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Analysis of the inflammatory response to titanium and PTFE implants in soft tissue by macrophage phenotype quantification

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Implants of commercially pure titanium and polytetrafluoroethylene (PTFE) were inserted in the rat abdominal wall for 1, 6 or 12 wk. The foreign-body reaction was evaluated by immunohistochemical quantification of monocytes/macrophages and by the thickness of the foreign-body capsule. At all time intervals, the majority of interfacial cells were ED1-positive while ED2-positive cells were localized deeper in the tissue. Neither titanium nor PTFE displayed a significant change in capsule thickness over time. The total cell numbers decreased over time for both types of material. At 12 wk the PTFE implants, compared to titanium, were surrounded by a significantly thicker reactive capsule with larger total cell numbers. No significant differences were seen in the macrophage subset response between the two types of implants. Thus, the present study showed differences between titanium and PTFE at 12 wk but not at earlier time points.

1. Introduction

Titanium has successfully been used in bone implants for several decades, and it has also shown superior biocompatibility in the adjacent soft issue [1, 2]. A fundamental question is whether these results depend on the material characteristics of titanium or on other factors not directly related to the material *per se*. Several studies have shown that different biomaterials elicit different inflammatory reactions [3–7]. The biological environment at an implant is complex [8] and easily disturbed by various factors. The crucial mechanisms for the generation of the host foreign-body response to the implanted material are, at present, largely unknown. The purpose of the present study was to compare the soft-tissue response elicited by titanium implants to solid PTFE, which has widely different material characteristics compared to titanium. Both materials are non-toxic and stable in the biological environment and previous studies have indicated differences in the biological response, such as leukocyte activation [3] and protein distribution [7].

Some tissue parameters are readily quantitative, such as thickness of the foreign-body capsule and the density of cells adjacent the implant. Macrophages are crucial to the inflammatory response in general and especially at biomaterials [9, 10]. Subpopulations of rat macrophages can be identified immunohistochemically [11, 12], eliminating the uncertainty of differentiating macrophages from other cell types. The use of monoclonal antibodies allows studies on recruitment and localization of different macrophage phenotypes, such as newly recruited blood monocytes (ED1) and mature tissue macrophages (ED2) [11, 12]. These antibodies have previously been used to quantify the cellular response to biomaterials [5, 13–16]. Some studies show differences in cell distribution between implants with different chemical composition and with different surface structure [13, 14], whereas other results indicate that the macrophage enumeration is not related to the overall tissue response [17]. It is, however, conceivable that the numbers and activation state of macrophages adjacent to the implant reflects material properties relevant for the long-term fate of the implant material.

In the present study we have focused attention on subsets of macrophages and the formation of the foreign-body capsule, comparing the tissue responses elicited by two principally different materials, chemically pure (c.p) titanium and PTFE.

2. Material and methods

2.1. Animals

Male Sprague-Dawley rats ($n = 24$), weighing 200–250 g, fed on standard pellets and water ad libitum, were used. The rats were anaesthetized by intraperitoneal (i.p)

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injections of 1.0 m/100 g body weight of a solution containing sodium pentobarbital (60 mg m^{-1}) and NaCl (9 mg m^{-1}) in 1:9 volume proportions. The experimental protocol was reviewed and approved by the Animal Ethics Committee.

2.2. Implants

Two different implants of c.p. titanium, respectively of polytetrafluoroethylene (PTFE), were used. The implants consisted of a cylindrical rod (length 3 mm, diameter 2 mm) connected to the centre of a circular plate (thickness 1 mm, diameter 5 mm) [18]. One titanium and one PTFE implant were inserted on either side of the linea alba in each rat. Eight rats were used at each implantation period, i.e. at 1, 6 and 12 wk. Before insertion, the c.p. titanium implants were cleaned and sterilized for 15 min in a solution of 30% H_2O_2 and NH_2OH at room temperature and the PTFE implants were cleaned and sterilized in ethanol in an ultrasonic bath. The implants were rinsed and kept in sterile saline prior to surgery.

2.3. Implantation procedure

Details of the implantation procedure have been described previously [18]. In brief, the rectus abdominis muscle sheath was opened and the muscle moved laterally. A titanium implant and a PTFE implant were inserted on either side of the linea alba. The rod part was inserted through a small opening in the peritoneum. The rectus abdominis muscle was slipped back to cover the implant and a suture was placed in the muscle sheath to secure the position of the implant.

2.4. Tissue fixation

After 1, 6 or 12 wk the animals were re-anaesthetized and the implants with surrounded tissue were removed *en bloc*. The specimen was washed in ice-cold phosphate-buffered saline (PBS, pH 7.4), embedded in Tissue Tek® O.C.T. compounds 4583 (Histolab Products AB, Sweden) and snap frozen for 30 s in 2-methylbutane at $-70\,^{\circ}C$. The implant was then exposed by dissection through the peritoneal membrane. The implant was carefully removed mechanially without letting the specimen thaw. Longitudinal sections were cut in a cryostat (6 μm thick) and collected on chromium–alum treated slides and allowed to air dry. The slides were kept at $-70\,^{\circ}C$ until stained.

2.5. Immunohistochemistry

The staining procedures for the macrophage subclasses ED1 and ED2 were done according to Rosengren *et al.* [16]. In brief the staining procedure was as follows.

ED1 and ED2: after removal of the endogenous peroxidase activity and blocking of unspecific bindings, the slides were incubated with primary antibodies. The primary antibodies used were mouse anti-rat ED1 and ED2 (Serotec Ltd, Oxford, UK). The primary antibodies were exposed to a biotinylated horse anti-mouse IgG secondary antibody. The sections were then incubated with Vectastain ABC peroxidase standard PK-4000 (Vector Lab. Inc., Burlingame, CA, USA). The presence of peroxidase was detected using 3-Amino-9-Ethyl-Carbazole (Sigma Chemical Co, St Louis, USA). Thereafter, the sections were counterstained in Mayers HTX. For the control sections, mouse monoclonal antibodies directed to human cell surface antigens were used as primary antibody.

All images were obtained using a Kodac DSC-200 digital camera (Rochester, New York), mounted on a Nikon FXA microscope in transmitted light. The images were retrieved and marked using Photoshop 3.05 software (Adobe Photoshop, Mountain View, CA, USA) on a Macintosh 8500 computer (Cupertino, CA) and printed on a Kodak (ColorEase PS Print) sublimation printer.

2.6. Morphometry

One titanium and one PTFE implant were inserted in each rat on either side of the linea alba thereby allowing paired comparisons (see Section 2.3). Eight sections were enumerated for ED1 and ED2 positive cells, for each implant type and each evaluation period [16]. The sections were evaluated by the same person (A.R.), who did not know to which group an individual specimen belonged. The numbers of cells were determined by manual counting of the positive cells on sections stained for ED1 and ED2, respectively ($n = 8$ for each evaluated biological parameter at each time point). Further, the numbers of cell nuclei (counterstained cells) were used as a measure of total numbers of cells, which thus includes macrophages, fibroblasts and other cells. The quantification was done in a Leitz microscope in bright-field mode at $\times 25$ magnification. A 10×10 ocular square grid where each square covered a 42×42 μm large area was superimposed at the centre along the tissue border adjacent to the implant surface. The thickness (μm) of the reactive capsule was determined using the grid and was defined as the distance between the tissue border adjacent to the implant and the muscle border. The number of cells in the measured capsule were manually counted in five rows of squares from the implant surface to the border of the muscle. The cell numbers are given in numbers per mm^2 based on the actually counted area. In order to quantify the tissue reactions immediate to the implants, the number of cells in the interfacial area was defined as the row of five squares along the implant surface. Within these squares the numbers of cells were counted as described for the capsule.

2.7. Statistics

Non-parametric statistics were used in this study using the Statview 4.5 (Abacus Concepts, Berkeley, CA.) for the Macintosh. The design of the study allowed for paired comparisons. For comparison between titanium and PTFE implants in the same rat, the Wilcoxon signed rank test was used [19] based on a comparison between the counts for each implant

type ($n = 8$) at the three different evaluation periods. For evaluation of capsule thickness and cell density over time, the Kendall rank correlation test was done. The relevant p-values corresponding to a single comparison at $p < 0.05$ are given for each comparison. Because non-parametric statistics were used, all values are presented as median values with the interquartil range.

3. Results

3.1. General observations

Around both types of implants a reactive zone containing mainly monocytes/macrophages and other cells (mostly fibroblasts), was observed. Monocytes/macrophages with ED1 immunoreactivity predominated close to the implant, whereas ED2 positive cells were abundant more distantly from the implant (Figs 1a–d and 2a–d). At 1 wk after insertion, the tissue was unorganized and the border of the reactive zone was indistinct. After 6 wk the tissue became more organized and the inflammatory reaction declined successively. This is reflected in the wide range of the capsule thickness data observed earlier, and that the range decreased with time (Fig. 3).

3.2. Reactive capsule

The decrease in capsule thickness over time was not statistically significant (Fig. 3). The only statistically significant difference in capsule thickness was detected at the 12 wk time point, where titanium implants had a less foreign-body capsule as compared to PTFE ($p = 0.024$).

3.3. Cell distribution

The total cellularity (including macrophages, fibroblasts and other cells) in the interfacial zone (the row of five squares along the implant surface) and in the measured capsule decreased over time for the titanium ($p = 0.001$; $p = 0.001$) as well as for the PTFE implants ($p = 0.0018$; $p = 0.0029$). The cellular counts for the interfacial zone and the measured capsule are depicted in Figs 4 and 5, respectively. The enumeration of the ED1 and ED2 subpopulations of macrophages did not show any statistically significant differences over time, or when titanium and PTFE were compared to each other. The only statistically significant difference when comparing titanium to PTFE was for the total cellular density in the measured capsule at 12 wk ($p < 0.017$) (Fig. 5).

4. Discussion

The present study was performed to evaluate to what extent the excellent clinical results with titanium as an implant material are due to its material characteristics. We therefore compared the tissue response to titanium with the response to PTFE, a well-established non-metallic implant. The implants were inserted in rat abdominal wall for 1, 6 and 12 wk. We

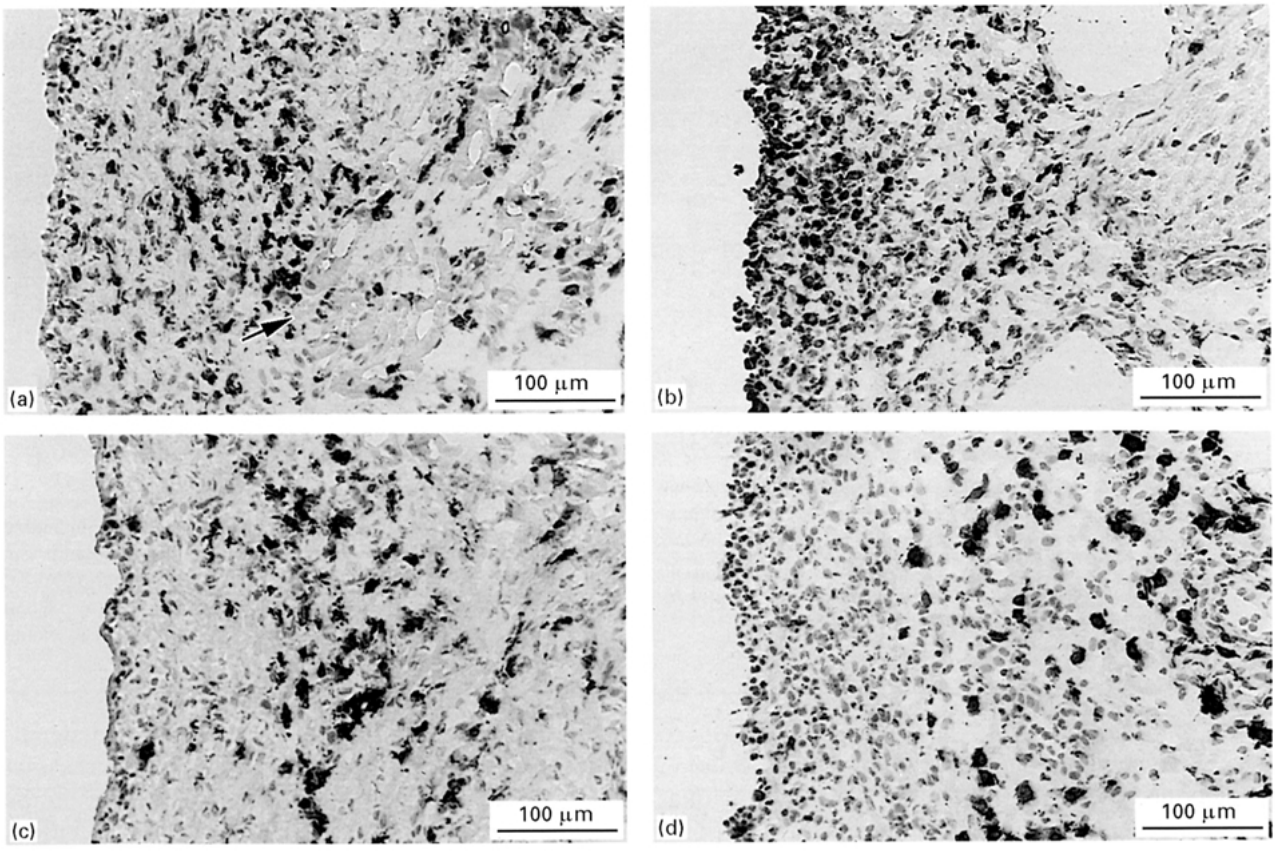

Figure 1 Microphotographs of immunohistochemically stained sections taken from the tissue adjacent to implants inserted in the rat abdominal wall for 1 wk. The specific staining appears black and cell nuclei are grey. The removed implant was to the left. (a) Stained for EDI cells, c.p. titanium (arrow indicates muscle border); (b) stained for ED1 cells, PTFE; (c) stained for ED2 cells, c.p. titanium; (d) stained for ED2 cells PTFE.

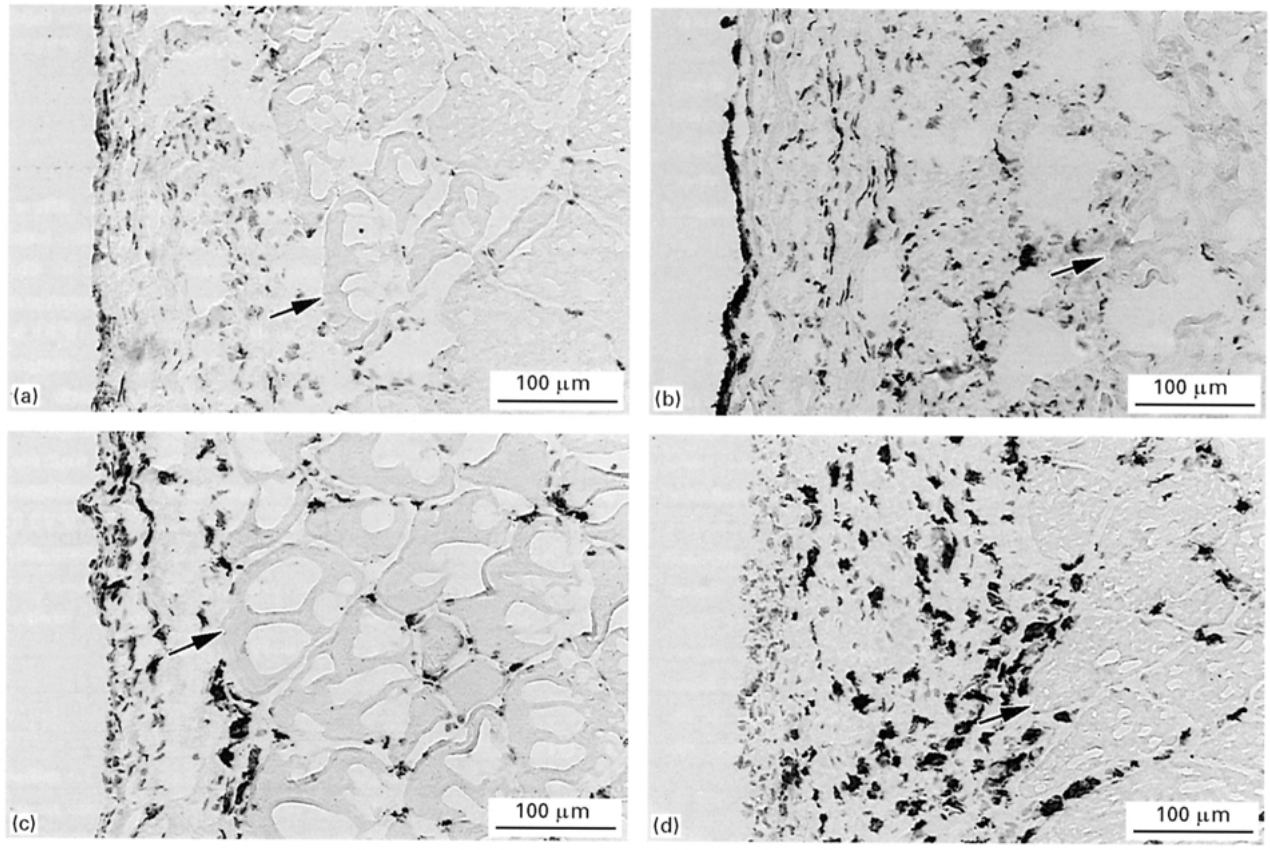

Figure 2 Microphotographs of immunohistochemically stained sections taken from the tissue adjacent to implants inserted in the rat abdominal wall for 12 wk. The specific staining appears black and cell nuclei are grey. The removed implant was to the left. The arrow indicates the muscle border. (a) Stained for ED1 cells, c.p. titanium; (b) stained for ED1 cells, PTFE; (c) stained for ED2 cells, c.p. titanium; (d) stained for ED2 cells, PTFE.

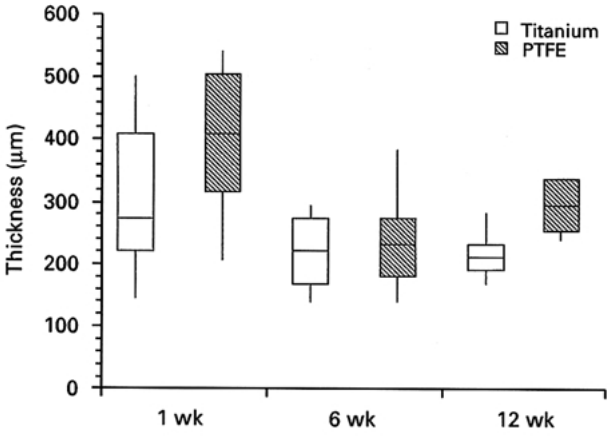

Figure 3 Box plot of the capsule thickness after 1, 6 or 12 wk implantation for c.p. titanium and PTFE implants in the rat abdominal wall ($n = 8$ for each time point and implant type). The box represents the 25 and 75 percentiles and the median value is indicated by the horizontal line within the box. The 10 and 90 percentiles are indicated by the vertical lines.

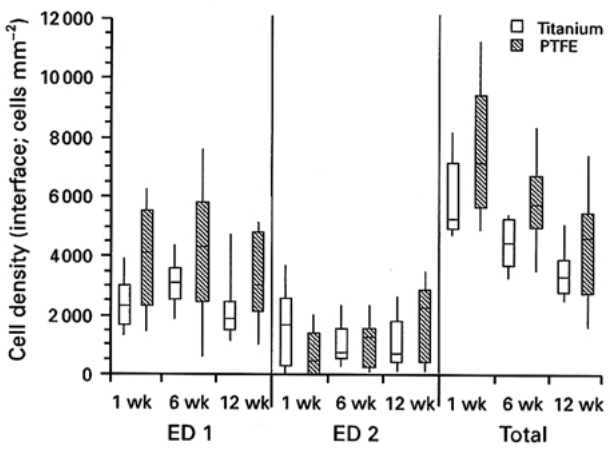

Figure 4 Box plot of the cell density at the interface enclosing the ED1 and ED2 positve cells as well as total cell numbers (including macrophages, fibroblasts and other cells) 1, 6 or 12 wk for c.p. titanium and PTFE implants ($n = 8$ for each time point and implant type). The box represents the 25 and 75 percentiles and the median value is indicated by the horizontal line within the box. The 10 and 90 percentiles are indicated by the vertical lines.

used solid PTFE to avoid the issue of porosity and to be able to compare the present findings with those of protein distribution using the same two types of material and implantation periods [7].

The cellular response was quantified by measuring the total number of cells and macrophage subsets throughout the reactive capsule as well as in the interfacial area close to the implant. The total cell numbers at the interface and in the capsule decreased significantly over time for both materials. There were, however, no significant material-related differences in the number of total cells or macrophage subpopulations at the early time periods (1 and 6 wk). Further, there was no difference in capsule thickness at these early time points. At the 12 wk time point, there was a statistically significant material-related difference for both

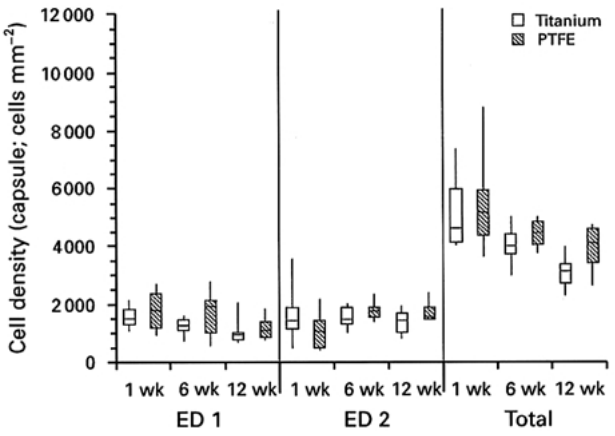

Figure 5 Box plot of the cell density in the reactive capsule enclosing the ED1 and ED2 positive cells as well as total cell numbers (including macrophages, fibroblasts and other cells) at 1, 6 or 12 wk for c.p. titanium and PTFE implants. ($n = 8$ for each time point and implant type). The box represents the 25 and 75 percentiles and the median value is indicated by the horizontal line within the box. The 10 and 90 percentiles are indicated by the vertical lines.

capsule thickness and total cell density but not with respect to macrophage subsets. Thus, PTFE induced only a thicker capsule and higher total cell number compared to that of titanium at the latest investigated time point. It was noted that there were no significant changes in capsule thickness over time, which is in accordance with Therin *et al.* [20] who reported that the foreign-body membrane thickness is a stable parameter for metallic and ceramic materials.

One possible explanation for the lack of significant differences at early time points could be that material-specific tissue responses are overshadowed in the early phase by other inflammatory factors, like the surgical trauma and those imposed by the stiff implant in the flexible living tissue. These factors may subside with the reorganization and adjustment of the foreign-body capsule making material-specific properties relatively more important at later times.

The present study indicates that the cellular density differences found at later time points between titanium and PTFE are due to other cell types than monocytes/macrophages, because no significant changes could be detected in the numbers of ED1 or ED2 positive cells. However, this does not rule out the importance of the monocyte/macrophages for the biocompatibility of an implant, because differences in macrophage number may not be obligately related to macrophage function. It is conceivable that the monocytes/macrophages close to the PTFE implants were functionally altered compared to titanium implants, which resulted in total cell number differences at later time points for the two materials tested.

The regulating mechanisms for the recruitment of macrophages to implants are at present not clearly understood. An established idea is that the composition of proteins at the implant surface and in surrounding tissue will influence further cell response [21–23]. The accumulated proteins may modify the functional response of the macrophages such as the metabolic activation, phagocytosis and secretion of mediators, which in turn influence secondary events in the reactive tissue adjacent to the implant [24]. Different materials have been shown to induce different protein adsorption patterns as well as cell responses *in vitro*. Recently, we have shown that it is possible to investigate the protein localization in a satisfactory preserved tissue-to-implant topography *in vivo* [25]. In the following study it was demonstrated that the distribution of extracellular proteins, especially fibronectin and collagen I varied between titanium and PTFE at the 6 and 12 wk implantation time point [7]. The differences in the protein distribution were mainly that fibronectin and collagen I were located at a further distance from the implant surface for PTFE implants compared to titanium implants. Thus, there was a wider unreactive cellular zone (not stainable for the proteins) for PTFE implants. It is tempting to speculate that the total cellularity differences detected in the present study, and the protein distribution pattern in our previous study [7] are related. It is not inconceivable that the differences in total cellular density (which will include fibroblasts) will elicit differences in protein distribution and it is also possible that the chain of events is the opposite. The question is, what factors or mechanisms triggered these events?

In conclusion, solid implants of PTFE will induce an increased soft-tissue response compared to titanium. The differences are relatively small and will appear at later time points. They are probably material-specific, but the underlying cellular and molecular mechanisms are, at present, largely unknown.

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References

1. K. M. HOLGERS, P. THOMSEN, A. TJELLSTROM and L. E. ERICSON, *Biomaterials* **16** (1995) 83.
2. P.-I. BRÅNEMARK, J. LAUSMAA, L. E. ERICSON, P. THOMSEN, R. BRÅNEMARK and R. SKALAK, in "Reconstructive preprosthetic oral and maxillofacial surgery" edited by R. J. Foneseca and W. H. Davies (Saunders, Philadelphia, PA, 1995) p. 165.
3. A. S. ERIKSSON and P. THOMSON *Biomaterials* **12** (1991) 827.
4. T. RÖSTLUND, P. THOMSEN, L. M. BJURSTEN and L. E. ERICSON, *J. Biomed. Mater. Res.* **24** (1990) 847.
5. J. A. HUNT, K. R. ABRAMST and D. F. WILLIAMS, *Anal. Cell. Pathol.* **7** (1994) 43.
6. J. M. BELLÓN, J. BUJÁN, L. CONTRERAS and A. HERNANDO, *Biomaterials* **16** (1995) 381.
7. A. ROSENGREN, B. R. JOHANSSON, N. DANIELSEN, P. THOMSEN and L. E. ERICSON, *ibid* **17** (1996) 1779.
8. L. TANG and J. W. EATON, *Am. J. Clin. Pathol.* **103** (1995) 466.
9. J. M. ANDERSON and K. M. MILLER, *Biomaterials* **5** (1984) 5.
10. J. M. DAVIDSON, in "Inflammation: Basic principles and clinical correlates" edited by J. I. Gallin, I. M. Golstein and R. Snyderman (Raven Press, New York, NY, 1992) p. 809.

11. C. D. DIJKSTRA, E. VAN VLIET, E. A. DÖPP, A. VAN DER LELIJ and G. KRAAL, *Immunology* **55** (1985) 23.
12. C. D. DIJKSTRA, E. A. DÖPP, P. JOLING and G. KRAAL, *ibid* **54** (1985) 589.
13. D. G. VINCE, J. A. HUNT and D. F. WILLIAMS, *Biomaterials* **12** (1991) 731.
14. J. A. HUNT, P. J. DOHERTY, D. ANNIS and D. F. WILLIAMS, *ibid.* **13** (1992) 651.
15. D. CAMPOCCIA, J. A. HUNT, P. J. DOHERTY, S. P. ZHONG, M. O'REGAN, L. BENEDETTI and D. F. WILLIAMS, *ibid.* **17** (1996) 963.
16. A. ROSENGREN, N. DANIELSEN and L. M. BJURSTEN, *ibid.* **18** (1997) 979.
17. J. M. BELLÓN, J. BUJÁN, L. A. CONTRERAS, A. HERNANDO and F. JURADO, *J. Biomed. Mater. Res.* **31** (1996) 1.
18. P. THOMSEN, L. M. BJURSTEN and L. E. ERICSON, *Scand. J. Plast. Reconstr. Surg.* **20** (1986) 173.
19. S. SIEGEL and N. J. CASTELLAN, "Nonparametric statistics for the behavioural sciences" (McGraw-Hill, New York, 1988).
20. M. THERIN, P. CHRISTEL and A. MEUNIER, *J. Biomed. Mater. Res.* **28** (1994) 1267.
21. B. KASEMO and J. LAUSMAA, *CRC Crit. Rev. Biocompat.* **2** (1986) 335.
22. T. A. HORBETT and J. L. BRASH, in "Proteins at interfaces" edited by J. L. Brash and T. A. Horbett, (American Checop, ACS Symposium Series, Washington, DC, 1987) p. 1.
23. J. D. ANDRADE, in "Surface and interfacial aspects of biomedical polymers" edited by J. D. Andrade, (Plenum Press, New York, NY 1988) p. 1.
24. P. THOMSEN and L.E. ERICSON, in "The bone-iomaterial interface" edited by J. E. Davies, (University of Toronto Press, Toronto, 1991) p. 153.
25. A. ROSENGREN, B. R. JOHANSSON, P. THOMSEN and L. E. ERICSON, *Biomaterials* **15** (1994) 17.

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