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Biodistribution of titanium dioxide from biologic compartments

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Abstract The layer of titanium dioxide (TiO_2) of the implant is chronically exposed to the internal electrolyte milieu in the peri-implant biological compartment. Corrosion results from electrochemical attack and ensuing gradual degradation of the metallic materials and is thus of biological interest when these biomaterials are employed in clinical implantology. Herein we evaluated and compared the chronic effect and the biodistribution of TiO_2 administered subcutaneously or intraperitoneally. We propose that the compartmentalization of titanium in the area of subcutaneous injection would reproduce the biological compartment of the implant and its microenvironment from which metal ions could be released and migrate systemically. Potential TiO_2 deposits were identified and characterized in skin, liver and lung by histological and EDX analyses. After both treatments, the skin, liver, and lungs exhibited histological evidence of TiO_2 deposits. In order to characterize in situ macrophage-like cells, tissue sections were immunohistochemically stained for CD68. Tissue specimens from all organs assayed showed positive staining for anti-macrophage monoclonal antibody CD68 (PGM1). Despite the compartmentalization of titanium within nodular areas in rats treated subcutaneously, systemic migration occurred. We concluded that systemic migration of TiO_2 occurred regardless of the administration route.

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1 Introduction

Titanium implants are widely used in dental, orthopedic, and cardiovascular therapy and in restorative and plastic surgery [1]. Titanium is a very reactive metal that develops a layer of titanium dioxide (TiO_2) on its surface when it comes into contact with air and/or liquids. This layer delimits the interface between the biological milieu and the implant, passivating it and partially avoiding corrosion [2–4].

The layer of TiO_2 of the implant is subjected to prolonged exposure to the internal electrolyte milieu in the peri-implant biological compartment. Corrosion results from electrochemical attack and ensuing gradual degradation of the metallic materials and is thus of biological interest when these biomaterials are employed in clinical implantology [2]. When a metal implant fails due to multiple local and/or systemic causes [5, 6], an inflammatory granulation tissue or a fibrous layer may develop in the peri-implant area. Several studies have reported the presence of titanium particles in tissues in the vicinity of failed dental or orthopedic implants. Corrosion is one of the possible causes of implant failure [7–11].

Williams and Williams [12] established that "whether noble or passivated, all metals will suffer a slow removal of ions from the surface, largely because of local and temporal variations in microstructure and environment. This need not to be continuous and the rate may either increase or decrease with time, but metal ions will be released into that environment upon prolonged implantation".

The release of particles/ions from the metal structure of the implant to the surrounding biological compartment, their biodistribution in the organism and their final destination are issues that lay at the center of studies of biocompatibility and biokinetics. These particles/ions can be phagocytosed by macrophages, remain free in the intercellular spaces of the peri-implant biological compartment [9], or spread systemically to other target organs. Within this context, Urban et al. [13] showed dissemination of metallic particles from hip and knee replacements to organs such as liver, spleen and lymph nodes in 27 autopsies.

We previously showed that TiO_2 administered intraperitoneally disseminates systemically to liver, spleen and lung [14–16]. However, to the best of our knowledge, no experimental studies have been performed to date employing a route of administration in which dissemination is greatly reduced or prevented altogether.

Within this context, the aim of the present study was to evaluate and compare the chronic effect and the biodistribution of TiO_2 administered subcutaneously or intraperitoneally.

2 Materials and methods

2.1 Chemicals

TiO_2 (Anatasa) and all histological dyes and reagents employed were purchased from Sigma-Aldrich Co., (St. Louis, MI, USA). Antibody CD 68 (clone PG-M1), polymer-alkaline phosphatase anti-mouse and anti-rabbit antibody were purchased from DAKO (Carpinteria, CA, USA).

2.2 Animal treatment

Male Wistar rats ($n = 20$) of $\sim$100 g body wt were divided into two groups. Rats were injected either intraperitoneally (i.p.) or subcutaneously (s.c.) with a single injection of a suspension of TiO_2 (Anatasa) at a dose of 1.60/100 g body weight in 5 ml saline solution. TiO_2 particles were sphere-like and about 1 μm in diameter.

At 12 months of treatment the animals were sacrificed and the skin at the injection site, liver and lung were analyzed macroscopically. Skin, liver and lung samples were routine processed for light microscopy observation.

The guidelines of the National Institute of Health (NIH; NIH Publication No. 85-23, Rev. 1985) and the Statement of Ethics Principles of the Faculty of Dentistry, University of Buenos Aires [Res (CD) 325/02 and Res (CD) 694/02] for the use and care of laboratory animals were observed.

2.3 Histological analysis

Skin, liver and lung were processed for histological evaluation. The samples were fixed in 10% formalin and routine processed for paraffin embedding and staining with hematoxylin-eosin or Grenacher Carmin to facilitate the identification of phagocytosed material. The samples were treated with picric acid to avoid the possible presence of formalin pigments.

2.4 Immunocytochemistry

To clearly identify the presence of phagocytic cells in skin, liver and lung, the expression of the monoclonal antibody to CD68 antigen, which has a high affinity and specificity for cells of the mononuclear phagocyte lineage, was detected by immunocytochemistry [17–19].

Formalin-fixed tissue sections prepared from skin, liver and lung blocks, were deparaffinized, rehydrated in distilled water and then incubated with monoclonal mouse anti-human CD68 PGM1 antibody (1:50 dilution) during 1 h at 20°C. Citric acid buffer (pH: 6) was used for antigen retrieval before the addition of mouse anti-human CD68 during 10 min. Tissue sections were incubated with labeled polymer-alkaline phosphatase antimouse and anti-rabbit antibody and revealed with Fast Red to visualize the reaction. After incubation, sections were washed in distilled water and counterstained with hematoxylin.

2.5 Microchemical analysis

The deposits in organs were chemically identified by EDX (energy dispersive X-ray analysis) using a Philips scanning electron microscope (Eindhoven, The Netherlands) equipped with an EDX system (EDAX Falcon PV 8200 [3.0], Mahwah, NJ).

3 Results

All the treated animals failed to show changes in body weight or behavior throughout the experimental period.

3.1 Macroscopic analysis

The animals injected i.p. exhibited widespread, whitish, diffuse deposits of TiO_2 in all intraperitoneal organs, in particular on the surface of the liver. No tissue changes were observed at the administration site. Conversely, when

TiO_2 was administered s.c., macroscopic examination revealed the presence of nodular areas with deposits of TiO_2 at the injection site (Fig. 1a–c). Skin dissection evidenced the presence of the injected material within a pseudomembrane with inwards projections (nodular area), creating an actual compartment (Fig. 2).

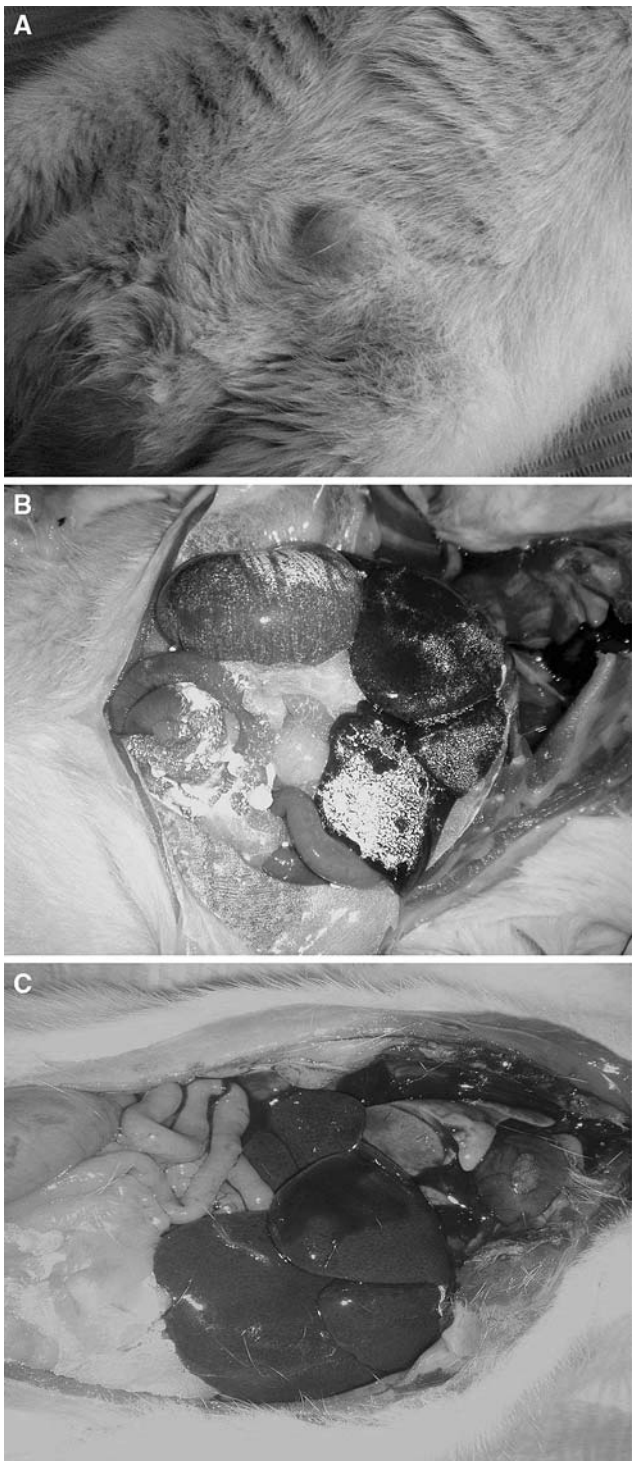

Fig. 1 (**a**) Nodular area at the site of subcutaneous injection. (**b**) Note the widespread distribution in the abdominal organs of the material injected i.p. (**c**) Note the absence of deposits in animals injected s.c.

3.2 Histological and immunocytochemical analysis

The histological analysis of the nodular areas of the skin of animals treated s.c., confirmed that the injected material was surrounded by a capsule of fibrous connective tissue that emitted projections towards the interior of the capsule (Fig. 3). The interior connective projections exhibited abundant blood vessels (Fig. 4a, b). At the deposit-capsule and deposit-projections interfaces we observed the presence of deposits of massive material, either free and/or phagocytosed by macrophage-like cells, and the absence of inflammatory infiltrate and multinucleated giant cells (Figs. 4a, b and 5). Conversely, when animals were treated i.p., no histological alterations were observed in the skin.

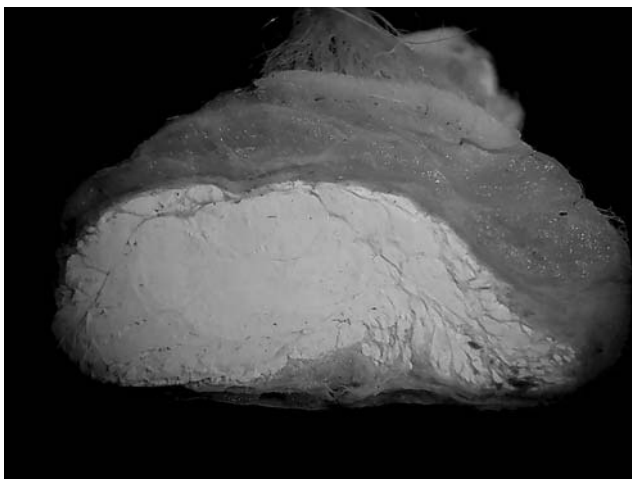

Fig. 2 Macroscopic view of the cutaneous nodular area. Note the material contained within a pseudomembrane that emits projections inwards

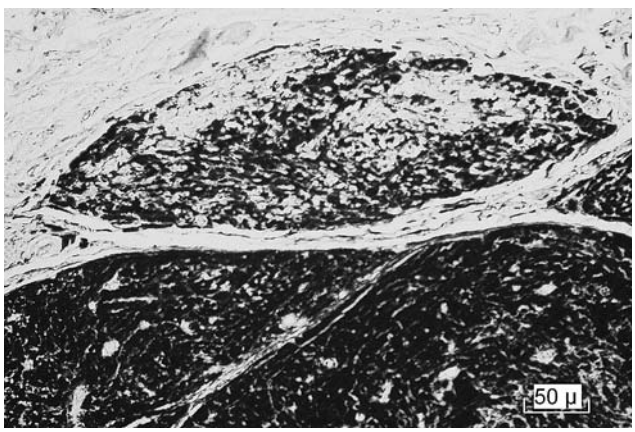

Fig. 3 *Nodular area*. Note the injected material in black within the fibrous capsule that emits projections inwards (hematoxylin-eosin; original magnification 50×)

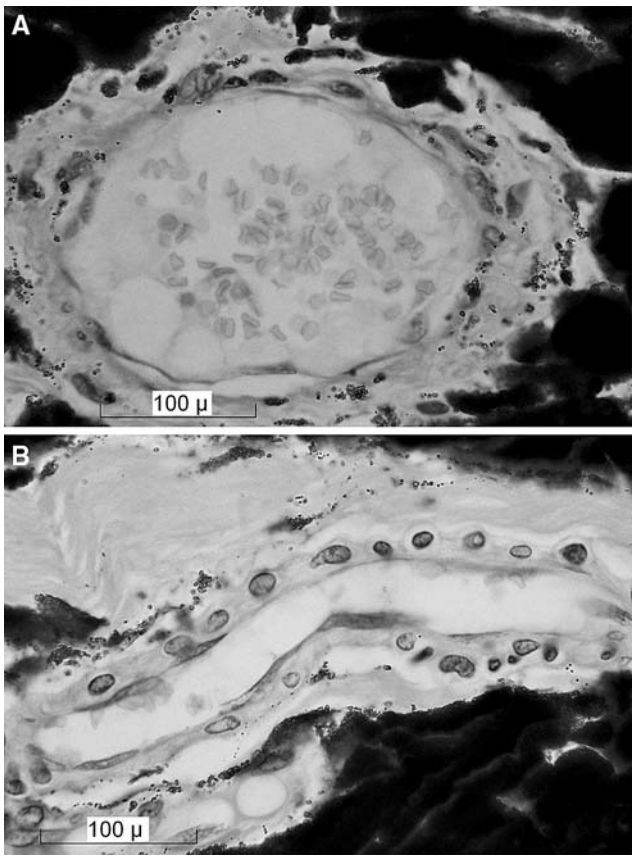

Fig. 4 (**a** and **b**) Angiogenesis in the inner walls. Note the presence of the injected material on the periphery of the blood vessels (Grenacher Carmin stain; original magnification 1,000×)

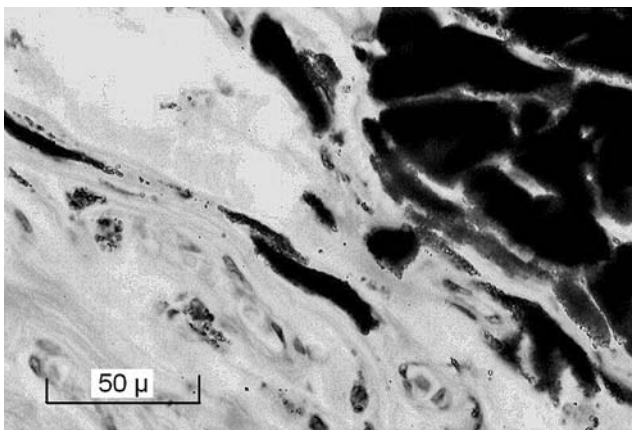

Fig. 5 *Interface material-capsule*. Note the detail of the fibrous capsule, the presence of material, free or phagocytosed by macrophages and the absence of inflammatory infiltrate (hematoxylin-eosin; original magnification 1,000×)

It is noteworthy that, regardless of the administration route employed (i.p. or s.c.), the histological analysis revealed the presence of deposits of material phagocytosed by cells of the mononuclear phagocytic system in the parenchyma of both liver and lung (Figs. 6 and 7). Phagocytosis in the liver was also performed by the hepatocytes themselves (Fig. 6). Moreover, histological analysis revealed that the amount of deposits in liver and lung was similar in both treated groups.

Tissue specimens from all organs assayed showed positive staining for anti-macrophage monoclonal antibody CD68 (PGM1). In the liver, macrophages were identified in the periphery and in the area corresponding to the central vein (Fig. 8a), whereas in the lung macrophages were distributed homogeneously in all the parenchyma (Fig. 8b). In the area of subcutaneous injection, macrophages were observed in the connective tissue of the capsule and amongst the deposits of material (Fig. 8c). Although the samples exhibited a widespread positive reaction, some negative areas were observed.

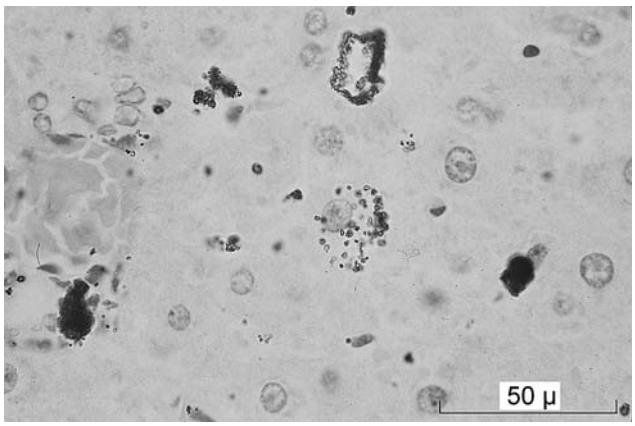

Fig. 6 *Liver*. The presence of material can be observed free or phagocytosed by macrophages. Note a prominent hepatocyte featuring a cytoplasm loaded with particles (Grenacher carmin stain; original magnification 1,000×)

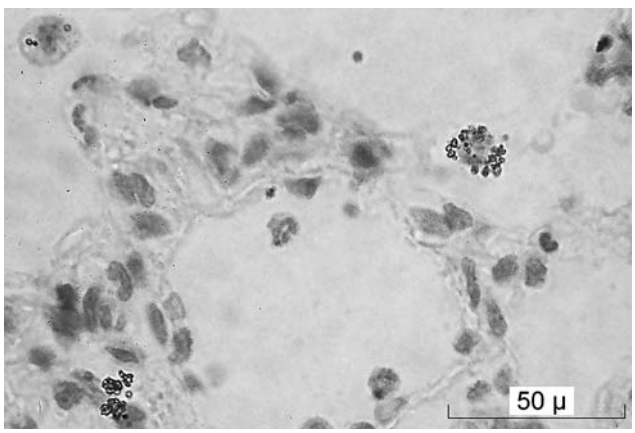

Fig. 7 *Lung*. Note the presence of alveolar macrophages loaded with particles in their cytoplasm (Grenacher carmin stain; original magnification 1,000×)

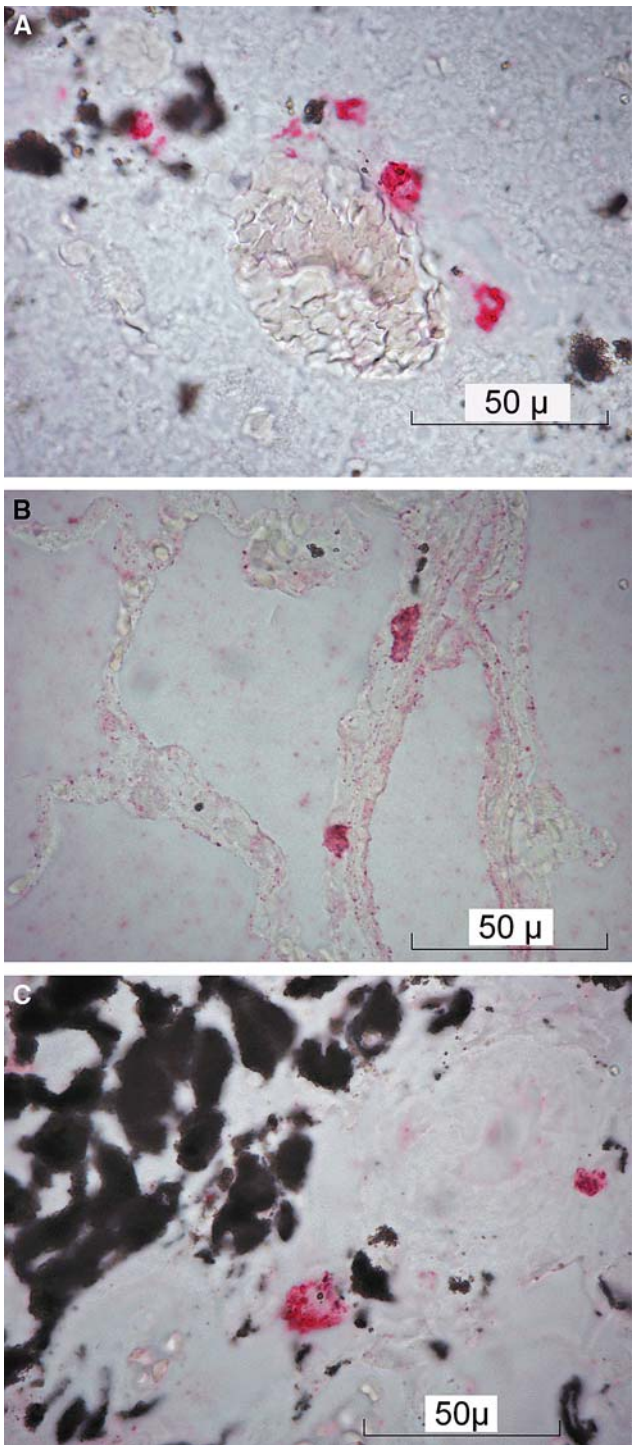

Fig. 8 *Antibody to CD68*. Photomicrographs show CD86-positive macrophage-like cells. (**a**) Liver, (**b**) Lung and (**c**) Skin (original magnification 1,000×)

3.3 Microchemical analysis

Analysis of the massive deposits by EDX confirmed the presence of titanium. Other elements naturally present in tissues such as Ca, Na, and Cl were also detected. The Ag peak corresponded to the metal employed for sample coating (Fig. 9).

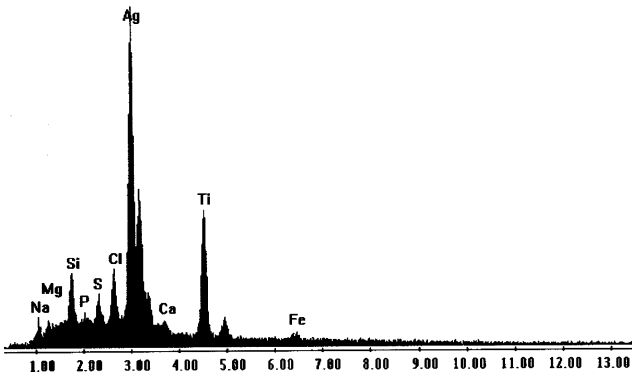

Fig. 9 *EDX*. Titanium spectrum corresponding to the organs under study

4 Discussion

All the metallic materials employed in surgery as permanent implants are liable, to a certain degree, to corrosion due to variations in the internal electrolyte milieu. The risk of clinical complications related to corrosion of implants in place today must be understood [2].

Biomaterials of potential clinical interest can trigger an inflammatory response at the material-tissue interface [20]. Tissue response to biomaterials depends on the chemical and physical properties of the implant [21]. Macrophages are cells that respond rapidly to in vivo implantation of a biomaterial, including metals, ceramics, cement, and polymers. Their response depends mainly on the size and structure of the material, among other variables [20–24]. Those particles that are smaller than the macrophages themselves (<10 µm) can be easily phagocytosed. However, the larger particles (10–100 µm) are ingested by giant, multinucleate cells [25].

The biomaterial employed in the present study was composed of 1 µm in diameter particles. It is well known that the particles can be free or form agglomerates [26, 27], favoring the appearance of giant cells. However, in the present study we found these particles free in the interstitial spaces and not only within phagocytes like CD68+ macrophages, but also in the cytoplasm of hepatocytes.

As we have previously shown [14], i.p. injection of TiO_2 resulted in widespread distribution of the material in the abdominal area, with deposits in liver, spleen and lung. In that study we employed different experimental doses (0.0016 g, 0.16 g or 1.6 g TiO_2/100 g BW). With the lower doses (0.0016 g and 0.16 g TiO_2/100 g BW) we observed titanium deposits at a histological level, particularly in blood-filtering organs such as liver and spleen. However, in

the case of other target organs such as lung, the deposits (particularly those found in alveolar macrophages) were optimally visible at the higher dose (1.6 g TiO_2/100 g BW). Although this is a high dose in terms of a "normal" in vivo situation, our group develops experimental pathology models with doses that allow for the rapid observation of the adverse effects in tissues of different materials.

In patients bearing large coxofemoral implants, the implant surface exposed to the bioenvironment is significant, increasing the probability of corrosion and release of ions/particles. The conditions of the bioenvironment are also relevant, in particular when a granulomatous process induces a fall in local pH, favoring corrosion even more. Jacobs et al. [28] found in a retrospective study that the concentration of titanium in the serum of 21 patients who had a loose titanium containing total hip-replacement component was increased approximately twofold compared with the level in 21 controls.

We have previously shown at shorter experimental times—1 and 3 months post-i.p. injection—with the same dose employed herein (unpublished data), titanium deposits in organs with marked macrophagic activity such as liver, spleen and lung, in keeping with the fact that macrophages respond rapidly to in vivo implantation of a biomaterial. We must point out that, according to our findings, the minimum time for histological observation of TiO_2 deposits simultaneously in the three organs we routinely evaluate in our studies (liver, spleen and lung), is 3 months post-injection.

The biological response of TiO_2 is clearly evident as from 6 months post-intraperitoneal injection. Because the metal implants employed in Orthopedics and Dentistry remain in the patient's body for long periods of time, we evaluated the chronic biological effect of TiO_2, prolonging the experimental time to 12 months ($\sim$1/3 of the animal's life).

It is known that titanium implants form a passivating oxide layer composed mainly of anatase and rutile. According to Effah et al. [29] after 4 weeks of immersion in simulated physiological fluids, this oxide layer consists of anatase alone or anatase and rutile. Moreover, Sul et al. [30], showed the influence of oxidized implants on bone response working with titanium oxide crystals composed of anatase and mixtures of anatase and rutile. It is noteworthy that TiO_2 with crystallographic features of *rutile* compared to TiO_2 with *anatasa* crystallography showed better biocompatibility [16]. Taking into account that the TiO_2 with crystallographic features of anatasa is always found on the implant surfaces, we consider that the studies with TiO_2-*anatasa* have added relevance. Moreover, we employed TiO_2-*anatasa* to evaluate potentially greater adverse effects than with TiO_2-*rutile* in a chronic time-frame.

Interestingly, the present study showed that when TiO_2 was injected s.c., despite the fact that the material was contained by a fibrous capsule at the site of injection, systemic migration was observed from this biological compartment.

The histological analysis of the organs revealed that the titanium particles were phagocytosed by cells of the mononuclear phagocyte system. Although macrophages can be identified by light microscopy analysis of hematoxylin-eosin stained sections, we employed the antibody to CD68 for specific immunohistochemical identification of this cell type.

Rae [31] reported that peritoneal macrophages and fibroblasts exposed to pure titanium released small and significant amounts of lactate dehydrogenase, suggesting the induction of some degree of cell damage which in turn, could result in cell lysis and death. It seems likely that the cycle of death and re-phagocytosis of titanium particles by macrophages induces immuno-inflammatory responses and fibrous encapsulation [32]. Specifically, engulfing macrophages release cytokines such as IL-1, b-FGF and TGF-β that are potent fibroblastic stimulators of the production of more collagen [21, 33, 34].

The monoclonal antibody to CD68 antigen has a high affinity and specificity for cells of the mononuclear phagocyte lineage [17, 18]. Although CD68-positive macrophages were observed in all organs, the range of reaction intensity revealed different degrees of expression. This heterogeneity in reaction intensity could be due to variations in the maturation state of the macrophages [35] and/or in the degree of activation induced by different stimuli such as phagocytosis [36]. CD68 could be expressed in certain conditions and thereby macrophages subsets. Thus, the phenotype of these cells depends upon the nature of the stimulus [37].

The angiogenesis observed in the inner fibrous walls of the capsule conceivably allowed for systemic dissemination of the particles and deposition in the target organs under study. The biodistribution of the particles administered by both routes could have occurred by cells of the monuclear phagocytic lineage as we have previously shown [38] and/or by blood plasma [28, 39–41]. Although the deposits of titanium in liver and lung were not quantified, histological observation revealed they were similar in both treated groups. This finding would suggest that the levels of titanium transported in plasma would be similar regardless of the administration route (i.p. and s.c.).

Although we did not actually identify lymph vessels, the particles may have migrated by this potential dissemination route as previously described by other authors [13, 42].

In the liver, the systemic biodistribution of titanium resulted in the presence of TiO_2 particles both in macrophages and hepatocytes. Previous studies have evidenced

the phagocytic capacity of these cells [43, 44] and their selective phagocytosis [44]. The accumulation of particles in the liver could compromise liver function as described by Urban et al. [13]. These authors associated the presence of titanium particles in a patient to granulomatous reactions and hepatomegalia. Various studies reported the presence of macrophages related to failed prostheses, be they orthopedic or odontological [9, 10, 13, 45, 46].

Our results allow us to postulate that titanium compartmentalization at the site of subcutaneous injection could be considered as the biological compartment of an implant and its micromilieu from which ions/metal particles could be released, remain in the peri-implant milieu or migrate systemically.

Dental implants, and particularly orthopedic implants, are often performed in elderly patients who frequently exhibit mild underlying disorders. We must stress that their condition must be taken into account because the effect of particles that result from a corrosion process could have clinical implications.

Evidently, implant corrosion would not be restricted to a local problem. The fact that the evolution of metallic particles in the different target organs is to date unknown warrants future studies.

5 Conclusions

The biological effects of implant corrosion would not be restricted to a local phenomenon. Titanium compartmentalization at the site of subcutaneous injection could be considered as the biological compartment of an implant and its micromilieu from which ions/metal particles could be released, remain in the peri-implant milieu or migrate systemically.

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