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The response of primary rat and human osteoblasts and an immortalized rat osteoblast cell line to orthopaedic materials: comparative sensitivity of several toxicity indices

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When studying the biocompatibility of orthopaedic biomaterials it is often necessary to discriminate between responses which show mild cytotoxicity. It is therefore essential to use a very sensitive index of toxicity. We have compared the sensitivity of four well-established indices of toxicity: total cell protein content, leakage of lactate dehydrogenase (LDH), reduced glutathione content and the MTT assay, with that of a novel index, alkaline phosphatase (ALP) activity. Comparisons were made by detecting nickel chloride toxicity in osteoblasts. ALP activity, the novel method, proved the most sensitive index of toxicity and it provides a convenient automated assay for assessing the interactions of materials with osteoblasts. The responses to nickel chloride and to aqueous extracts prepared from carbon fibre reinforced epoxy and polyetheretherketone (peek), two candidate materials for orthopaedic implants, were compared in primary and immortalized rat osteoblasts, and in primary human osteoblasts. Although the immortalized rat osteoblast cell line, FFC, was consistently the most sensitive cell type, the responses of the human cells and the FFC cell line were similar in terms of ALP activity throughout the range of nickel concentrations studied. Neither peek nor epoxy material extracts showed a significant decrease in the MTT or ALP responses in any of the three cell types. Our data suggest that immortalized rat osteoblasts may provide an *in vitro* model system for screening the biocompatibility of orthopaedic polymers.

1. Introduction

In vitro cell culture systems are increasingly being used to assess the biocompatibility of biomaterials using quantitative, objective protocols. For biocompatibility testing, it is essential to establish a very sensitive index of cytotoxicity. The materials being tested often provoke only slight toxicity, and hence it is frequently necessary to differentiate between these mild cellular responses to select the most compatible materials. We have compared the sensitivity of four well-established indices of cytotoxicity: total cell protein content, intracellular reduced glutathione (GSH) content, leakage of cytosolic lactate dehydrogenase (LDH) and the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. These standard indices were used to judge the sensitivity of alkaline phosphatase (ALP) activity as a relatively novel index of orthopaedic material interactions with osteoblast derived cells.

Both the MTT and the ALP assays can be read directly in cells growing in microtitre wells using an automatic microplate reader. This offers considerable advantage in terms of speed, simplicity and convenience. The MTT assay is used extensively to test chemicals that elicit considerable toxicity, and is one of the favoured methods used by the National Cancer Institute for its drug screening programme [1, 2]. However, there are many problems associated with the method which can give rise to irreproducibility and inaccuracy both intra- and inter-laboratory. The best known of these is the difficulty in dissolving the formazan reaction product [3, 4]. However, the assay is also prone to misinterpretation of results because cell density, pH and MTT concentration affect the absorption spectrum [4, 5]. ALP activity is being investigated as an alternative, convenient microplate assay which may improve upon the MTT method for detecting the interaction of biomaterials with osteoblast-derived cells.

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Measurement of intracellular GSH content can reveal useful mechanistic information on the toxicity of materials. Depletion of GSH, prior to loss of cell viability, can be caused by several mechanisms [6]. Electrophilic species released by materials may be conjugated with GSH either spontaneously, or more commonly, enzymatically by glutathione-S-transferase. Redox reactions initiated by a material may oxidize GSH to the dimer, GSSG, which is secreted from cells. These represent the most common interactions of foreign chemicals with GSH although there are many potential sites of interaction with this ubiquitous thiol.

To compare the indices of toxicity we have used the responses of the immortalized rat osteoblast cell line, FFC, to nickel chloride. This metal is known to be released from orthopaedic implants [7, 8] and has been shown previously to inhibit the growth of fibroblasts [9, 10] and to decrease proteoglycan synthesis in chondrocytes [11]. The sensitivities of the MTT and ALP assays were further compared in the three cell types using extracts prepared from carbon fibre reinforced polyetheretherketone (peek) and epoxy two candidate biomaterials for development of an isoelastic hip joint [12].

In addition to the choice of index to be used for detecting cytotoxicity, a major consideration in biocompatibility testing is the selection of the cell type. This should be representative of the cell population within the tissue exposed to the biomaterial in clinical use. Many cell lines are available for screening biocompatibility, but most have de-differentiated and lost the majority of their tissue-specific characteristics. On the other hand, primary cells are not always readily available for such tests. We believe that the answer to this problem may lie in the development of immortalized differentiated cell lines obtained by transfecting primary cells with viral DNA. In our laboratory we have developed this approach using primary cultures of rat hepatocytes transfected with SV40 viral DNA. The resulting immortalized rat hepatocytes retain several aspects of differentiated liver function and respond to hepatotoxins to the same extent as the primary cultures [13, 14]. We have now applied these methods to primary cultures of osteoblasts and obtained an immortalized osteoblast cell line which may provide a readily available *in vitro* model system for assessing biomaterial compatibility. This paper reports results of the comparison of the responses of neonatal rat primary calvarial osteoblasts, immortalized rat osteoblasts and primary human adult osteoblasts to orthopaedic materials.

2. Materials and methods

2.1. Preparation and culture of cells

Primary cultures of rat osteoblasts were isolated from the frontal, parietal and occipital regions of 24-hour-old neonatal rat calvariae using a modification of a published method [15]. The tissues were washed with tris buffered saline (TBS), finely minced and incubated for 90 min with a 1 : 1 mixture of 0.25% (w/v) trypsin and 0.02% (w/v) versene. After incubation, the cells were harvested by centrifuging at 700 g for 8 min, suspended in Dulbecco's modification of Eagle's medium (DMEM) supplemented with 10% (v/v) foetal calf serum (FCS) and seeded in 35 mm Petri dishes at a density of 2×10^4 cells/cm^2. Primary cultures of human osteoblasts were obtained from Dr David Jones, Department of Experimental Orthopaedics, Westfalische Wilhelms University, Munster, Germany. The osteoblasts used in this study were obtained from adult jaw bone and are spongiosal cells derived from the neural crest.

Rat osteoblasts at passage 4 were transfected with pUK42, a plasmid (10.9 kb) containing the complete viral SV40 genome (except for a 6 bp deletion at the origin of replication) and the complete sequence of pRSV neo which contains the gene coding for neomycin resistance. On the day prior to transfection a confluent 75 cm^2 flask of osteoblasts was split 1 : 3. Transfection was carried out by calcium phosphate precipitation using 100 μg/10^6 cells of pUK42 DNA [16]. Cells were incubated with DNA for 16 h, then the monolayer was washed with serum-free medium to remove the precipitate, before the medium was replaced with DMEM containing 10% FCS. To establish that the transfection procedure had resulted in expression of the SV40 sequences, the presence of large T antigen was detected using immunofluorescence as previously described [16]. This procedure was carried out 7 weeks after transfection.

Both primary and immortalized rat osteoblasts were routinely grown in DMEM, supplemented with 10% FCS, and for passaging, the split ratios were 1 : 3 and 1 : 10, respectively. Primary human osteoblasts were cultured in Ham's F-10 medium supplemented with 10% FCS and 50 mg/1 L-ascorbic acid, and were split 1 : 3. The primary cells were used for cytotoxicity experiments at passage 3, and the immortalized cells at the 14th passage after immortalization.

2.2. Preparation of the materials and extracts

The cytotoxicity of the orthopaedic polymers, peek and epoxy, was assessed by preparing aqueous extracts as detailed by the British Standard method, BS 5736, part 10. Silescol, silicone rubber (Bibby Sterilin Ltd., Stone, Staffs), was used as the non-toxic negative control material and tin-stabilized poly vinyl chloride (Portex Ltd., Kent) as the toxic control material. Discs of peek and epoxy, with carbon fibre reinforcement, were prepared by the Department of Aeronautical Structures and Materials, The Royal Institute of Technology, Stockholm. The control materials were tested as standard discs of 12 mm diameter and 1.5 mm thickness. The peek and epoxy discs were of 15 mm diameter and 3.0 mm thickness. Materials were supplied as non-sterile discs, and were extracted in an unsterilized state.

Discs of all materials were soaked in 70% (v/v) ethanol for 30 min, followed by treatment with phosphate buffered saline (PBS), pH 7.4, containing 100 units/ml penicillin and 100 μg/ml streptomycin for 2 h. The discs were then washed with sterile media,

and extracts prepared according to the recommendations of the British Standard, BS 5736, part 10. The discs were extracted at a surface area to extractant volume of 1 cm^2/ml in sealed 100 ml conical flasks in a shaking water bath at 37 °C for 48 h. The extractant used was DMEM (for rat cells) or Ham's F-10 medium (for human cells) supplemented with 10% FCS and the antibiotics as detailed above. At the end of the extraction period, the extracts were passed through a 0.22 μm filter to remove any debris and stored at − 70 °C until required.

Undiluted extracts of the materials were added to the cells at the time of seeding. Exposure time was 48 h. At the end of this period toxicity was assessed by the different parameters detailed below.

Stock solutions of 50 mM nickel chloride in water were sterile filtered through 0.22 μm filters. Nickel was added (at the same time as the cells were seeded) at concentrations ranging from 5 to 500 μM in media containing 10% FCS. Cells were exposed to the nickel for 48 h before toxicity was assessed.

2.3. Measurement of cytotoxicity

Measurements of protein, GSH content and LDH leakage were carried out on cells grown on 24-well plates at a density of 10^5 cells/cm^2 after exposure to either the material extracts or to nickel chloride for 48 h. LDH activity was measured in 100 μl aliquots of the medium as described previously [17]. Cell monolayers were washed once with TBS, and the intracellular GSH extracted with 200 μl of 10% (w/v) trichloroacetic acid. These acidic extracts were stored at − 70 °C until analysis of GSH fluorimetrically by the method of Hissin and Hilf [18]. The cell monolayer was subsequently digested at 37 °C for 18 h using 80 μl of 1 M NaOH and these samples were stored at − 20 °C for measurement of total cell protein by the method of Lowry and co-workers [19].

The MTT and ALP assays were carried out on cells grown on 96-well plates at a seeding density of 10^5 cells/cm^2 after 48 h exposure to either the extracts of materials or to nickel chloride. For the MTT assay, the medium was aspirated and 50 μl of 10 mM MTT in PBS, pH 6.75, added to each well. After an incubation period of 4 h at 37 °C, the liquid was aspirated and the formazan reaction product dissolved in 200 μl dimethylsulphoxide. The optical densities were measured in a Biorad Model 450 Microplate reader at 540 nm. ALP activity was measured using kit no. 245–20 purchased from Sigma Chemical Company, Poole, Dorset, England. For the ALP assay, the medium was aspirated and 60 μl of the ALP reagent added. The optical density, at 405 nm, was read immediately (time 0), and again after incubating the plates for 20 min at room temperature. The readings at time 0 were subtracted from those after 20 min, to determine the ALP activity. All statistical comparisons were made by ANOVA, followed by Dunnett's test.

3. Results

Fig. 1 shows the morphology of rat and human primary cultures, and immortalized rat osteoblasts. The primary cells from the two species are similar in size (15–20 μm diameter), whereas the immortalized cells are smaller in size (10–12 μm diameter). Fig. 2 shows the expression of the T antigen in the immortalized rat osteoblasts detected by immunofluorescent staining.

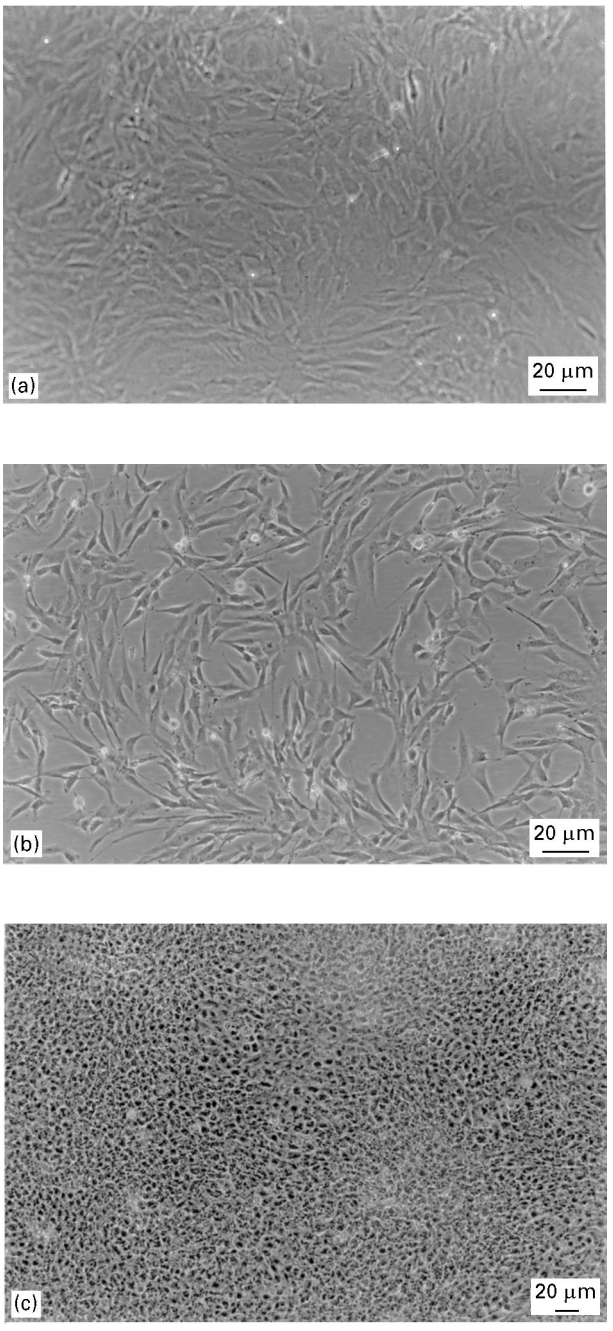


Figure 1 Morphology of (a) primary rat; (b) human osteoblasts, and (c) immortalized rat osteoblasts.

Immortalized rat osteoblasts were used to compare the sensitivities of the five toxicity tests; measurement of total cell protein, GSH content, leakage of LDH, the MTT assay and ALP activity. This comparison was carried out using concentrations of nickel chloride between 5 and 500 μM, and the results are shown in Fig. 3. The least sensitive indices of toxicity were LDH and GSH, which only detected significant responses to nickel at concentrations of 250 μM and above. The Lowry assay for total protein content detected significant toxicity at 100 μM nickel and

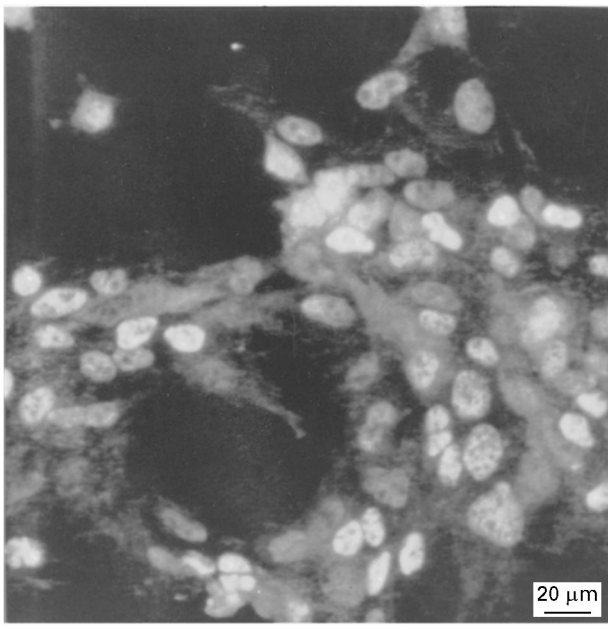


Figure 2 Immunofluorescent localization of large T antigen in immortalized rat osteoblasts.

above. The data show that both the MTT assay and ALP activity were sensitive methods, detecting significant responses to 5 μM nickel. In fact, ALP was more sensitive than MTT and could detect significant toxic responses to 1 μM nickel (data not shown). It is important to note that nickel does not directly inhibit ALP activity. In the presence of 500 μM nickel chloride, homogenized FFC osteoblasts retained over 85% of their ALP activity (results not shown).

Using the MTT and ALP methods as those demonstrated to be most sensitive, the responses of primary and immortalized rat osteoblasts and primary human cells to nickel were compared. These results are shown in Fig. 4. Once again the superior sensitivity of the ALP activity compared with the MTT assay is apparent from these data. The sensitivity of the cell types to nickel chloride varied, with the FFC immortalized rat osteoblasts being most sensitive and the primary osteoblasts least sensitive. The responses of the two rat-derived cell types were compared with those of the human cells. With the MTT test, only at the two highest concentrations of nickel, 250 and 500 μM, was there a significantly different response in the rat cells. Although the FFC immortalized rat cell line was more sensitive to nickel toxicity than the human cells, the responses of the human cells and FFC cells were similar in terms of ALP activity throughout the range of nickel concentrations studied. However, primary rat cells were significantly less sensitive to nickel at 25 and 250 μM.

The effects of undiluted carbon fibre reinforced peek and epoxy extracts were measured in each cell type in terms of both ALP activity and the MTT assay. These results are illustrated in Fig. 5. The peek and epoxy material extracts caused no significant decrease in either ALP or MTT responses in any of the three cell types. The only significant difference observed was an increase in ALP activity in response to exposure to the epoxy extracts in the human primary osteoblasts.

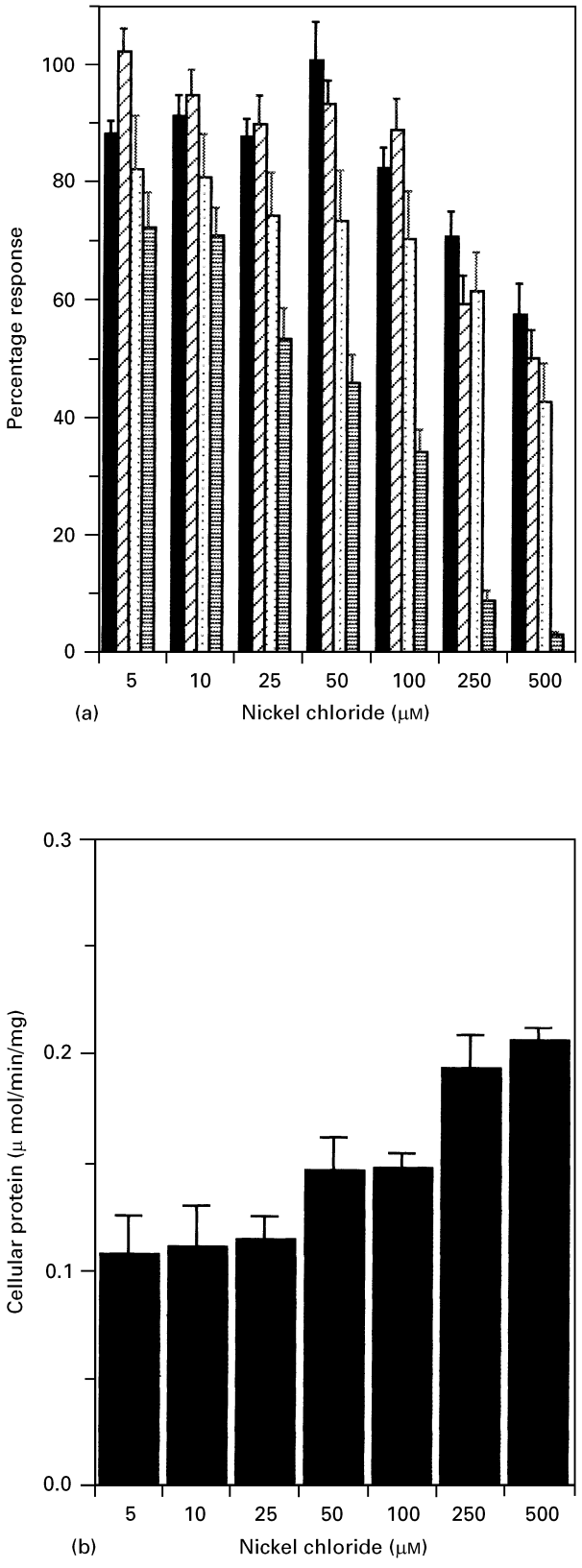
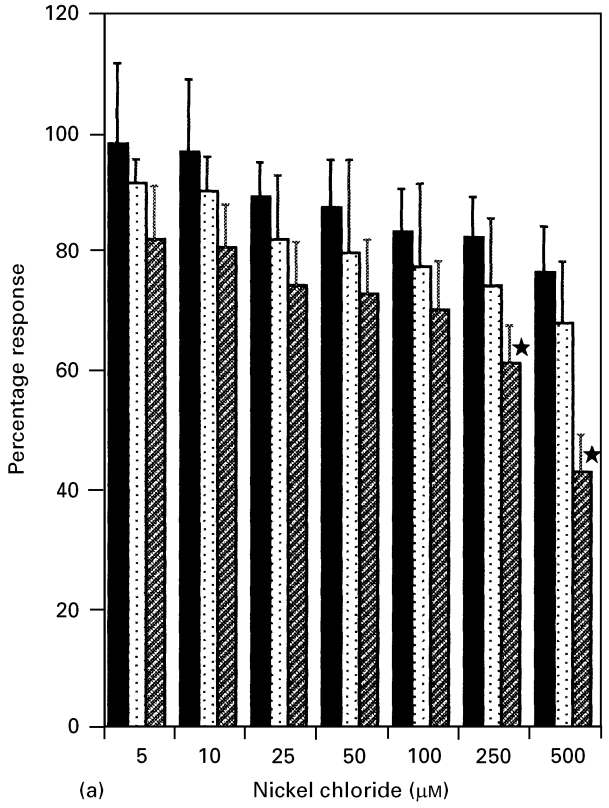


Figure 3 Comparative sensitivity of the different indices of toxicity in nickel chloride-treated FFC immortalized rat osteoblast cells. To facilitate inter-assay comparisons on Fig. 3a, all results are expressed as a percentage of the values obtained in control cultures treated with medium only. Control values were assigned 100% for each individual assay. Results are means ± SEM, of a least nine experiments. The LDH values are shown separately on Fig. 3b because LDH increases in response to toxic insult, whereas all the other indices decrease in value. The results of each assay were significantly different ($p < 0.05$) from controls as follows: LDH and GSH assays at and above 250 μM; Lowry (protein content) at and above 100 μM; MTT and ALP at and above 5 μM. ■ Lowry; ▨ GSH; ▤ MTT; ▤ ALP.



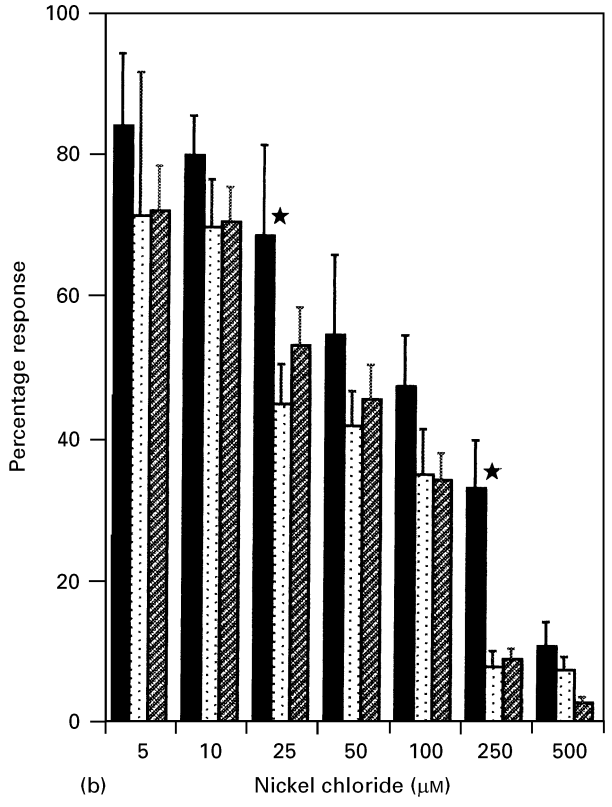
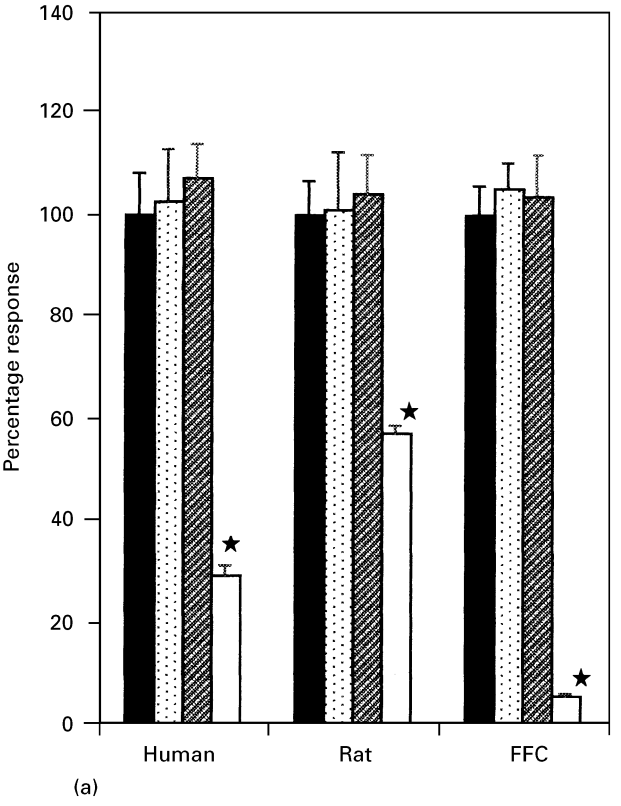


Figure 4 The response of primary and immortalized rat osteoblasts and primary human osteoblasts to nickel chloride measured by (a) the MTT assay and (b) ALP activity. To facilitate comparison between the MTT and ALP assay sensitivities within each cell type, the results are expressed as a percentage of the values obtained in control cultures treated with medium only. Control values were assigned 100% for each individual assay. Results are means ± SEM, of at least 12 experiments. ■ Rat; ▦ Human; ▨ FFC. $*p < 0.05$, compared with the responses of human cells.



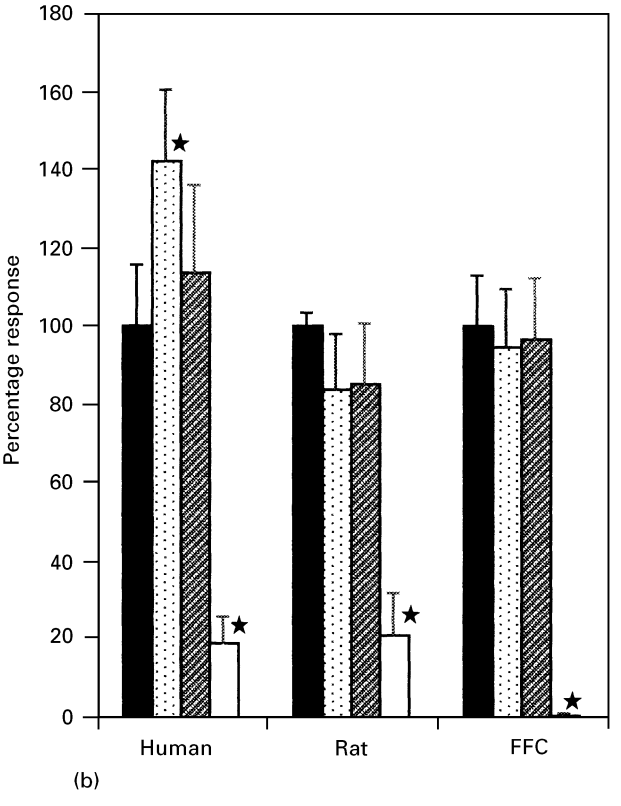


Figure 5 The response of primary and immortalized rat osteoblasts and primary human osteoblasts to material extracts detected by (a) the MTT assay and (b) by ALP activity. Results are expressed as a percentage of the response to the negative control material extracts (assigned 100%) for each cell type, and are the means ± SEM, of at least 16 experiments. ■ neg; ▦ epoxy; ▨ peak; □ pos. $*p < 0.05$, compared with negative control extracts.

4. Discussion

The responses of the FFC cell line to nickel illustrate the marked variation in sensitivity between different parameters commonly used to detect the toxicity of chemicals. Measurement of LDH activity is one of the most widely used indices of toxicity, partly because, since the activity can be measured in the culture medium, the viability of a monolayer culture can be measured non-invasively and conveniently over a prolonged period. This enables chronic testing of chemicals to be undertaken using minimal cell numbers. However, we have demonstrated here that this method is inferior in terms of sensitivity.

Detection of nickel toxicity by GSH depletion was also rather insensitive, but this may reflect the mechanism of toxicity. Previous studies have shown that GSH does not protect against nickel toxicity in fibroblasts [9], and the interaction of osteoblasts with this metal may not involve a significant change in the redox balance of the cell under the conditions used in our experiments. Where the toxicity of a chemical involves oxidative stress and/or disruption of GSH levels by conjugation, then GSH depletion is a more useful parameter.

Measurement of total cell protein content was a more sensitive index of toxicity than LDH leakage, and detects loss of dead cells due to detachment from the culture flasks. The MTT assay relies on the ability of the viable cells to reduce a water-soluble yellow dye, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, to a water-insoluble purple formazan product. From our results disruption of reduction enzyme activities occurs before loss of adhesion in response to nickel.

Finally, the most sensitive parameter, among those we measured, was ALP activity. 1 μM nickel chloride caused a significant decrease in osteoblast ALP activity. This was not due to a direct inhibition of ALP enzyme activity by nickel. Measurement of ALP activity may therefore provide a convenient, automated *in vitro* assay with improved sensitivity for detecting the interaction of chemicals with osteoblasts. This will have particular relevance for biocompatibility testing of orthopaedic materials where we are often trying to discriminate between materials exhibiting only slight cytotoxic interactions.

We have previously used histochemical localization of ALP in osteoblasts to investigate biocompatibility of orthopaedic polymers [12]. However, the method described in the present paper is superior in that it requires minimal manipulation and is easily automated. Harmand and co-workers [20] also used ALP activity to assess the biocompatibility of an orthopaedic polymer, epoxy, in osteoblasts. They found that homogenates prepared from cells cultured on the resin had lower ALP activity than those cultured on a negative control material. A recent review by Pizzoferrato and colleagues [21] has recommended that assessment of osteoblast responses to biomaterials should include assays of the processes involved in the bone mineralization process, including ALP activity. The improvement in sensitivity provided by the ALP activity test for detecting osteoblast interactions with nickel in our experiments reinforces this recommendation. Although ALP activity changes in osteoblasts according to the differentiated state, we have found that its expression is stable in FFC cells between passages 2 and 28 (Macnair, unpublished data).

The end-point to be quantified for assessment of biocompatibility is one of the main issues to be decided in any study, but another matter of paramount importance is the choice of cell type. For orthopaedic materials it is essential to determine the interaction with bone-forming cells. Osteoblasts have been shown to be more sensitive to biomaterial interactions than fibroblasts [12, 20]. Many people believe that only human cells should be used to test the biocompatibility of clinical materials. However, human cells, particularly primary differentiated cells, are not always readily available. We recently developed an immortalized rat osteoblast cell line in our laboratory and propose that such cell lines may provide a convenient *in vitro* model system for screening biomaterial toxicity. The sensitivity of primary and immortalized rat, and primary human, osteoblasts to nickel was compared to assess the predictive value of studying interactions of chemicals with the FFC immortalized cells. After immortalization, rat osteoblasts were markedly more sensitive to nickel-induced toxicity than the primary rat cells. The ALP activity measurements were similar for FFC cells and human primary cells throughout the range of nickel concentrations. With the MTT assay, FFC cells were significantly more sensitive than human cells to 250 and 500 μM nickel. The toxicity of nickel was greatest in cells which had the fastest growth rate (the FFC immortalized cells) and least in the slowest growing primary rat osteoblasts. On the basis of these data, immortalized rat osteoblasts merited further validation for screening the biocompatibility of orthopaedic materials.

The responses of the three cell types to extracts prepared from carbon fibre reinforced epoxy and peek, two candidate biomaterials for the development of an isoelastic hip joint, were then compared using the MTT and ALP methods. As observed with the responses to nickel chloride, the ALP method was more sensitive than the MTT assay. Also, comparing the effects of the positive control extracts, FFC was the most sensitive cell type, followed by the human primary cells. Neither polymer extract caused a significant reduction in MTT assay responses or in ALP activity in any cell type. This indicates that neither material was overtly toxic. Human primary cells showed greater ALP activity when cultured in extracts of epoxy; the significance of this is not clear at present. We have previously shown that extracts of epoxy were slightly cytotoxic to fibroblasts, and depleted GSH in immortalized rat osteoblasts [12]. Harmand and co-workers have found that when cultured on epoxy, ALP activity is disturbed in human primary osteoblasts and the material is slightly cytotoxic [20]. Previous workers have demonstrated that peek exhibits excellent biocompatibility [22, 23]. From our previous studies [12] on the biocompatibility of these two materials, and from the present data, it would appear that peek may be the material of choice as regards

biocompatibility for further development of an isoelastic hip joint.

The use of the FFC immortalized rat osteoblast cell line will be further validated in our laboratory and we believe it may provide a suitable *in vitro* cell culture model system for screening orthopaedic materials. We are presently growing FFC cells, and primary human osteoblasts on peek and epoxy to assess the effects of direct material contact on osteoblast growth, function and morphology.

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References

1. J. CARMICHAEL, W. G. deGRAFF, A. F. GAZDAR, J. D. MINNA and J. B. MITCHELL, *Cancer Res.* **47** (1987) 936.
2. M. C. ALLEY, D. A. SCUDIERO, A. MONKS, M. L. HURSEY, M. J. CZERWINSKI, D. L. FINE, B. J. ABBOT, J. G. MAYO, R. H. SHOEMAKER and M. R. BOYD, *ibid.* **48** (1988) 589.
3. H. WAN, R. WILLIAMS, P. DOHERTY and D. F. WILLIAMS, *J. Mater. Sci. Mater. Med.* **5** (1994) 154.
4. M. D. SMITH, J. C. BARBENEL, J. M. COURTNEY and M. H. GRANT, *Int. J. Art. Org.* **15** (1992) 191.
5. J. A. PLUMB, R. MILROY and S. B. KAYE, *Cancer Res.* **49** (1989) 4435.
6. A. MEISTER. In "The liver: biology and pathobiology", edited by I. M. Arias, W. B. Jakoby, H. Popper, D. Schachter and D. A. Shafritz (Raven Press, New York, 1988) p. 401.
7. L. D. DORR, R. BLOEBAUM, J. EMMANUAL and R. MELDRUM, *Clin. Orthop. Res.* **261** (1990) 82.
8. U. E. PIZZAGLIA, C. MINOIA, L. CECILIANI and C. RICCARDI, *Acta. Orthop. Scand.* **54** (1983) 574.
9. M. H. GRANT, C. NUGENT and R. BERTRAND, *Toxicol. In Vitro* **8** (1994) 191.
10. L. J. BEARDEN and F. W. COOKE, *J. Biomed. Mater. Res.* **14** (1980) 289.
11. C. J. KIRKPATRICK, W. MOHR and O. HAFERKAMP, *Res. Exp. Med.* **181** (1982) 259.
12. C. MORRISON, R. MACNAIR, C. MACDONALD, A. WYKMAN, I. GOLDIE and M. H. GRANT, *Biomaterials* **16** (1995) 987.
13. C. MACDONALD, M. VASS, B. WILLETT, A. SCOTT and M. H. GRANT, *Human Exp. Toxicol.* **13** (1994) 439.
14. M. H. GRANT, N. GRANGER and C. MACDONALD, *Brit. J. Clin. Pharmacol.* **38** (1994) p. 172.
15. I. BINDERMAN, D. DUKSIN, A. HARELL, E. KATZIR and L. SACHS, *J. Cell Biol.* **61** (1974) 427.
16. U. KREUZBURG-DUFFY and C. MACDONALD, *Immunology* **72** (1991) 368.
17. D. C. ANUFORO, D. ACOSTA and R. V. SMITH, *In Vitro* **14** (1978) 981.
18. R. J. HISSIN and R. HILF, *Anal. Biochem.* **74** (1976) 214.
19. O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR and R. J. RANDALL, *J. Biol. Chem.* **193** (1951) 265.
20. N. F. HARMAND, L. BORDENAVE, R. BAREILLE, A. NAJI, R. JEANDOT, F. ROUAIS and D. DUCASSOU, *J. Biomater. Sci. Polymer Edn* **2** (1991) 67.
21. A. PIZZOFERRATO, G. CIAPETTI, S. STEA, E. CENNI, C. R. ARCIOLA, D. GRANCHI and L. SAVARINO, *Clin. Mater.* **15** (1994) 173.
22. L. M. WENZ, K. MERRITT, S. A. BROWN, A. MOET and A. D. STEFFEE, *J. Biomed. Mater. Res.* **24** (1990) 207.
23. K. A. JOCKISH, S. A. BROWN, T. W. BAUER and K. MERRITT, *ibid.* **26** (1992) 133.

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