JOURNAL OF MATERIALS SCIENCE: MATERIALS IN MEDICINE 5 (1994) 18–24

Physico-chemistry and cytotoxicity of ceramics

Part II *Cytotoxicity of ceramics*

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General cytotoxicity was assayed for ceramic (Al_2O_3, ZrO_2/Y_2O_3, AlN, B_4C, BN, SiC, Si_3N_4, TiB_4, TiC, TiN) diamond and graphite powders, using 3T3 Balb/c permanent cell lines. Neutral red test was carried out in order to establish cell viability. Further investigations were undertaken on human differentiated cells (human umbilical venous endothelial cells): cell behaviour (MTT assay, total cell protein content) and differentiation (immunofluorescence) were studied. In both cases, no cytotoxic effect has been noticed. All the impurities contained at low concentration in these powders do not seem to present any effect. The correlation which has been previously observed between cytotoxicity–cell culture response and blood haemolysis for polymers has not been established here for ceramic powders. We conclude that all the ceramic powders tested here and therefore the corresponding bulk ceramics or ceramic coatings do not induce any cytotoxic effect.

1. Introduction

Ceramics, which are more and more under consideration for biomedical applications, are widely used in the orthopaedic field: alumina (Al_2O_3), zirconia (ZrO_2/Y_2O_3) and titanium nitride (TiN [1]). Many other ones could be selected for such applications: titanium carbide (TiC), silicon nitride (Si_3N_4), aluminium nitride (AlN), boron nitride (BN), boron carbide (B_4C), . . . , if they were biocompatible.

Moreover, ceramics have begun to appear as constitutive materials of blood-contacting devices: silicon carbide (SiC, [2]), diamond-like carbon (DLC, [3]) and TiN [4] for prosthetic heart valves and TiN for a left ventricular assist device [5].

The cytotoxicity of these ceramics must be studied *in vitro* in the early stages before animal experiments [6], in order to discard highly cytotoxic candidate materials and to reduce the amount of *in vivo* testing to a minimum.

The purpose of this study is to evaluate the *in vitro* general cytotoxicity of ceramic [Al_2O_3, ZrO_2/Y_2O_3, AlN, B_4C, BN, SiC, Si_3N_4, titanium diboride (TiB_2), TiC, TiN], diamond and graphite powders with 3T3 Balb/c permanent cell lines. The use of such permanent cell lines can represent only a step in screening biomaterials [7]. The use of differentiated cell cultures is more justified and provides more information if a human cell system characteristic of the tissue which it will confront *in vivo* is involved in the *in vitro* test. As we are considering these ceramics as potential materials for blood-contacting devices, we chose human umbilical venous endothelial cells (HUVEC) as differentiated cells. Cell behaviour was studied in this case too, as well as differentiation.

All these cytotoxicity results were analysed considering the physico-chemistry results [8] and compared to those obtained with the blood haemolysis test [9].

2. Materials and methods

2.1. Materials

Iscove's modified Dulbecco's medium (IMDM) containing 4.5 $g\,l^{-1}$ D-glucose was obtained from Gibco (BRL Life Technologies Sarl, 1 rue du Limousin, BP 7050, F 95051 Cergy Pontoise Cédex, France) and foetal calf serum (FCS) from Boerhinger-Mannheim, France (2 avenue du Vercors, BP 59, F 38242 Meylan Cédex, France), 25 cm^2 culture flasks were from Corning (NY, USA) and multiwell dishes (0.32 cm^2 wells) were obtained from Nunc (Roskilde, Denmark). Phenol was from Merck (5–9 Rue Anquetil, F-94731 Nogent sur Marne Cédex, France).

2.2. Ceramic powders

Alumina (Al_2O_3), zirconium oxide/yttrium oxide (ZrO_2/Y_2O_3) and silicon carbide (SiC) were supplied by Lonza France–Martinswerk GmbH (10–12 rue des Trois Fontanot, F 92000 Nanterre, France). Aluminium nitride (AlN), boron carbide (B_4C), boron nitride (BN) and titanium diboride (TiB_2) were provided by Comaip-Esk (68 Avenue G. Bizot, F 75012 Paris,

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France), silicium nitride (Si_3N_4) by Metabap-HCST (17 rue Eugène Delacroix, F 75116 Paris, France). Titanium carbide (TiC) and titanium nitride (TiN) were purchased from Cerex (24A rue de la Résistance, F 74108 Annemasse, France). Diamond powder was elaborated by de Beers Industrial Diamond Division–Eskenazi SA (24 rue Joseph Girard, CH-1227 Genève, Switzerland) while graphite powder was supplied by Superior Graphite Co. (120 South Riverside Plaza, Chicago, IL 60606, USA).

All powders were sterilized at 120 °C.

2.3. Cell culture models

The permanent cell line used was 3T3 Balb/c (American Type Culture Collection, Rockville, MD, USA; nb CCL 163) mouse embryo cells. The differentiated cells were Human Umbilical Venous Endothelial Cells (HUVEC), cultured [10] and identified as previously described [11].

Experiments were carried out on monolayers in 96-well microtitre plates. Cells were seeded at a density of 10^5 cells cm^{-2} in these 96-well assay culture plates (Nunc) in growth medium (IMDM supplemented with either 10% v/v or 20% v/v FCS for 3T3 Balb/c and HUVEC, respectively). They were placed for 24 h at 37 °C in a humidified atmosphere containing 5% CO_2.

2.4. Obtention of material extracts

The indirect test is subordinated to the obtention of possible released products from the ceramic powders under defined conditions [12]. A specific weight of powder, with respect to the ratio 5 cm^2 of sample surface ml^{-1} of extraction vehicle, was incubated in an extraction vehicle, composed of culture medium (IMDM supplemented with FCS). The extraction was performed in borosilicated glass tubes at 37 °C for 120 h without agitation. At the end of the incubation period, ceramic powders were removed after centrifugation and the so-called extracts were used. Other borosilicated glass tubes containing the same extraction vehicles without any powder were obtained under the same conditions to provide controls for the testing procedure.

2.5. Contact between cells and material extracts

Twenty-four hours after cell seeding the medium was removed and replaced by material extracts or controls at various concentrations (100%, 50%, 10%, 1%) in the culture medium for 3 days at 37 °C. A phenol solution at a concentration of 64 $\mu g\,ml^{-1}$ was used to obtain reproducible cytotoxicity for 3T3 Balb/c cells.

At the end of the incubation time, viability and cell metabolic activity tests were performed.

2.6. Cell viability test–neutral red assay

The neutral red assay is a quantitative colorimetric measure of cell viability. The method has already been described [13]. Briefly, the medium was removed and replaced by a neutral red solution (50 $\mu g\,ml^{-1}$ of medium). After a 3 h incubation at 37 °C, the neutral red solution was replaced by a formol solution (10%; 200 $\mu l\,well^{-1}$) for a 15 min incubation. An acetic acid solution (10%; 200 $\mu l\,well^{-1}$) replaced the formol one and the incubation went for a further 30 min. The optical density was read at 490 nm. The density of the red colour is directly proportional to the viability of the cell population and inversely proportional to the toxicity of the test substance.

This test was only performed for 3T3 Balb/c cells line.

2.7. Cell metabolic activity test–MTT assay

The MTT assay is a colorimetric analysis of the metabolic activity of the cell: viable cells have the ability to metabolize a water-soluble tetrazolium dye 3(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) into an insoluble formazan salt. Briefly, the medium was removed and cell layers were rinsed with phosphate buffer (0.2 $ml\,well^{-1}$). MTT solution (0.125 ml; 5 $mg\,ml^{-1}$) prepared with Hanks buffer (1 g glucose l^{-1}) was added for an incubation at 37 °C for 3 h. The supernatant was discarded and the insoluble formazan crystals were dissolved by pure DMSO (0.100 $ml\,well^{-1}$). The optical density was read at 540 nm. The intensity of the blue colour is directly proportional to the metabolic activity of the cell and inversely proportional to the toxicity of the test substance.

This test was carried out for HUVEC.

2.8. Cell protein content

Cells were rinsed and scraped using a rubber policeman with PBS (0.1 M, pH 7.4), then sonicated at 0 °C for 30 s. Total protein content was estimated using the Lowry method [15] ($\mu g\,10^5$ cells $^{-1}$).

2.9. Evaluation of differentiated function–immunofluorescence

The presence of Von Willebrand Factor (vWF) was demonstrated by indirect immunofluorescence staining of HUVEC. Cells were fixed with 80% acetone and incubated in the presence of the primary antibody (rabbit antiserum to human Factor VIII antigen; Nordic Immunological Laboratories, the Netherlands) at a dilution of 1:50 for 30 min. After rinsing, the cells were incubated in the presence of the second antibody (FITC-conjugated goat antiserum to rabbit IgG; Nordic) at a dilution of 1:30 for 30 min. All incubations were carried out at 37 °C, HUVEC were examined by epifluorescence and photographed.

3. Results

3.1. 3T3 Balb/c cell lines–cell viability test

Results following incubation of cells with extracts were obtained as a mean of the absorbance values obtained from cells incubated in the presence of control extracts and from cells incubated in the presence

TABLE I Percentage of BALB 3T3 cells at day 3

Material extract	Percentage of viable cells			
	100% Extract concentration	50% Extract concentration	10% Extract concentration	1% Extract concentration
Negative control	100	100	100	100
Al_2O_3	103 ± 3	103 ± 2	101 ± 2	98 ± 2
ZrO_2/Y_2O_3	92 ± 7	98 ± 2	98 ± 1	98 ± 4
AlN	95 ± 2	93 ± 2	95 ± 2	92 ± 2
B_4C	100 ± 2	100 ± 3	100 ± 2	101 ± 3
BN	86 ± 2	87 ± 4	100 ± 2	99 ± 1
SiC	100 ± 5	101 ± 7	101 ± 7	103 ± 3
Si_3N_4	99 ± 4	100 ± 3	99 ± 4	101 ± 3
TiB_2	78 ± 6			
TiC	98 ± 5	101 ± 3	100 ± 3	100 ± 3
TiN	94 ± 1	96 ± 5	98 ± 4	100 ± 3
Diamond	100 ± 1	95 ± 5	94 ± 2	96 ± 2
Graphite	101 ± 1	101 ± 1	100 ± 2	100 ± 1
Phenol (65 $\mu g\,ml^{-1}$)	5 ± 3			

of material extracts. The percentage of viable cells was calculated as follows: $(\bar{E}/\bar{C}) \times 100$ for each extract at a given concentration.

The standard error on the percentage was calculated by a formula [16] derived from [17]:

$$S = [S_E^2 \times 1/\bar{E}^2) + (S_C^2 \times \bar{E}^2/\bar{C}^4)]^{1/2}$$

where $\bar{E}$ is the mean absorbance obtained from material extracts, $\bar{C}$ is the mean absorbance obtained from control extracts, S is the standard error of the $\bar{E}/\bar{C}$ ratio, S_E is the standard deviation in the calculation of $\bar{E}$ and S_C is the standard deviation in the calculation of $\bar{C}$.

Table I gives all the results obtained for 3T3 Balb/c cells. The associated histograms showed (Fig. 1) that for the 100% extract concentrations, the percentage of viable cells was never lower than 75%, whatever the material powder was. It can be seen from Fig. 2 that, for the twelve histograms corresponding to the twelve powders, the results were very close for all extract concentrations (100%, 50%, 10%, 1%).

Fig. 3 illustrates the cell confluency and the red staining of the 3T3 Balb/c cells after the neutral red test had been carried out. Phenol induced the cytotoxicity we were expecting [18]. Whatever the ceramic powders, no cytotoxicity has been noticed.

3.2. Human endothelial cells–cell metabolic activity test

Calculations were carried out in the same way as described above. Table II presents the results obtained with differentiated cells: no material extract induced a cytotoxic response; all percentages are higher than 75% (Fig. 4). All the extract concentrations gave the same results (Fig. 5).

Cell confluency can be clearly seen: the cells were homogeneous, large and polygonal, and exhibited the typical cobblestone pattern (Fig. 6).

Fig. 7 shows the insoluble formazan crystals.

As for 3T3 Balb/c cells, no cytotoxic effect has been detected for HUVEC.

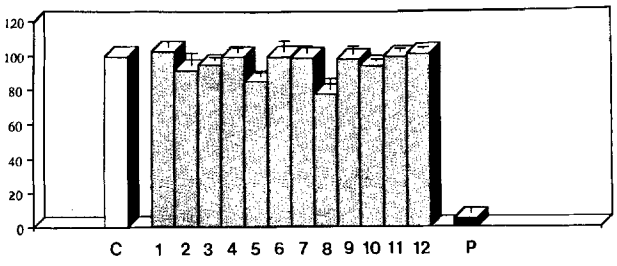

Figure 1 Percentage of viable cells (3T3 Balb/c Cells). □ Control, ▦ material extract, ■ Phenol, 1–Al_2O_3, 2–ZrO_2/Y_2O_3, 3–AlN, 4–B_4C, 5–BN, 6–SiC, 7–Si_3N_4, 8–TiB_2, 9–TiC, 10–TiN, 11–diamond, 12–graphite.

3.3. Cell protein content

The total cell protein content was very close for the negative control and the extracts, whatever the extract concentration (100%, 50%, 10%) (Table III).

3.4. Evaluation of differentiated function–immunofluorescence

The immunofluorescence study showed numerous bright fluorescent dots in the cytoplasm of endothelial cells (Fig. 8), whichever the extract considered.

4. Discussion

The percentage of viable cells was higher than 75% for 3T3 Balb/c cells or for HUVEC at 100%, 50%, 10% and 1% extract concentrations. HUVEC showed, by total cell protein content, a normal metabolic activity and the presence of vWF was clearly demonstrated. Consequently, no cytotoxicity has been found.

All these powders have been previously characterized [8]. Despite their different morphologies, their different crystallographic characteristics, their different specific areas, their different compositions and their various impurities (either common, such as O, Si, K, . . . , or more exotic, such as Hf, Pt, . . .), all these powders produced the same result: no cytotoxic effect.

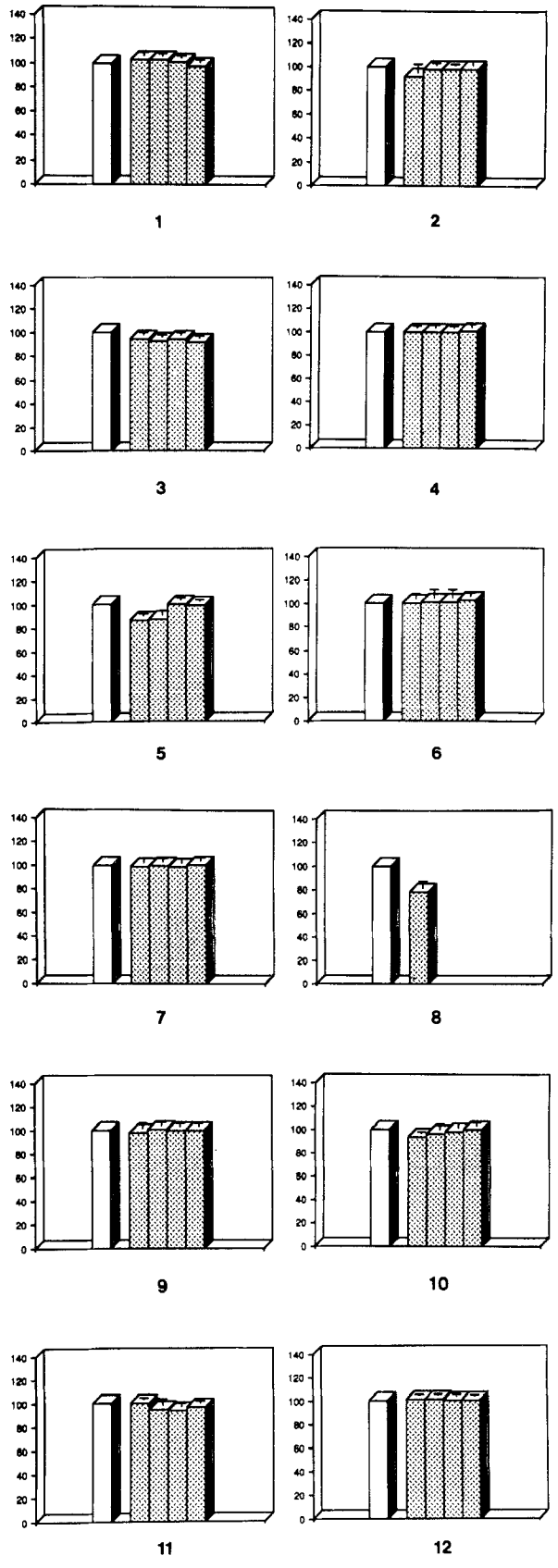

Figure 2 Twelve histograms for the twelve powder extracts at four concentrations. □ Control, ⊠ powder extracts (3T3 Balb/c cells).

As a correlation has been established [19] between the haemolytic activity and tissue culture response, as well as between the haemolytic activity and *in vivo* acute toxicity, for polymeric materials, we tested in a parallel study, the blood haemolytic activity with respect to the recommended test ratio [20], i.e. 0.5 g of powder ml^{-1} of extracting fluid (diluted blood). Under

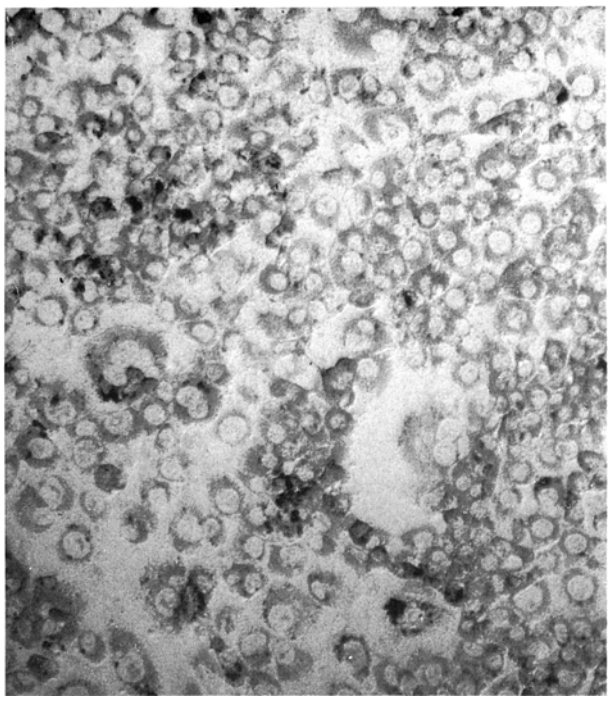

Figure 3 Cell confluency and colouration with the neutral red.

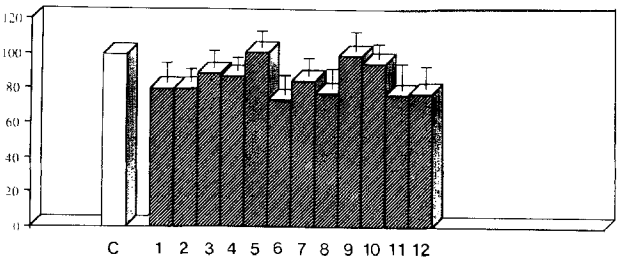

Figure 4 Percentage of viable cells (HUVEC). □ Control, ▨ powder extracts. 1–Al_2O_3, 2–ZrO_2/Y_2O_3, 3–AlN, 4–B_4C, 5–BN, 6–SiC, 7–Si_3N_4, 8–TiB_2, 9–TiC, 10–TiN, 11–diamond, 12–graphite.

such conditions, AlN and TiB_2 powders exhibited blood haemolytic activity [9].

Such haemolysis results infer that AlN and TiB_2 should induce at least a slight cytotoxic effect. As shown by the cytotoxicity results, none was noticed. Accordingly, the correlation which was expected between the haemolysis test results and the cytotoxicity test results was not observed for all the ceramic powders.

We then tried to assess the blood haemolytic activity for the same powders following the same schedule [20] but using the recommended cytotoxicity test ratio of 5 cm^2 of material ml^{-1} of extracting fluid. In this case, none of the powders under test induced blood haemolysis [9].

The complexity of the *in vivo* situation can never be adequately simulated *in vitro* [20] and further experiments should be under consideration in order to conclude definitely: cytocompatibility tests [21] involving cells in direct contact with material samples, and also *in vivo* toxicity tests. TiN, which exhibits neither blood haemolytic activity nor a cytotoxic effect, was submitted to an *in vivo* acute toxicity test (the detailed results of which will be reported in

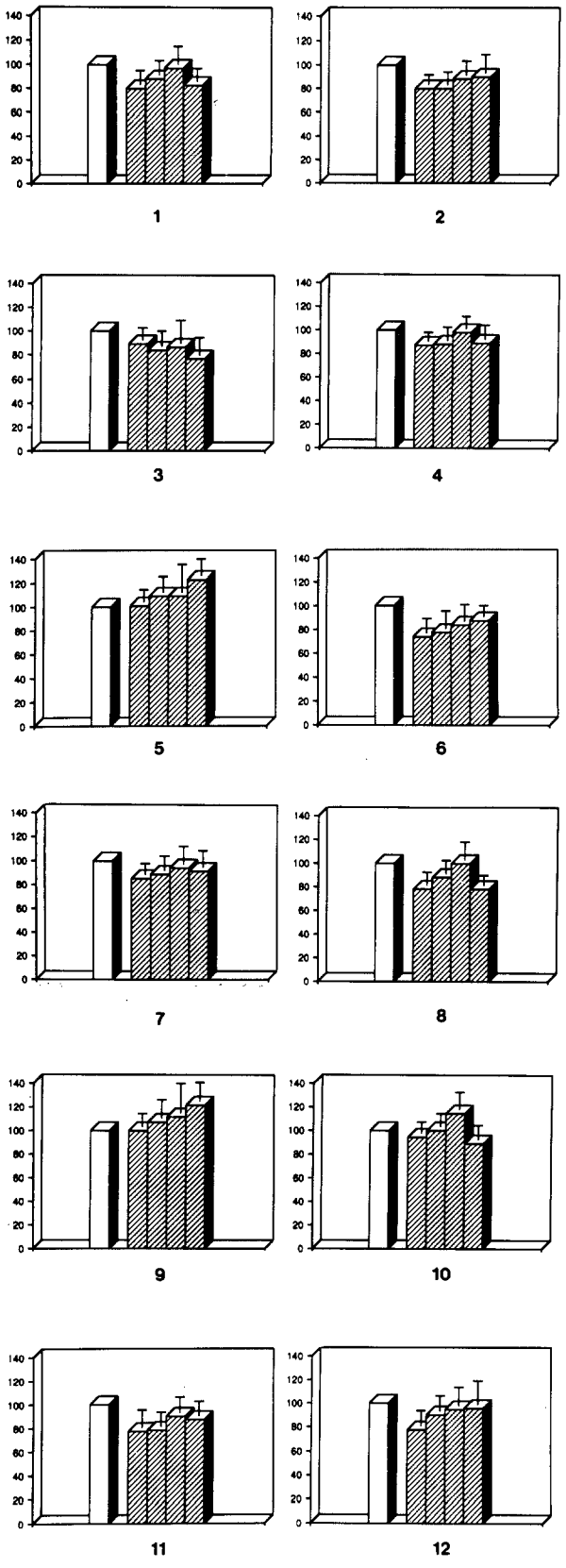

Figure 5 Twelve histograms for twelve powder extracts at four concentrations. □ Control, ▨ powder extracts (HUVEC).

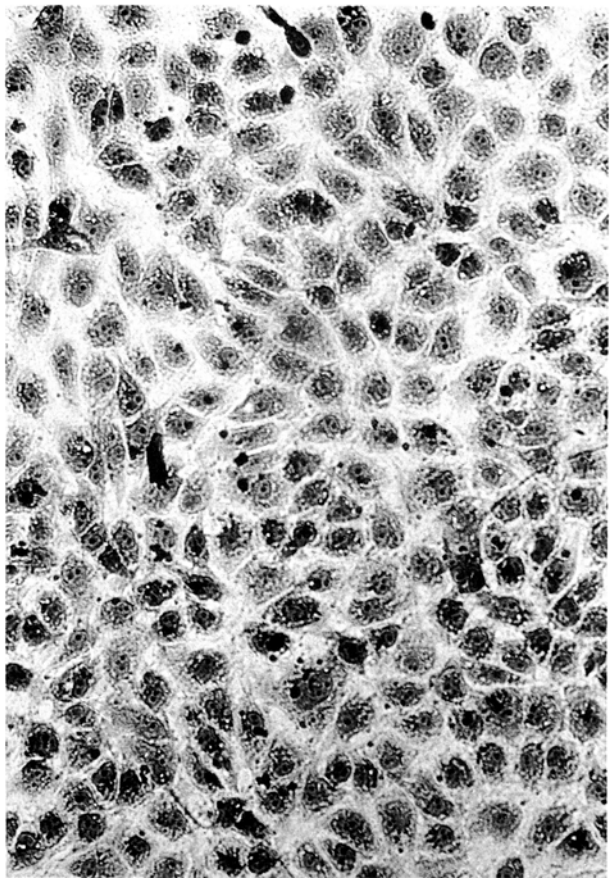

Figure 6 HUVEC at confluency.

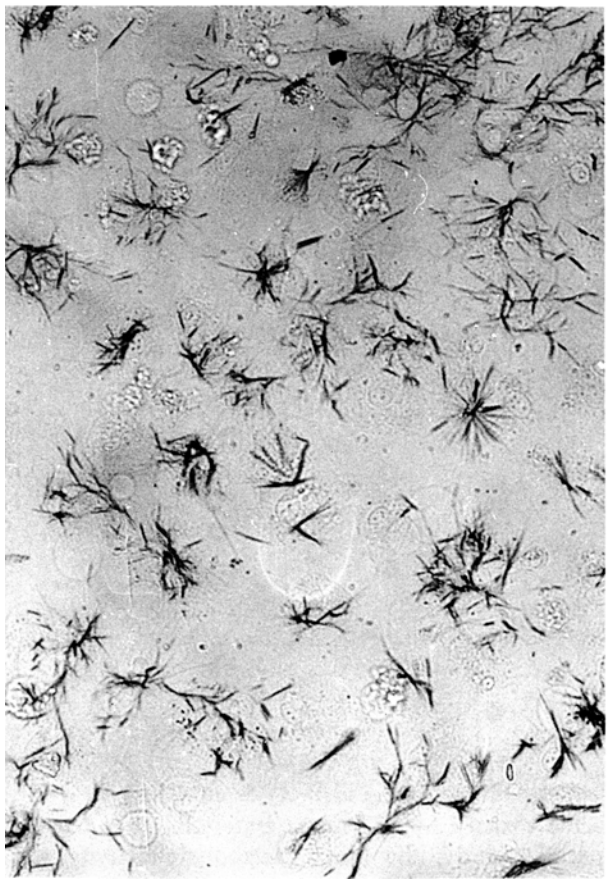

Figure 7 Insoluble formazan crystals in HUVEC.

another paper). No *in vivo* toxicity response was observed. A correlation between blood haemolytic activity and *in vivo* toxicity could then be pointed out.

The proposed correlation between haemolytic activity and cytotoxicity and toxicity could be a subject of controversy and, in the future, we will be concentrating further on *in vivo* toxicity tests which will be

TABLE II Percentage of human endothelial cells at day 3

Material extract	Percentage of viable cells			
	100% Extract concentration	50% Extract concentration	10% Extract concentration	1% Extract concentration
Negative control	100	100	100	100
Al_2O_3	80 ± 12	88 ± 12	97 ± 15	86 ± 11
ZrO_2/Y_2O_3	80 ± 8	80 ± 11	88 ± 12	90 ± 15
AlN	89 ± 10	84 ± 13	87 ± 19	77 ± 14
B_4C	87 ± 8	88 ± 11	98 ± 10	92 ± 12
BN	101 ± 10	109 ± 13	109 ± 23	123 ± 13
SiC	74 ± 11	77 ± 15	84 ± 13	87 ± 9
Si_3N_4	85 ± 10	89 ± 12	95 ± 14	94 ± 13
TiB_2	78 ± 11	88 ± 11	100 ± 15	78 ± 8
TiC	100 ± 11	107 ± 16	112 ± 24	122 ± 15
TiN	95 ± 9	100 ± 11	115 ± 14	92 ± 12
Diamond	77 ± 15	78 ± 12	90 ± 13	87 ± 12
Graphite	78 ± 12	90 ± 13	95 ± 15	96 ± 20

TABLE III Total cell protein content (HUVEC)

Material	µg proteins 10^5 cells^{-1}		
	100% Extract concentration	50% Extract concentration	10% Extract concentration
Negative control	169	156	156
Al_2O_3	200	187	231
ZrO_2/Y_2O_3	200	194	262
AlN	231	312	244
B_4C	178	169	175
BN	187	219	162
SiC	219	287	219
Si_3N_4	187	187	219
TiB_2	250		256
TiC	162	144	169
TiN	187	175	169
Diamond	175	219	319
Graphite	231	187	256

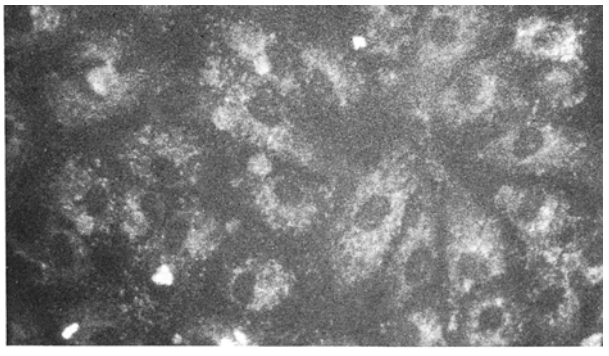

Figure 8 Immunofluorescence staining of endothelial cells for von Willebrand Factor.

undertaken on the ceramic powders discussed in this paper.

5. Conclusion

The results obtained here for permanent cell lines or for differentiated cells allowed us to conclude that all the powders tested here do not induce a cytotoxic effect and all the corresponding ceramics materials, either coatings or bulk materials, could be under consideration for use in the human body and particularly for blood-contacting devices.

Acknowledgements

This work was partly supported by TERACOR, ZI Toulon-Est, BP 238, 80 Avenue Lagrange, 83089 Toulon, France. We would like to thank Mrs Gonfier (Service de la Stérilisation, Hôpital Pellegrin, Place Amélie Raba Léon, 33000 Bordeaux, France) for sterilization of powders and glass tubes, and Mrs M. Rouais for typing the manuscript.

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Received 19 June 1992
and accepted 22 February 1993