliferation but a low migration. Thus, the explant culture on semi-solid agar medium allowed the differentiation of the alloys with respect to biological properties of the cell sheet newly synthesized. With regard to migration parameter and adhesion strengths, Pd alloy exhibited the best performance while Ag alloy the least. Au alloy displayed better migration potential than Au-Pd alloy, although it showed less resistance to corrosion. A non-precious alloy like Ni-Cr showed a poor cytocompatibility. The toxicity of alloys which contain Ni, Cr and Fe had also been reported by Exbrayat *et al.* (1987) and Meryon *et al.* (1987). Both surface properties and pureness of alloy determined the degree of toxicity.

## Conclusion

Culture of human epithelium explants layered on agar nutrient medium and covered with tested substratum enabled the growth of a pluristratified epithelial tissue which was endowed with biochemical and morphological properties characteristic of *in vivo* tissue, mainly the expression of terminal differentiation CKs. The quantification of biological parameters such as cell proliferation, migration, adhesion and viability allowed a comparative assessment of the cytocompatibility of dental materials. This experimental model may be useful to investigate fundamental mechanisms at the interface dental material/ gingival epithelium. Further experimentation are required to better improve the freezing process of storage and the incubation conditions to extend the cultures beyond 14 days.

## Acknowledgements

This study was supported by a grant from INSERM (CRE 91013) and the authors wish to thank J.L. Duval for scanning electron microscopy. They are grateful to Metalor (Neufchatel, Switzerland) for providing dental metals.

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## Discussion with Reviewers

**J.M. Schakenraad**: Why does CK2 remain unchanged?
**Authors**: To answer this question requires further quantitative analysis. From our observation, it may be suggested that early CK2 was first found in the round cells released from explants. The second wave of cells got through the differentiation process which induced the sequential synthesis of CKs responsible for late synthesis of CK2. The progressive cell growth might contribute to maintain a constant level of CK2.

**J.M. Schakenraad**: Since your culture system can only be used for 14 days (depletion of nutrition) and your epithelial cell outgrowth takes 12 days, can you think that this model is still useful and adds to already established test methods.
**Authors**: Our culture model allowed cell outgrowth as soon as day 6-8 and cell growth continued for 14 days. Thus, a comparative observation of cell behavior on various substratum is possible. The main advantage of the technique is to maintain the three-dimensional structure of the tissues which preserve the interaction between the different cell phenotypes. Thus, it might reproduce the *in vivo* healing process involving the whole tissue. Phase contrast microscopy clearly showed the outgrowth of two cell populations which appeared at different levels and might originate from different tissue layers. Improvement of the technique to refeed nutrient agar and to prolong the incubation period is being investigated.

**J.M. Schakenraad**: Why is the CK5 not expressed in frozen cultures?
**Authors**: Since the freezing procedure retarded the growth of the second wave cells, the CK pattern observed by day 14 was comparative to that provided by fresh explant culture carried out for 10 days. In this extract, CK5 was poorly represented. Freezing and thawing procedures are under investigation to improve performance.

**R. Todescan**: How were the metal substrates treated/cleaned before their use in the experiment?
**Authors**: Metal substrates were thoroughly rinsed in distilled water then immersed in 90% ethanol and sonicated for 15 minutes at full power in Transonic 275 (Prolabo, Paris, France).

**R. Todescan**: If Cu was so effective in causing cell rounding and/or death, how can you explain that the cell density on Cu was so much higher than the other metals?
**Authors**: In our culture technique, it is usually observed that cells, which first emerged from explant grown in contact with a toxic substratum, accumulated around the explant but did not migrate. Since nutrient agar prevented cell detaching, cell density maintained high values but the viability was weak.

**M. Mednieks**: Randomization of selecting samples for representation in micrographs should be described: e.g., how many specimens, how many experiments? What is the repeatability of the culture characteristics from experiment (primary culture) to experiment?
**Authors**: The explant culture method yielded reproduc-

ible results provided that gingival specimens were extracted from young (19-25 years old) patients' tuberositor pocket during extraction of healthy wisdom teeth and that they were submitted to similar protocols of pre-incubation. In each experiment, cytocompatibility of metals was assessed by reference to Thermanox® in order to normalize the results.

**E. Pissiotis**: Depletion of medium is increased every day. How do the authors explain the fact that cell growth resumed by day 12, while it was decreased by day 10?
**Authors**: Culture medium contained a high percentage of serum (20%) which maintained a sufficient amount of growth factors. Table 1 shows that in fresh explant culture, cell growth increased continuously with incubation time beyond day 12 but the cell density decreased since the migration increased. Thus, depletion of medium would not be responsible for the cell decrease observed by day 10 in frozen explant culture.

**E. Pissiotis**: Cytotoxicity testing of dental materials is mainly done with tissue culture methods. Why did the authors chose organ culture methodology where they have to consider that different cell subpopulations exist?
**Authors**: The organ culture is representative of the tissue/material interface since the integrity of the tissue is preserved. The different cell phenotypes may interact and reproduce the *in vivo* implantation site. Cell line cultures, currently used by others, only consider the interaction between a substratum and a specific cell phenotype, excluding the environmental tissue cells.

**D. Arenholt-Bindslev**: How was fibroblast contamination avoided and can you be sure that contaminating fibroblasts did not affect your data on, i.e., adhesion?
**Authors**: Indeed, we cannot be sure that fibroblast contamination has been avoided. The epithelium was thoroughly separated from the underlying connective tissue under a dissection microscope. Both immunostaining and microscopic observation of the cell layer revealed the presence of a majority of epithelial cells. Whether or not some fibroblasts, at late incubation time, may appear as elongated cells, their growth would still be restricted by depletion medium which caused the degeneration of epithelial cells.

**D. Arenholt-Bindslev**: Please comment on the relevance of agar as growth surface for cell adhesion studies compared to, i.e., collagen or even fibroblast-enriched collagen gel?
**Authors**: The agar medium played a double function for supporting explant and material specimens and for supplying nutrients. Cells will neither adhere nor grow on agar which is not favorable to monolayer cell cultures. They covered tested material layered over explant depending on its cytocompatibility.

**D. Arenholt-Bindslev**: Are you sure that the use of human explant cultures results in different cytotoxicity ranking than could have been obtained with more standardized cell types, i.e., continuous cell lines, if used in identical studies, in particular, as long as rather basic endpoints are regarded (cell counts, neutral red (NR)-staining, outgrowth area)?
**Authors**: Human explant cultures provide comparative results in different cytotoxicity ranking than continuous line cells as long as one compares precious and semi-precious alloys. Human explant cultures add a complementary aspect regarding proliferative and migratory potential of the cells released from the tissue in direct contact with material and cell adhesion strength. The sensitivity of the response was enhanced compared with continuous cell line cultures (see results for titanium).

**D. Arenholt-Bindslev**: How was the epithelium for explantation dissected? Were cases of impacted or semi-impacted molars (or both?) used? Could inflamed tissue be accidentally included? Did the authors distinguish between sulcular epithelium and junctional epithelium? If not, this might influence the results (Salonen *et al.*, 1989). How was fibroblast contamination/outgrowth prevented? If it cannot be prevented, how can the influence of fibroblast data on, i.e., the adhesion results be avoided?
**Authors**: Gingival biopsies originated from the attached gingiva removed from the tuberositor pocket during extraction of impacted wisdom teeth only. Neither sulcular nor junctional epithelium were considered and inflamed tissues were systematically excluded.

**D. Arenholt-Bindslev**: Even though reference is made to Duval *et al.* (1988), up to 75 minutes trypsinization at 37° seems to be a rather long time to disperse the thin epithelial cell sheet. Does agar prevent cell detachment when compared to other relevant growth surfaces? For cell adhesion studies, is agar relevant as growth substratum in comparison to collagen or even fibroblast-enriched collagen?
**Authors**: As stated in the text, cell trypsinization and counting proceeded in the following steps: 10 test specimens covered with cell sheet were removed from the agar layer, washed in PBS and incubated in trypsin/EDTA for 10 minutes. The trypsin/EDTA solution containing the cells released was taken off and the cells were counted. Cultures were further incubated for 10 minutes in a fresh trypsin/EDTA solution and treated repetitively, for up to 75 minutes, to get to a total

dispersion of the epithelial sheet. The numbers of cells released at each step were summarized to calculate the percentages of cells released and to set up the kinetic curves.

**D. Arenholt-Bindslev**: On Figure 1b, degenerative changes seem to dominate the peripheral cells already at day 8 (i.e., vacuolization); please comment? A vertical section showing the light microscopy histology of the epithelial would be much more interesting.

**Authors**: Figure 1 points out the release of two waves of cells. First cells released did not migrate over the substratum. They commit to terminal differentiation. Degenerative changes may occur later. The second wave of spread cells, shown on the Figure 1b, might proceed differently. They have a high mitotic index as revealed by $^{3}$H-thymidine labelling, depending on their spatial localization on the cell sheet. Further experiments are currently being carried out to study their metabolism and their differentiation process.