"IN VITRO" COMPARATIVE IMMUNE EFFECTS OF DIFFERENT TITANIUM COMPOUNDS.

L. DI GIAMPAOLO[1], M. DI GIOACCHINO[1], J. PONTI[2], E. SABBIONI[2], M.L. CASTELLANI[3], M. REALE[3], E. TOTO[1], N. VERNA[1], P. CONTI[3], R. PAGANELLI[1] and P. BOSCOLO[1*]

[1]Department of Medicine and Science of Ageing, Section of Occupational Medicine, Allergy and Clinical Immunology, G. D'Annunzio University, Via dei Vestini, 66013 Chieti, Italy. [2]European Commission, Institute for Health and Consumer Protection, ECVAM Unit, Ispra, Italy. [3]Department of Oncology and Neurosciences, Immunology Division, G. D'Annunzio University, Via dei Vestini, 66013 Chieti, Italy

Exposure to Ti compounds is today an occupational and environmental health hazard. Object of this study was to determine "in vitro" effects of different Ti salts on cultured human peripheral blood mononuclear cells (PBMC) proliferation and cytokine release. 10^{-4} and 10^{-7} M Ti compounds did not modify spontaneous PBMC proliferation. Ti dioxide (a biocompatible material and sunscreen component) did not exert effects on phytoemagglutinin (PHA) stimulated PBMC proliferation and on PHA stimulated IFN-γ and TNF-α release from PBMC. On the other hand, 10^{-4} M Ti oxalate (with wide industrial applications) and Ti ascorbate (used mainly in agriculture) inhibited about 70 % the PHA stimulated PBMC proliferation; both these Ti compounds at 10^{-4} and 10^{-7} M concentrations significantly inhibited TNF-α release, while only Ti oxalate inhibited that of IFN-γ. Titanocene (used in chemotherapy) did not exert effects on PBMC proliferation but markedly inhibited IFN-γ and TNF-α release. On the whole, this study demonstrates that Ti dioxide is not immunotoxic; Ti oxalate shows marked immunotoxicity; titanocene exerts selective toxicity on cytokine release but not on PBMC proliferation, while Ti ascorbate affects TNF-α release from PBMC but not IFN-γ release. In conclusion, these data show that immunotoxicity of Ti depends on speciation.

Titanium (Ti) and its compounds play an important role in occupational and environmental exposure. Ti is a component of hard alloys used in aircraft and car industries, in house building and in other activities. Ti oxalate is mainly used in the production of paints and pigments; Ti ascorbate is utilized in agriculture; Ti dioxide is not only a component of orthopedic and orthodontic prothesis but also of sunscreens, while other Ti compounds (e.g. titanocene) are used as chemotherapeutic agents for treatment of cancer diseases.

Ti ascorbate and other Ti compounds are utilised in agriculture both as foliar spray to plants (e.g. tomato) and as fertilizers added to the root zone; they either exert bacteriostatic and bactericidal effects or act as a fertilizers playing a role in the nutrition of crop plants (1,3)

Ti was shown to form biocomplexes with cellular constituents (4); rats i.p. injected with Ti as [^{44}Ti] ammonium oxalotitanate showed increased Ti in tissues (mainly spleen, femur and kidney) after 19 days from the injection; at this time Ti was excreted via urine and feces and plasma; Ti (mainly associated with proteins) was three times lower than after 16 hours from the treatment; Ti compounds were found bound both to macromolecules of the liver cytosol and to plasma proteins. In another study on rats

Key words: titanium, lymphocytes, cytokines, occupational exposure, environmental exposure

Mailing address: Prof. Paolo Boscolo, Medicina del Lavoro, Università "G. D'Annunzio", Via dei Vestini, 66013 Chieti Scalo (Italy) Tel and Fax: +39 871 3556704; e-mail boscolo@unich.it

0394-6320 (2004)

exposed to Ti ascorbate, it was shown that this compound mainly existed as complex with transferrin and that ^{45}Ti accumulated in inflamed regions of the kidney (5).

Different investigations demonstrated that occupational exposure to Ti dioxide and Ti tetrachloride may induce respiratory disorders including pulmonary fibrosis as well as plaques and diffuse thickening of the pleura; however a role of occupational Ti exposure in inducing lung cancer was not found in several studies (6-8).

Ti and Ti dioxide were evaluated as biocompatible materials (9). Implants containing T did not present corrosion effects unless fluoride compounds were present (10); maxillo-facial miniplates were well tolerated for up to 13 years (11). However, periprothesic inflammation with consequent ostheolisis was described after long-term from the implant of the prothesis (12). In this regard, it was demonstrated that Ti modulates "in vitro" the release of bone associated cytokines by human peripheral blood mononuclear cells (PBMC) (13); moreover, Ti inhibited both proliferation of T and B lymphocytes and production of immunoglobulins in mice both *"in vitro"* and *"in vivo"*(14).

There was concern for the utilization of Ti dioxide as main component of sunscreens and cosmetic creams since it was shown that this compound could cause oxidative damage to nucleic acid through free radical formation under photoxidation (15). Other studies showed that Ti dioxide exerts properties of photocathalitic "killing" under aerial conditions; this was increased by the superoxide dismutase (16). It was also demonstrated that Ti dioxide induced crossing-over and micronuclei formation in hamster ovary cells (17). Another study demonstrated that Ti dioxide may induce necrosis of tumor cells by modifying the Ca^{2+} metabolism (18).

Several compounds containing Ti, as a metal center, exert a wide spectrum of antitumor effects. These Ti compounds (including titanocene) have been used in chemotherapy for 20 years (19); the high affinity of Ti for phosphate groups may also be important for explaining its biological activity (20).

Although it is known that Ti compounds exert immune effects, there are no studies for comparing the immunotoxicity of different Ti compounds. Object of this investigation are the immune effects of four Ti compounds with a role in occupational and environmental exposure.

MATERIALS AND METHODS

Isolation of human peripheral blood mononuclear cells (PBMC)

Nine healthy men volunteers (mean age 34 years, range 24-58 years) were recruited for this study. They were not taking any drug and showed routine blood analyses in the normal range. Fasting EDTA-treated whole blood samples were obtained from each subject at 8 a.m. and human peripheral blood lymphocytes (PBMC) were purified by Ficoll-Hypaque (BioSpa Milan, Italy) density gradient centrifugation, 20 min at 400 x g. After three washings with Hank's balanced solution (HBSS), PBMC were resuspended in RPMI 1640 added 10 % FCS, 2 mM L-glutamine, 25 mM HEPES, 100 U/ml penicillin and 100 μg/ml streptomycin (Sigma Chem. Co., Italy). The culture is designated as complete medium.

Cell proliferation

PBMC were suspended at 10^6 cells/ml in complete medium. 100 μl aliquots of cell suspension were placed in each well of a standard 96-well microtiter plate (Falcon, St. Louis, MO, USA). Cells were then incubated at 37°C in humidified atmosphere with 5% CO_2 for 78 hours in the following conditions:

a) no other reagent added (control sample) .
b) with 20 μg/ml phytohemagglutinin (PHA) (Sigma Chem. Co., Italy) only.
c) with 10^{-4} or 10^{-7} M Ti dioxide (TiO_2), Ti ascorbate $(C_2H_5)_2TiCl_2$, Ti oxalate $TiO(C_2O_4)_2$ and titanocene (Alfa Aesar, Cologno Monzese, Milano, Italy) both in presence and absence of PHA.
d) with 10^{-4} or 10^{-5} M oxalic acid both in presence and absence of PHA.

Ti salts were added from the 10^{-3} and 10^{-6} M stock solutions previously prepared. None of the reagents used contained endotoxin, as judged by Limulus amebocyte assay (minimum detection level 0.1 μg/ml).

Enzymatic immunoassay for the quantification of cell proliferation.

Cell proliferation was evaluated using 5'-bromo-2'-deoxiuridine (BrdU) cell proliferation assay

(Oncogene research products, Darmstadt, Germany). BrdU was added to wells of the microtiter plate during the final 24 hours culture. Cells were fixed and permeabilized as well as the DNA was denaturated by treatment for 30 min at room temperature with fixative/denaturing solution. Anti-BrdU monoclonal antibody was pipetted into the wells and allowed to incubate for one hour. Unbound antibody was washed away and horseradish peroxidase-coniugate goat anti-mouse was added for 30 min at room temperature. Contents of wells were removed by inverting over sink and tapping on paper towels. Chromogenic substrate solution tetramethylbenzidine (TMB) was added to each well and incubated in the dark at room temperature for 15 min. Stop solution was added to each well in the same order as the previously added substrate solution. All reagents were provided with the kit and used following the manufacturer's instructions.

All experiments were performed in triplicate. Absorbance in each well was measured using a spectrophotometric plate reader at dual wavelengths of 450-540 nm. The color intensity was proportional to the amount of incorporated BrdU in the cells and this to the degree of cell proliferation.

Production and Measurement of cytokines.

Cultures were set up in 1 ml/well 24-wells Costar plastic plates, using 0.8 ml of PBMC containing 10^6 cells/well in complete medium, and the following conditions:

a) no other reagent added (control sample)
b) with 10 μg/ml phytohemagglutinin (PHA) (Sigma Chem. Co., Italy) only.
c) with 10^{-4} or 10^{-7} M TiO_2, Ti ascorbate $(C_2H_5)_2TiCl_2$, Ti oxalate $TiO(C_2O_4)_2$ and titanocene (Alfa Aesar, Cologno Monzese, Milano, Italy) in presence of PHA

The cultures were then incubated at 37°C in humidified athmosphere with 5% CO_2 for 48 hours; cells were then checked for viability by trypan blue dye exclusion by inverted Leica microscope analysis. Supernatants were collected and stored at –70 °C in aliquots until analysis. Interferon (IFN) γ, and Tumor-Necrosis–Factor (TNF) α levels in culture supernatants were determined by Quantikine colorimetric ELISA kits (Benfer-Scheller, Key-Stone Laboratories, USA) following the manufacturer's instructions. All experiments were performed in triplicate.

Statistical analysis of the data.

Statistical analysis was performed with Statistica, Release 4.5. Kolmogorov-Smirnov tests was used to shown the data distribution.

RESULTS

Kolmogorov-Smirnov tests showed that most of the data conformed to a non-parametric distribution. In particular, the values of Ti salts conformed more to a normal distribution expressed as % (in relation to the corresponding controls without Ti compounds) than as absorbance units.

The values of spontaneous and PHA stimulated PBMC proliferation in presence of Ti salts are reported either in table 1 (expressed as absorbance units) or in table 2 (expressed as %). Table 2 reports the values of PBMC proliferation in presence of oxalic acid expressed as absorbance units, while table 3 reports those of the PHA stimulated IFN-γ and TNF-α release from PBMC expressed as %.

Cell Proliferation

Addition of 10^{-4} and 10^{-7} M Ti compounds to PBMC cultures did not modify their spontaneous proliferation (Tab.I). Non-significant changes were found in PHA-stimulated PBMC proliferation in presence of 10^{-4} and 10^{-7} M Ti dioxide and titanocene (Tab.I). On the other hand, 10^{-4} M Ti oxalate and Ti ascorbate inhibited about 70% PHA-stimulated PBMC proliferation (tab.I). Non-significant inhibitory effects were also found in PHA-stimulated PBMC in presence of all Ti compounds at 10^{-7} M concentration (Tab.I).

The stimulation index (S.I.) of proliferation (ratio between PBMC proliferation stimulated/ non-stimulated by PHA) was significantly inhibited only in presence of 10^{-4} M Ti oxalate and ascorbate (Tab. I)

10^{-4} M (but not 10^{-5} M) oxalic acid increased spontaneous PBMC proliferation, while both 10^{-4} and 10^{-5} significantly inhibited (about 30 %) the PHA stimulated PBMC proliferation (Tab. II).

Cytokine production.

The spontaneous TNF-α release from PBMC

Tab. I. *Spontaneous and PHA stimulated proliferation of PBMC incubated with and without 10^{-4} and 10^{-7} M Ti compounds.*

Spontaneous proliferation	10^{-4} M	10^{-7} M
Control without metal	176 ± 29	
Ti oxalate	190 ± 41	178 ± 34
Titanocene	179 ± 18	193 ± 22
Ti dioxide	183 ± 21	190 ± 15
Ti ascorbate	155 ± 12	167 ± 14
PHA stimulated proliferation		
Control without metal	1621 ± 166	
Ti oxalate	491,46 ± 355*	1566 ± 526
Titanocene	1493 ± 201	1654 ± 221
Ti dioxide	1598 ± 144	1594 ± 154
Ti ascorbate	439 ± 81 *	1679 ± 170
Stimulation index (S.I.) : ratio between PHA stimulated/spontaneous PBMC proliferation		
Control without metal	9.12 ± 1.73	
Ti oxalate	2.65 ± 2.15*	9.01 ± 1.67
Titanocene	8.4 ± 1.63	8.65 ± 1.34
Ti dioxide	8.91 ± 1.12	8.39 ± 1.79
Ti ascorbate	2.71 ± 1.21*	10.15 ± 1.02

Values (expressed as absorbance) are mean ± S.D.
Mann Whitney U test. Difference statistically significant in relation to the control cultures:
**p< 0.001.*

was (mean ± S.D) 78 ± 42 pg/ml and the PHA stimulated 1422 ± 351 pg/ml; the spontaneous IFN-γ release from PBMC was of the order of the detection limit (22 ± 11), while the PHA stimulated release was 1178 ± 284 pg/ml.

10^{-4} and 10^{-7} M Ti dioxide did not affect IFN-γ and TNF-α release from PBMC (Tab.III). On the contrary, 10^{-4} and 10^{-7} M Ti oxalate significantly inhibited both IFN-γ and TNF-α release with a dose-response effect (Tab. III). Titanocene affected cytokine release (as Ti oxalate) but it did not significantly inhibit IFN-γ release at 10^{-7} M concentration. Furthermore 10^{-4} and 10^{-7} M Ti ascorbate inhibited the release of TNF-α from PBMC but it did not affect IFN-γ release (Tab. III).

DISCUSSION

Ti dioxide did not show immunotoxicity also at high concentration (10^{-4} M), while Ti oxalate and ascorbate demonstrated to be more

Tab. II. *Spontaneous and PHA stimulated proliferation of PBMC incubated with and without 10^{-4} and 10^{-5} M oxalic acid*

	Controls	10^{-4} M	10^{-5} M
Without PHA	178 ± 25	237 ± 30*	181 ± 22
With PHA	1581 ± 192	1013 ± 132**	9985 ± 141**
Stimulation index	9.09 ± 1.45	4.48 ± 0.97**	5.65 ± 1.12**

Values (expressed as absorbance) are mean ± S.D.
*Mann Whitney U test. Difference statistically significant in relation to the control cultures: *p<0.01; **p< 0.001 .*

	10^{-4} M	10^{-7} M
TNF-α		
Ti oxalate	44.9 ± 27.9 **	63.6 ± 34.6 *
Titanocene	21.3 ± 27.5 ***	85.6 ± 12 *
Ti dioxide	93.6 ± 14.5	90.2 ± 14.2
Ti ascorbate	29.9 ± 10.2 ***	44 ± 13.6 **
IFN-γ		
Ti oxalate	8.16 ± 6 ***	61.6 ± 31.1 *
Titanocene	6.1 ± 5.8 ***	77.7 ± 31.3
Ti dioxide	86.4 ± 25.4	88.8 ± 17.2
Ti ascorbate	96.2 ± 9.3	96.1 ± 9.4

Tab. III. *Percentage (in relation to control cultures) of PHA stimulated TNF-α and IFN-γ release from PBMC stimulated by PHA in presence of 10^{-4} M e 10^{-7} M Ti compounds*
Values (expressed as percentage) are mean ± S.D.
TNF-α release from PHA stimulated PBMC is 1422 ± 351 pg/ml; PHA stimulated IFN-γ release from PHA stimulated PBMC is 1178 ± 284 pg/ml.
*Mann Whitney U test. Difference statistically significant in relation to the control cultures: **p<0.01 ***p< 0.001 .*

immunotoxic than titanocene. Therefore, the results of this study demonstrate that Ti dioxide can be considered a biocompatible material. At this regard, Ti oxide alloys showed lower cytotoxicity than intact Ti alloys with respect to survival or growth rates in either cells or chicken embryonic femurs (9). Ti dioxide layer on the surface of alloys used for implants was found to protect from an inflammatory response (21). A hydroxiapatite layer formed on an anodic Ti oxide film had also hardly any effect on LPS induced proliferation of human PBMC and IL-1a production by these cells (22). Therefore, a hyper-reactivity to the implant of Ti alloys may be mainly induced by non-specific mitogen activators stimulating monocytic and lymphoid cells (12).

Our study, demonstrating that Ti dioxide is not immunotoxic, suggests that sunscreens containing Ti provide a good alternative to chemical sunscreens in protection against ultraviolet (UV) B-induced immunosuppression

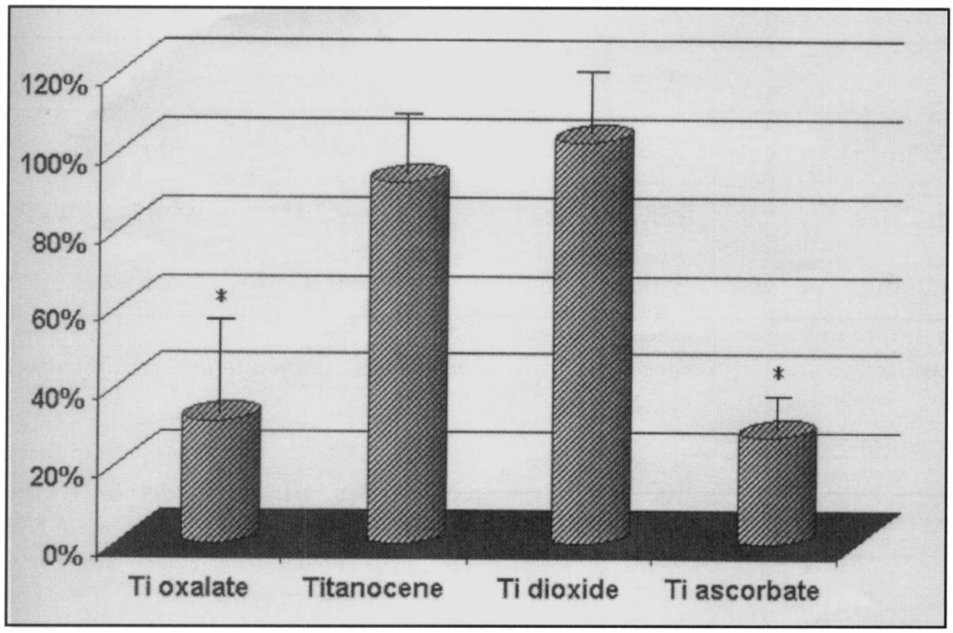

Fig.1 *Percentage (in relation to control cultures) of PHA stimulated proliferation of PBMC incubated in presence of 10^{-4} M Ti salts. Values (expressed as %) are mean ± S.D. Mann Whitney U test. Difference statistically significant in relation to the control cultures: *p<0.001.*

(23). However, it was shown that Ti dioxide could cause free radical formation under photoxidation (15) and exert a photocathalitic "killing" of tumor cells (16). Therefore, an accurate screening of the synthesis of sunscreens containing Ti is a prerequisite for their safe use (24). Moreover, it cannot be excluded that Ti compounds may exert adverse effects (including those carcinogenic) to the skin following long exposure to UV light .

Titanocene, at 10^{-4} and 10^{-7} M concentrations, inhibited IFN-γ and TNF-α production by PBMC, but did not inhibit PBMC proliferation. This result shows that titanocene, used as chemioterapic drug, can have a specific immunomodulatory effect, because PHA stimulated PBMC proliferation needs the activation of several metabolic mechanisms including involvement of IL-2 (25).

Ti oxalate, with a wide industrial utilization, presented a highly toxic effect. This compound inhibited PBMC proliferation at 10^{-4} M concentration and was able to reduce IFN-γ and TNF-α release both at 10^{-4} and 10^{-7} M. With regard to this, the immune effects of Ti oxalate may be in part induced by oxalic acid which (at 10^{-4} M concentration) enhanced spontaneous PBMC proliferation and inhibited PHA stimulated PBMC proliferation with a lower percentage (35 %) than Ti oxalate (70 %).

Ti ascorbate, largely used in agriculture, inhibited lymphocyte proliferation at 10^{-4} M concentration; this compound inhibited TNF-α release by PBMC, while it did not affect IFN-γ release. TNF-α is produced by a variety of cell types including T and B lymphocytes, NK cells, macrophages, astrocytes and dendritic cells. This cytokine is known to exert several important biological activities including those involved in septic shock and rheumatoid arthritis; in particular it was shown to be cytotoxic for many tumor cells also by stimulating apoptosis (26). On the other hand, IFN-γ production (mainly by T lymphocytes) may be considered the expression of a Th-1 cell response (27).

On the whole, this study demonstrates that Ti compounds have different effects on PBMC proliferation and cytokine production depending

on their chemical speciation. This may modify the absorption of this element into cells as well its intracellular metabolism and binding to intracellular components.

ACKNOWLEDGEMENTS

This study was supported by the EU contract No. 14660-1988-12F1ED ISP IT and by a 40% support (2003-2004) of the Italian Ministry for Education, University and Research (MIUR).

REFERENCES

1. **Ram N., M.Verloo and A.Cottenie.** 1988. Influence of foliar and soil-supplied titanium on tomato. *Acta Agronomica Hungarica. 37:43.*
2. **Yaghoubi S., C.W.Schwietert and J.P. McCue.** 2000. Biological roles of Titanium. *Biol. Trace Elem. 78:205.*
3. **Stefanovits-Banyai E., L. Simon-Sarkadi, K. Lesko, I. Kerepesi, E. Sardi and I. Pais.** 2001. Effects of titanium ascorbate on heavy matal induced stress-effects in plants. *3rd Int. Symposium on Trace Elem. in human: new perspectives.* Proceeding book: p.139.
4. **Edel J., E. Parafante and E. Sabbioni.** 1985. Retention and tissue binding of titanium in the rat. *Human Toxicol. 4:177.*
5. **Kimura S., K. Saitoh, M. Kawamura, T. Ido and K. Ishiwata.** 1990. In vivo studies on titanium ascorbate. *Proc of 4th Int Trace Elem. Symposium*, Budapest.Ed. I. Pais: p. 325.
6. **Garabrandt D.H., L.J. Fine, C. Oliver, L. Bernstein and J.M. Peters.** 1997. Abnormalities of pulmonary function and pleural disease among titanium metal production workers. *Scand. J. Work Health. 13:47.*
7. **Fayerweather W.E., M.E. Karns, P.G. Gilby and J.L. Chen.** 1992. Epidemiologic study of lung cancer mortality in workers exposed to Titanium Tetrachloride. *Amer. Occup. Environ. Med. 34:164.*
8. **Boffetta P., V. Gaborieau, L. Nadon, M.F. Parent, E. Weiderpass and J. Siemiatycki.** 2001. Exposure to titanium dioxide and risk of lung cancer in a population-based study from Montreal. *Scand. J. Work Environ. Health. 27:227.*
9. **Kanematu N., K. Shibata, S. Kurenuma, K. Watanabe, A.Yamagami, Y. Nishio and T. Fujii.** 1990. Cytotixicity of oxide anodized titanium alloy evaluated by cell and organic culture study. *J. Gifu. Dent. Soc. 17:583.*
10. **Strietzel R., A. Hosch, H. Kalbfleish and Buch D.**1998. *Biomaterials. 19:1495.*
11. **Landford R.J. and J.W. Frame.** 2002. Tissue changes adjacent to titanium plates in patients. *J. Craniomaxillofac. Surg. 30:103.*
12. **Kohilas K., M. Lyons, R. Lofthouse, C.G. Frondoza, R. Jinnah and D.S. Hungerford.** 1999. Effects of prostethic titanium wear debries on mitogen-induced monocyte and lymphoid activation. *J. Biomed. Res. 47:95.*
13. **Wang J.Y., D.T. Tsukayama, B.H. Wicklund and R.B. Gustilo.** 1996. Inhibition of T and B cell proliferation by titanium, cobalt, and chromium: role of IL-2 and IL-6. *J. Biomed. Mater. Res.32:655.*
14. **Wang J.Y., B.H. Wicklund, R.B. Gustilo and D.T. Tsukayama.** 1997. Prosthetic metals impair murine immune response and cytokine release in vivo and in vitro. *J. Orthop. Res. 15:688.*
15. **Wamer W.G., Y. Iun-Jie and W. Rong Rong.** 1997. Oxidative damage to nucleic acids photosensitised by titanium dioxide. *Free Rad. Biol. Med. 23:851.*
16. **Cai R., K. Hashimoto, Y. Kubota and A. Fushishima.** 1992. Increment of photocatalytic killing of cancer cells using TiO_2 with the aid of Superoxide Dismutase. *Chem Letters 27:427.*
17. **Pei-Ju Lu, I-Ching Ho and Te-Chang Lee.** 1998. Induction of sister chromatid exchange and micronuclei by titanium dioxide in Chinese hamster ovary-K1 cells. *Mutat. Res. 414:15.*
18. **Sakai H., E. Ito, R.X. Cai, T. Yoshioka, Y. Kubota, K. Hashimoto and A.Fujishima.** 1994. Intracellular Ca^{2+} concentration change of T24 cell under irradiation in the presence of TiO_2 ultrafine particles. *Biochim. Biophys. Acta. 1201:259.*
19. **Melendez E.** 2002. Titanium complexes in cancer treatment. *Crit. Rev. Oncol. Hematol. 42:309.*
20. **Guo M., Z. Guo and P.J. Sadler.** 2001. Titanium(IV) targets phosphoesters on nucleotides: implications for the mechanism of action of the anticancer drug titanocene dichloride. *J. Biol. Inorg. Chem. 6:698.*
21. **Eisenbarth E., D. Velten, K. Shenk-Meuser, P. Linez, V. Biehl, H. Duschner, J. Breme and H. Hildebrand.** 2000. Interactions between cells and titanium surfaces. *Biom. Eng. 19:243.*
22. **Takebe J., S. Itoh, T. Ariake, H. Shioji, T. Shioyama, K. Ishibashi and H. Ishizawa.** 1998. The effects of immunocytes of anodic oxide titanium after hydrothermal treatment. *J. Biomed. Mater. Res. 42:272.*
23. **Van Der Molen R.G., H.M. Hurks, C. Out-Luiting,**

F. Spies, J.M. Van't Noordende, K.H. Koerten and A.M. Mommaas. 1998. Efficiency of micronized titanium dioxide-containing compounds in protection against UVB-induced immunosuppression in humans in vivo. *J. Photochem. Photobiol. 10:143.*

24. **Lademann J., H. Weigmann, H. Schafer, G. Muller, and W. Sterry.** 2000. Investigation of the stability of coated titanium microplates used in sunscreens. *Skin Pharmacol. Appl. Skin. Physiol. 13:258.*
25. **Colombetti S., F. Benigni, V. Basso and A. Mondino.** 2002. Clonal anergy is mantained independently of T cell proliferation. *J. Immunol. 169:178.*
26. **Fujiki H., M. Suganuma, S. Okabe, M. Kurusu, K. Imai and K. Nakachi.** 2002. Involvement of TNF-alpha changes in human cancer development, prevention and palliative care. *Mech. Ageing. Dev. 123:1655.*
27. **Romagnani S.** 2000. T-cell subsets (Th1 versus Th2). *Ann. Allergy Asthma Immunol. 85:9.*