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# Cobalt-chromium-molybdenum but not titanium-6aluminium-4vanadium alloy discs inhibit human T cell activation *in vitro*

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**This study describes the effect of the presence of cobalt-chromium-molybdenum (CoCrMo) and titanium-6aluminium-4vanadium (Ti6AL4V) disc samples on the CD3-mediated *in vitro* response of human peripheral blood T lymphocytes. Lymphocyte proliferation in the presence and absence of these metal alloy discs was measured by [$^3$H]thymidine incorporation. Inhibition of lymphocyte proliferation was observed in the presence of CoCrMo disc samples. In contrast, the presence of the Ti6AL4V metal alloy discs had no effect on T cell proliferation. Ultrastructural studies using scanning electron microscopy revealed that the differences in the number of blast cells on uncoated CoCrMo and Ti6AL4V discs from a 4 day culture were consistent with the results observed in the proliferation experiments, i.e. fewer blast cells were seen on the CoCrMo than on the Ti6AL4V discs. In addition, a quantitative analysis of trace elements using total reflection X-ray fluorescence spectrometry in supernatants from 68 h *in vitro* cultures containing Ti6AL4V or CoCrMo disc samples was performed, revealing differences in the relative metal concentrations in the culture conditions tested. These differences point to the presence of cobalt in the supernatants as a possible determining factor of the inhibition observed. Because cell viability did not appear to change, a more complex mechanism involving the interaction of metals with T lymphocytes may account for the results obtained.**

**Keywords:** cobalt-chrome-molybdenum alloy, EXTRA II A multielement trace analyzer, T lymphocytes, titanium-aluminium-vanadium alloy, total reflection X-ray fluorescence (TXRF)

## Introduction

Cobalt-chrome and titanium alloys have been used extensively in orthopaedic prostheses surgery and dental restorations. They are the two major surgical metallic materials used along with stainless steel (Tengvall & Lundström 1992). Although metallic implants have structure and mechanical properties that yield strength and hardness, metals are not biologically inert (Edward *et al.* 1992). During the endurance of a metal orthopaedic implant, metal alloys suffer various forms of degradation such as pitting and fretting crevice corrosion. Biomaterial-related corrosion products may induce adverse biological effects such as cytotoxicity, hypersensitivity and immunogenicity (Tengvall & Lundström 1992), thereby impairing and affecting cellular functions, including immune functions (Pelletier *et al.* 1990).

Mounting evidence derived from experiments testing the *in vitro* effects of metals on immune responses (Hartmut *et al.* 1989), indicates that some metals can effectively modulate expression of T lymphocyte surface molecules and T cell responses (Treagan 1975, de Sousa & Nishiya 1978, Nishiya *et al.* 1980, Bryan *et al.* 1981, Warner & Lawrence 1986, Santos & de Sousa 1994, Arosa & de Sousa 1995). In this study an *in vitro* method was developed to determine whether T cell proliferation was affected in the presence of two metal alloy discs. This was done by [$^3$H]thymidine incorporation of T cells cultured over 3 days after activation via the CD3 pathway with an anti-CD3 antibody. In addition, metal elements released into the culture supernatants were measured. The results provide additional evidence for a differential susceptibility of T lymphocyte responses to the presence of different metals *in vitro*.

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## Materials and methods

*Metal alloy discs*

Cobalt-chromium-molybdenum (CoCrMo) and titanium-6aluminium-4Vanadium (Ti6AL4V) metal alloy discs (7.4 mm × 2 mm thick circular discs) were provided by Plasma Biotal Ltd (Tideswell, UK).

*Cell separation*

Heparinized venous peripheral blood was obtained from 45 healthy donors from the Biomedical Institute and St Antonio General Hospital. Peripheral Blood Mononuclear Cells (PBMC) were isolated by Lymphoprep (Nycomed, Norway) density gradient configuration. PBMC were then washed twice in Hank's balanced salt solution (HBSS; Gibco, Grand Island, NY), resuspended in complete RPMI 1640 medium (Sigma, St Louis, MO) supplemented with 10% heat-inactivated pooled human serum, 100 IU ml $^{-1}$ penicillin (Atral, Portugal) and 0.1 mg ml$^{-1}$ streptomycin (Azevedos, Portugal).

*Cell culture and proliferation assay*

PBMC were cultured in 24-multidish cell culture trays (Nunclon Delta, Denmark). PBMC ($1.2 \times 10^6$) in a 1 ml volume were placed in wells where the metal alloy disc under testing had been previously introduced. Control cultures were set in wells without a disc, or without the disc and the antibody. A monoclonal anti-CD3 antibody (Dakopatts, a/s Denmark; M 756) titrated previously was added to the cell culture at a final concentration of 1/1000. The cells were cultured for 68 h at 37°C in a 5% $CO_2$ incubator. At 68 h the cells were suspended by gentle pipetting in the well. Triplicate samples from each original well were transferred to round-bottom 96-well microtitre plates (Nunclon Delta, Denmark) in a volume of 200 µl well $^{-1}$, where they were pulsed with 0.2 µCi well$^{-1}$ of [$^3$H]thymidine (specific activity: 25 Ci mmol$^{-1}$; Radiochemical Centre, Amersham, UK) for the last 4 h of culture.

Cell viability was determined by the Trypan blue exclusion method in control cultures and cultures done in the presence of the two metal alloy discs [$^3$H]Thymidine incorporation was determined at 72 h in a liquid scintillation counter (LS 3801; Beckman USA) after harvesting by a semi-automatic multiple harvester (Skatron, Liebyen, Norway).

Proliferation results are expressed as counts per minute (c.p.m.) and were calculated from the median of the triplicate values from each experimental condition. The percent of decrease/increase of proliferation for each metal alloy in Figure 2 was calculated from the [$^3$H]thymidine incorporation in c.p.m. according to the formula:

$$\% = (\text{c.p.m. in presence of metal alloy disc} - \text{c.p.m. in absence of metal alloy disc} \times 100)/(\text{c.p.m. in absence of metal alloy discs})$$

*Scanning electron microscopy (SEM)*

It was of interest to carry out ultrastructural studies on CoCrMo and Ti6AL4V discs, to examine whether any morphological modification had occurred during the 4 day culture.

Discs were prepared for examination by fixation with 2.5% glutaraldehyde buffered in 0.2M sodium cacodylate (pH 7.2) for 2 h at 4°C. After washing the discs in 0.2M sodium cacodylate, discs were dehydrated through a series of graded ethanol solutions (50 -100%), passed through a (25 100%) series of isoamylacetate solutions in 100% ethanol (10 min in each concentration) then left to dry at room temperature. The discs were mounted and sputter-coated with gold, and viewed under a scanning electron microscope (JSM 35C; JOEL, Tokyo, Japan) at CEMUP at an accelerating voltage of 25 kV. The magnification varied from 500 × 8000×. Cells on the surface of these discs were identified and counted.

*Multielement analysis of supernatants*

Analysis of supernatants was done by total reflection X-ray fluorescence (TXRF) in an ATOMIKA EXTRA II A multielement trace analyzer. Simultaneous cell cultures were performed in the presence of the two uncoated metal discs from selected donors ($n = 10$). Control supernatants were obtained from lymphocyte cultures to which no anti-CD3 was added or from cultures activated in the absence of the discs.

Samples for the determination of the metal concentration in the supernatants were collected at 68 h of culture. A volume of 200 µl was transferred to a 96-well microtitre plate and centrifuged at 1100 r.p.m. (Omnifuge 2.0 RS, Heraeus SEPATECH) for 2 min. Supernatants were then collected from each microwell and placed in clean eppendorf tubes and stored at 4°C. Quantification analysis of trace elements using the EXTRA II A was performed by ATOMIKA Instruments München, Germany.

*Sample preparation*

Sample solutions (10 µl) were pipetted onto a sample support. Then, 50 ng Ga was added as an internal standard (5 µl of a 10 p.p.m. Ga solution, diluted from a commercial gallium standard solution), which yielded a concentration of 5 µg Ga ml$^{-1}$ per sample. The specimen was then dried on a hot plate and treated with 10 µl of concentrated $HNO_3$ to remove the halogens. After drying again, the sample was ready for measurement: excitation with molybdenum and tungsten anode tubes (50 kV, about 10 mA), measuring time: 2000 s. The metal elements measured were P, S, K, Ca, Ti, V, Cr, Mn, Fe, Co, Ni, Cu, Zn, Rb, Sr, Ba and Pb.

*Statistics*

Data on lymphocyte proliferation in all conditions showed a logarithmic distribution. For that reason and for the purpose of statistical analysis, the logarithmic transformation was used. Group means were compared by the paired $t$-test at a significance level of $P = 0.05$.

## Results

*Influence of the presence of metal alloy discs on T cell proliferation in vitro*

The results observed collectively for each of the

two metal alloy discs are shown in Figure 1. T Lymphocyte activation was significantly lower ($P$ = 3.940E-7) in the presence of the CoCrMo alloy discs ($n$ = 45, $X$ = 8.540, SD = 1.43) than in control cultures ($n$ = 45, $X$ = 9.091, SD = 1.43), and in the presence of the Ti6AL4V metal alloy disc ($P$ = 1.297E-4). Lymphocyte activation in the presence of the Ti6AL4V ($n$ = 45, $X$ = 8.887, SD = 1.36) was not significantly different from the control cultures.

Figure 2 shows the individual results as percent of decrease or increase in each metal condition (see Materials and methods for formula used). When the results were analyzed for each individual, CoCrMo discs almost always had an inhibitory effect on T cell proliferation, whereas Ti6AL4V discs showed a variable effect with no significant decrease or increase on T cell proliferation.

Cell viability in the presence of the metal alloy discs was similar to that seen in the control cultures and never less than 90%.

*Ultrastructural studies on uncoated CoCrMo and Ti6Al4V discs by SEM*

The morphological aspect of activated T cells in the presence of the CoCrMo metal alloy disc of one donor is represented in Figures 3 and 4. Surface topography by SEM of the discs revealed micro-grooves typical of being machine milled (Figure 3). 'Particles' exhibiting variation in size and morphology were seen dispersed on the surface of these discs. In order to see whether these particles were of biological material, an electron beam was focused directly on the particle (Figure 4c). The cracking effect (as exemplified in Figure 4d) constituted evidence to support that it was biological material. These particles of different sizes could therefore be identified as 'blast' cells at different stages of activation. The blast cells seen in response to anti-CD3 in the presence of Ti6Al4V or CoCrMo discs were so distinct that in one case (summarized in Table 1) it was possible to scan an area (20%) of the disc and count the number of blast cells seen of increasing sizes found attached to the two metal alloy discs. The results showed both increasing numbers of blast cells and of greater size on the Ti6AL4V discs as compared with the CoCrMo discs, thus corroborating morphologically the quantitative analysis of the T cell response for each of these metal alloy discs treated.

*Multielement concentrations in culture supernatants*

The concentrations of manganese, cobalt, nickel, copper, zinc, molybdenum and titanium were measured in the supernatants of culture removed at 3 days after anti-CD3 activation in the absence and presence of the Ti6AL4V and CoCrMo discs. As a control, in one experiment the metal concentration

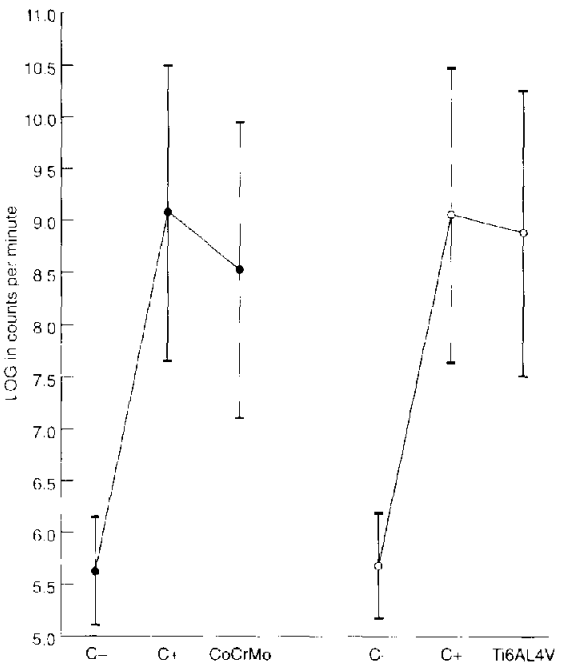


**Figure 1.** The response of CD3-mediated lymphocyte proliferation in the absence (C +) of presence of CoCrMo (●) and Ti6A14V (○) alloy discs ($n$ = 45). (C –: cell cultures not activated (5.64 ± 0.52). The

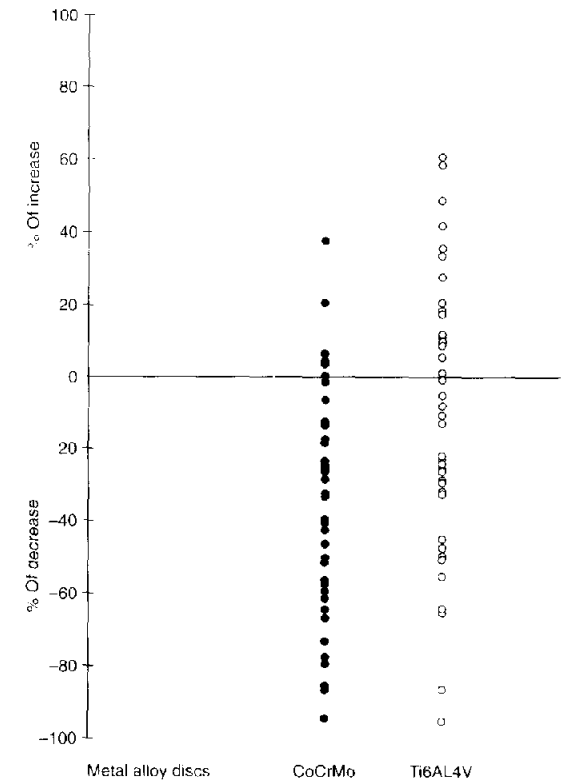


**Figure 2.** Percentage of inhibition/stimulation by CoCrMo and Ti6A14V alloys on individual lymphocyte proliferation ($n$ = 45).

in medium alone left for 3 days in the presence of the two discs was also estimated. The highest concentration of cobalt was seen in this culture (Table 2). The concentration levels of each element in the supernatants were approximately the same in all the donors, although the concentration levels varied among the conditions tested. In the control cultures where no disc was present (Figure 5A), the elements copper and zinc were detected in higher concentrations than other metal elements present. In general, higher concentrations of all the metals were seen in the supernatants of cultures in the presence of the metal alloy discs (Figures 5B and C). Here the concentration values of manganese, zinc and molybdenum were seen to increase 2- to 3-fold. The most striking increase in concentration was observed, however, in the presence of the CoCrMo metal alloy disc, where the cobalt concentration was 110 times greater than the concentration values obtained with the supernatants in the control cultures. However, the highest concentration noted was seen in the control supernatant in the presence of the CoCrMo disc alone in medium, without cells. With the Ti6AL4V discs (Figure 5C), the element

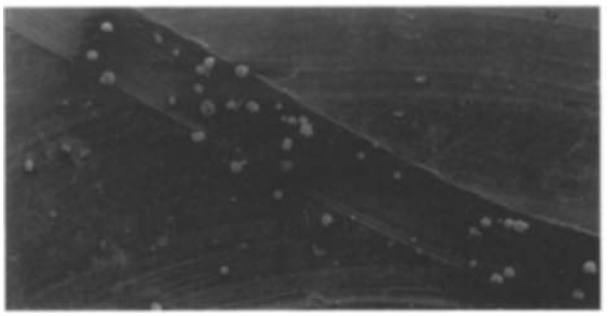

**Figure 3.** Blast cells seen dispersed on the surface of a CoCrMo disc under SEM at a magnification of 81 × at 25 k V.

titanium remained at low levels, yet copper was predominantly elevated as compared with other elements found in the supernatants.

*Discussion*

The present work was motivated primarily by an interest in testing whether T lymphocyte activation *in vitro* could be modified in the presence of solid metal alloys representative of alloys used as implants in medical practice.

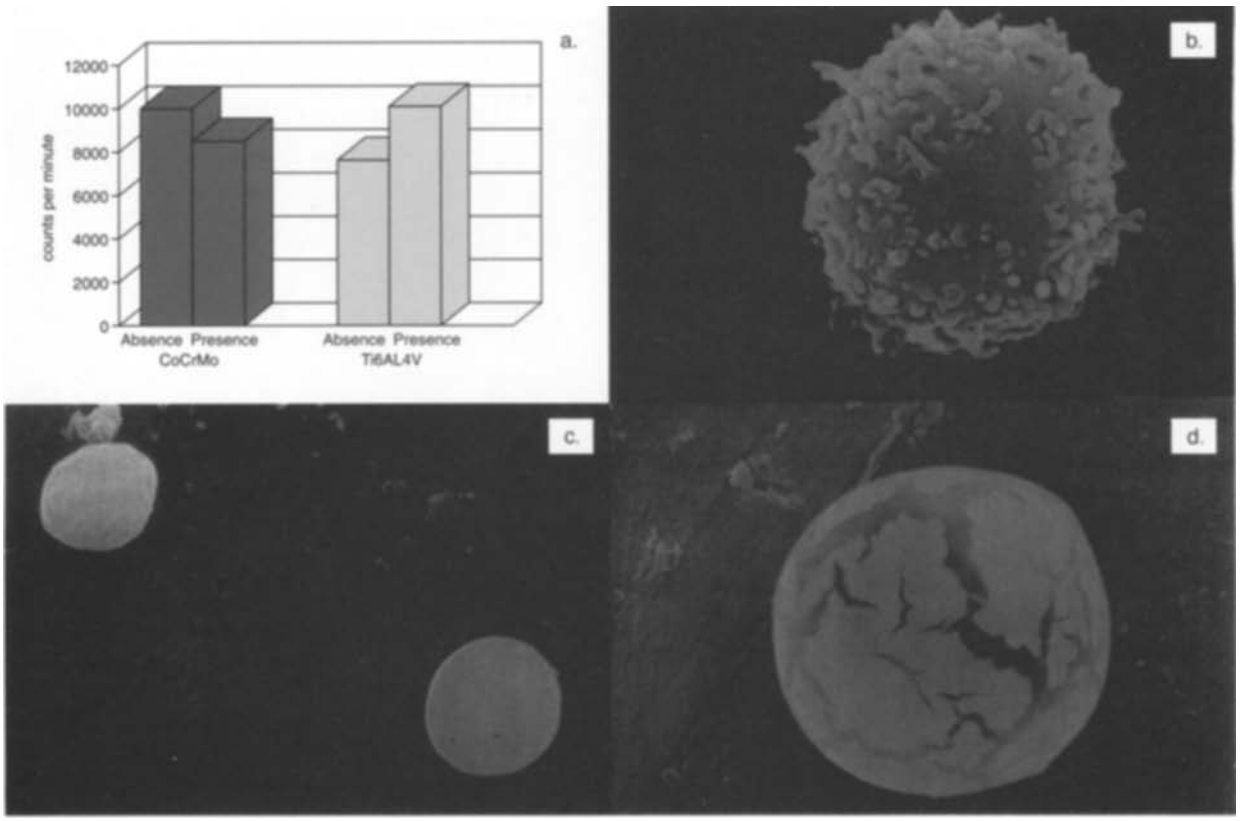


**Figure 4.** Morphological aspects of activated T cells in the presence of the Ti6A14V alloy disc seen under SEM (b–d) from one donor whose changes in response to anti-CD3 in the absence and presence of the metal alloy discs are shown in (a). (b) T lymphocytes at a magnification of 6560 ×. (c) Blast cells before an electron beam was focused (2460 ×). (d) A blast cell after an electron beam was focused (6150 ×).

**Table 1.** The numbers of blast cells counted under SEM on 20% of the surface of the metal alloy discs in one donor

| Metal alloy disc | Cell diameter (μm) | | | |
|---|---|---|---|---|
| 5 | 10 | 15 | 20 | |
| CoCrMo | 47 | 20 | 3 | 6 |
| Ti6A14V | 46 | 61 | 35 | 21 |

A new method was set up that permitted finding some answers to that question. T lymphocyte activation was found to be inhibited consistently when cultures were carried out in the presence of cobalt-chrome-aluminium discs, but not Ti6AL4V alloy discs. The inhibition was documented in two ways: (i) by a statistically significant decrease in the [$^3$H]thymidine incorporation and (ii) by the actual ultrastructural observation of smaller numbers of blast cells on the CoCrMo discs than on Ti6AL4V discs. Based on previous reports demonstrating the cytotoxicity of particulate cobalt towards human dermal fibroblasts (Evans & Benjamin 1987, Evans 1994) and murine macrophages (Garrett *et al.* 1983), it might be thought that the present results could have been attributed to the documented cytotoxicity of cobalt towards the T cells used in the present study. However, analysis of cell viability in the cultures carried out in the presence of the discs disregarded this possibility since viability was shown to be greater than 90%.

**Table 2.** Concentration of metal elements in supernatants, with and without cells

| Metal elements (μg ml$^{-1}$) | Metal alloy disc | | | |
|---|---|---|---|---|
| | CoCrMo | | Ti6A14V | |
| | Medium-RPMI cells **without** | Medium-RPMI cells[a] **with** | Medium-RPMI cells **without** | Medium-RPMI **with** cells[a] |
| Mn | 0.053 | 0.052 | 0.061 | 0.061 |
| Co | **1.204** | **0.568** | **0.034** | **0.026** |
| Ni | 0.021 | 0.016 | 0.017 | 0.068 |
| Cu | 0.437 | 0.401 | 0.817 | 0.696 |
| Zn | 0.093 | 0.258 | 0.411 | 0.276 |
| Mo | 0.185 | 0.093 | 0.106 | 0.020 |

[a]Donor was selected for comparison with the control.

Thus, we proceeded to analyze how the presence of the two metal alloy discs influenced the concentration of metal ions in the culture supernatants. A total of 21 metal elements were analyzed; of these, changes were noted with manganese, cobalt, nickel, copper, zinc and molybdenum.

In both cases the presence of the discs in the cultures resulted in increases of manganese, copper, zinc and molybdenum concentrations. The most notable increase exclusive to the CoCrMo alloy disc was, however, cobalt itself, from a concentration of 0.0066 μg ml$^{-1}$ in the control cultures to 0.7875 μg ml$^{-1}$ in the disc-containing cultures. Taking into account that the control experiment (with culture medium in the presence of the disc) had the highest

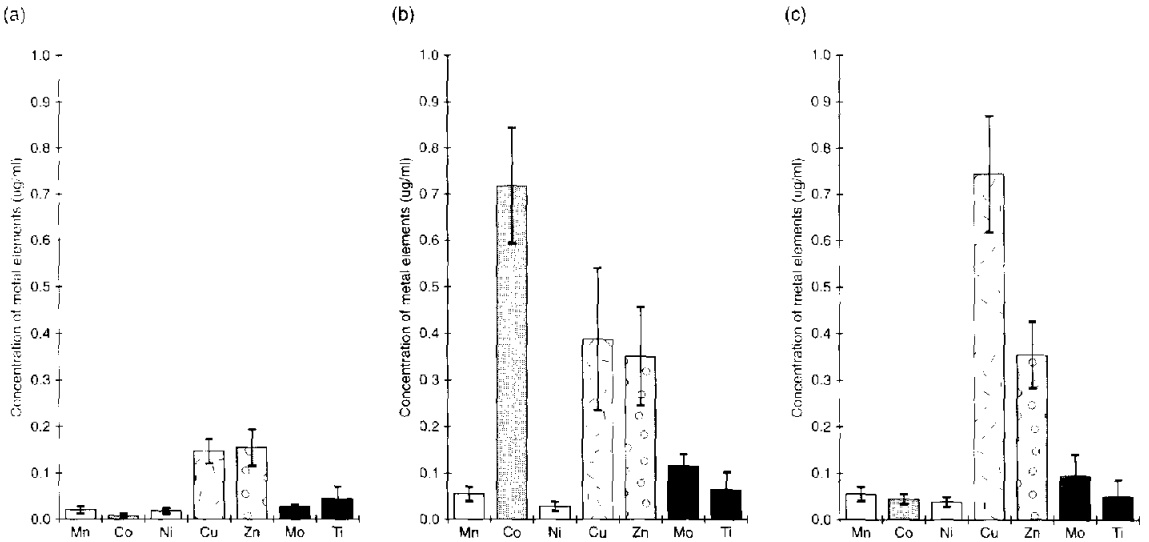


**Figure 5.** The concentration of manganese, cobalt, nickel, copper, zinc, molybdenum and titanium determined by TXRF found in supernatants from a 3 day culture. (A) Metal elements determined from the supernatants without the presence of a metal alloy disc. (B) Metal elements determined from the supernatants of the CoCrMo alloy disc. (C) Metal elements determined from the supernatants of the Ti6A14V alloy disc.

cobalt concentration, it is possible that chelating agents in the medium contributed to high metal concentrations in the cultures.

The mechanism whereby such concentrations of cobalt could affect lymphocyte activation remains elusive. In the literature the concentration at which cobalt has been found to be cytotoxic (Garrett *et al.* 1983) is 1000-fold higher than that detected in the present study. The metal could be interacting and affecting activation by (i) affecting the binding of the anti-CD3 antibody to CD3 itself or (ii) it could be affecting co-stimulatory signals known to be indispensible to trigger lymphocyte activation (Bravo *et al.* 1990, Pelletier *et al.* 1990, Carvalho *et al.* 1990). In a previous work testing the effect of exposure to ferric citrate, it was found that the presence of iron resulted in modulation of another T-cell-specific surface molecule, i.e. CD4 (Santos & de Sousa 1994), and that this in turn led to diminished $p56^{lck}$ activity in CD4 immunoprecipitates (Arosa & de Sousa 1995). It is therefore possible that cobalt could be acting directly on the cell surface. We cannot exclude, however, the possibility that the increase in cobalt in the CoCrMo disc cultures also affected the relative concentrations of the other metals.

It is known that titanium and titanium-based alloys can be used in a wide range of aqueous solutions without significant dissolution (Williams 1981). This property may be due to the formation of a protective oxide layer (Sousa & Barbosa 1993) thereby preventing the accumulation of corrosion products, such as metal ions into surrounding tissues (Sutherland *et al.* 1993) or culture medium. The data obtained in this study with the titanium alloy could support this observation, since neither the concentration of titanium in the supernatants between the different culture conditions (0.045–0.065 $\mu g\ ml^{-1}$) vary nor did the response of CD3 mediated T-lymphocytes in the presence of this metal alloy significantly alter in relation to the control.

The present results strengthen the wider conclusion that metal ions can have critical functions in regulating the expression of T lymphocyte functions (de Sousa *et al.* 1991).

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