

THE TOXICITY OF METALS USED IN ORTHOPAEDIC PROSTHESES

AN EXPERIMENTAL STUDY USING CULTURED HUMAN SYNOVIAL FIBROBLASTS

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Some of the component metals of the alloys used for total joint prostheses are toxic and dissolve in the body fluids. It is important to establish how toxic these metals are and to assess the risk of localised tissue necrosis around the prostheses. This has been investigated by incubating primary monolayer cultures of human synovial fibroblasts with various preparations of metals for periods up to 18 days. Morphological changes were evident after exposure to cobalt chloride at a concentration of 50 nanomoles per millilitre and to nickel chloride at 200 nanomoles per millilitre. Chromic chloride, ammonium molybdate and ferric chloride produced no changes up to 500 nanomoles per millilitre. Cultures exposed to particulate pure metals were poisoned by cobalt and vanadium but were not affected under the same conditions by nickel, chromium, molybdenum, titanium or aluminium. Particulate cobalt and vanadium were probably toxic due to their relatively high solubility (four and one micromoles per millilitre respectively after seven days incubation). Particulate nickel also dissolved (three nanomoles per millilitre after seven days) but not in sufficient quantities to be toxic.

It appears, therefore, that potentially the most harmful components are cobalt from cobalt-chromium alloy, nickel from stainless steel, and vanadium from titanium alloy. As far as can be estimated, the only combination of materials which is likely to give rise to toxic levels of metal under clinical conditions, is cobalt-chromium alloy articulating against itself to produce relatively high levels of cobalt.

It has been established that some of the component metals of the alloys used in total joint prostheses are toxic. Stainless steel powder has been shown to slightly lower the rate of growth of cells in culture, whilst particulate cobalt-chromium alloy has a much more marked effect (Mital and Cohen 1968; Pappas and Cohen 1968). Similarly, cobalt, nickel and cobalt-chromium alloy are toxic for macrophages *in vitro* (Rae 1975). Soluble salts of cobalt have also been shown to be toxic for various cultured cells (Heath 1954; Daniel *et al.* 1963) as have those of nickel (Basrur and Gilman 1967; Waters *et al.* 1975).

From the clinical point of view it is important to establish whether sufficient quantities of these potentially toxic elements are liberated from prostheses to cause local tissue reactions and produce clinical failure as a result of excessive tissue necrosis. The quantities of metal which dissolve *in vivo* are not known accurately, but estimates have been made (Swanson, Freeman and Heath 1973; Rae 1979). The concentration of metal to which tissues are exposed depends on factors such as the wear rates of the articulating surfaces, the rate of turnover of joint fluid, and the excretion rates of soluble metal. It is therefore difficult to quantitatively reproduce in an experimental system the conditions which

exist under clinical use. An approach is described here in which human synovial fibroblast-like cells were exposed to various concentrations of metal *in vitro* and the toxicity quantitatively measured. The threshold of toxicity for each metal can thus be established. This can then be compared to the concentration which has been estimated to occur around a prosthesis and the likelihood of necrosis predicted.

MATERIALS AND METHODS

Culture of human synovial fibroblasts. Small pieces of synovial tissue were obtained from patients undergoing operations on the knee for the treatment of ligament injury. Recently it has been found more convenient to use a small biopsy of normal synovium taken during arthroscopic examination of the knee. Tissue so obtained was maintained under aseptic conditions and finely chopped with a scalpel into approximately 0.2 millilitre cubes under calcium and magnesium-free Hanks' balanced salt solution. In some cases the resulting suspension of tissue was incubated in a solution of one per cent trypsin for 30 minutes at 37 degrees Celsius. After preparation the tissue was suspended in Medium 199 FC containing tissue culture Medium 199 plus Earles salts with 10 per cent foetal calf serum, 200 international units per millilitre of penicillin and 100 micrograms per millilitre of streptomycin. The suspension of tissue was then added to cell culture flasks of 25 square centimetres growing area and most of the supernatant removed so that the pieces of tissue were in firm contact with the substratum.

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After incubation overnight at 37 degrees Celsius, two millilitres of tissue culture fluid were gently added, care being taken not to disturb the adherent pieces of tissue. The flasks were incubated for a further 7 to 10 days and the medium was changed every two days. During this time cells migrated out of the explants to form a monolayer. At this stage, the flasks were treated with 0.25 per cent trypsin to remove the cells from the substratum. Any large pieces of tissue were allowed to sediment and the suspension of cells washed in calcium and magnesium-free Hanks' solution and resuspended in Medium 199 FC and seeded into clean flasks (the split ratio was about five to one). These stock monolayer cultures of cells were either used in experiments or were subcultured into new flasks when they had reached confluence. Stock cell lines were maintained for about four months; after this time the cells tended to exhibit a bizarre morphology and did not grow well.

Experimental procedure. Cells were seeded into flat-faced cell culture tubes at a density of 4×10^4 cells in two millilitres of Medium 199 FC on a growth surface area of 5.5 square centimetres. After 48 hours the medium was either changed to one in which the foetal calf serum was replaced with two per cent bovine serum albumin or the cells were maintained in Medium 199 FC. The former was used in experiments in which lactate dehydrogenase release was used to estimate cell damage (Rae 1975) and the latter in experiments where morphological changes were used to assess the toxicity. In appropriate cases the medium contained a preparation of the metal to be tested. Metal was presented to the cells in one of three ways: as a soluble salt (usually the chloride); in a pure particulate form; or as debris caused by wear generated from a total hip prosthesis. The following soluble salts of analytical grade were used: nickel chloride, cobalt chloride, ferric chloride, chromic chloride and ammonium molybdate. Pure particulate metals were of the following sizes in micrometres: aluminium <50; cobalt 1; chromium 5 to 30; molybdenum 5 to 6; nickel 1; titanium <150; and vanadium <75. Debris caused by wear was generated from total hip prostheses under sterile conditions as described previously (Rae 1979).

Replicate cultures were incubated with one of the metal preparations for a maximum of seven days for the enzyme release experiments and 18 days for the morphological studies. During the incubation periods the medium was changed every two days. In those experiments using soluble metal the fresh medium contained an identical concentration of metal, whereas in those experiments with particulate material the medium was carefully removed without disturbing the debris and replaced by fresh medium which was free from added metal.

The culture tubes used for morphological studies contained a glass cover slip. At the end of the appropriate incubation period this cover

slip was fixed for 15 minutes in methanol, and stained with May-Grünwald and Giemsa stains.

Determination of soluble metal. In those experiments in which the cells were exposed to pure particulate metal the culture medium was analysed for soluble metal at times during the incubation period. Flameless atomic absorption spectrophotometry was used to analyse samples of medium which had been filtered through a 0.22 micrometre Millipore filter. Depending upon the metal to be determined, 10 to 25 microlitres of untreated fluid were pipetted directly into the graphite tube furnace of a Perkin-Elmer HGA 74 heated graphite atomiser attached to a Model 360 atomic absorption spectrophotometer. At least two determinations of metal were carried out on each sample. The limits for detection of the metals refer to the lowest concentration in the original sample which could be expected to give a peak absorption of approximately twice the background level of noise. These are given as picomoles per millilitre: aluminium 92.7; cobalt 135.8; chromium 63.5; molybdenum 260.5; titanium 6890.4; and vanadium 1963.0.

RESULTS

The cells which were grown in monolayer culture and exposed to various metals at different concentrations were examined for evidence of necrosis. This was done by measuring the release of the enzyme lactate dehydrogenase which is found in the soluble cell fraction and is released into the extracellular fluid after injury to the cell membrane. Cell injury was also assessed by microscopical examination for morphological changes.

Stained preparations were examined microscopically and the effects of nickel and cobalt on the morphological appearance of the cells was classed as either type A, B or C. Cultures in which the cells exhibited a typical fibroblast morphology with normal nuclear and cytoplasmic staining were classed as type A. Those cultures in which approximately half of the cells showed morphological changes, such as the withdrawal of cytoplasmic processes and abnormal nuclear and cytoplasmic staining, were classed as type B; and cultures in which gross necrosis was evident were classed as type C. For a given combination of incubation time and metal concentration this score was marked on a diagram having the axes as shown in Figures 1 and 2. For

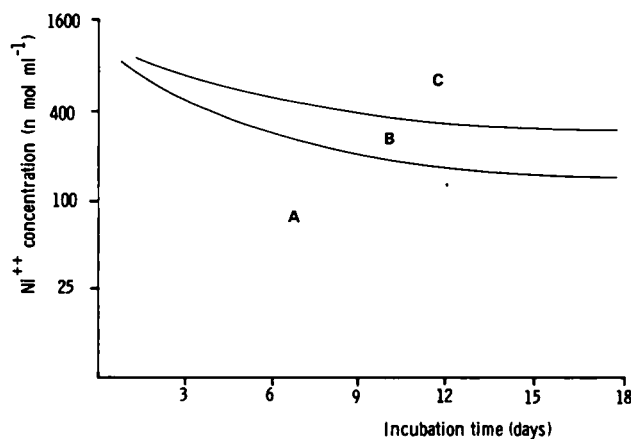


Fig. 1

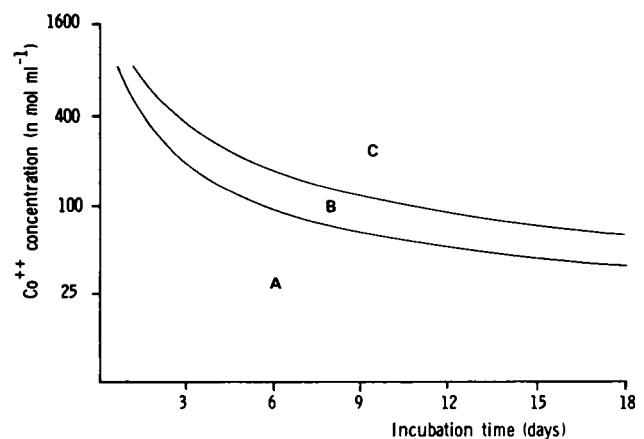


Fig. 2

Figure 1—Diagram to show the zones in which the combinations of nickel chloride concentrations and incubation time caused no visible morphological changes to the cells (A); changes to approximately 50 per cent of the cells (B); and gross necrosis (C). Figure 2—Diagram to show the zones where combinations of cobalt chloride concentrations and incubation time caused no visible morphological changes to the cells (A); changes to approximately 50 per cent of the cells (B); and gross necrosis (C).

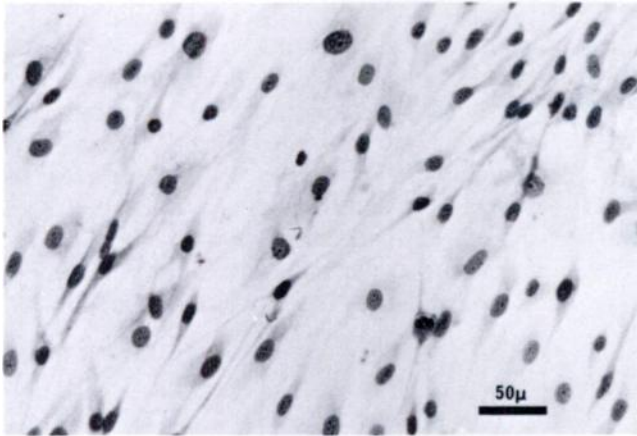


Fig. 3

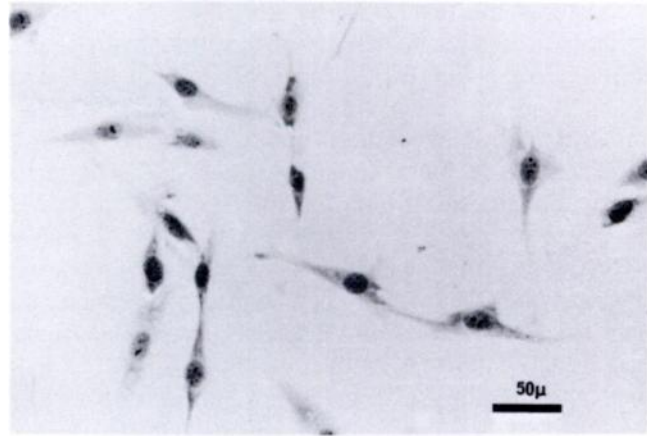


Fig. 4

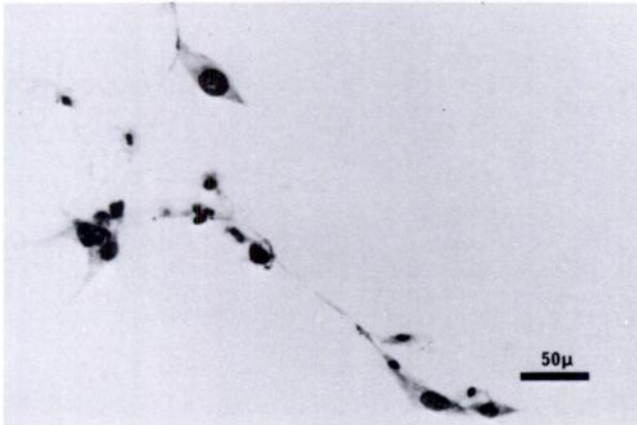


Fig. 5

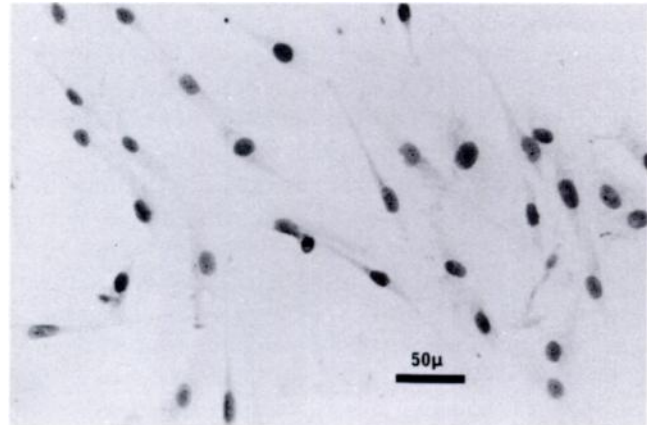


Fig. 6

Photomicrographs stained with May-Grünwald and Giemsa stains. Figure 3—A monolayer of human synovial fibroblasts maintained in culture for 120 days in medium which was free from added metal. Figure 4—Fibroblasts exposed to 200 nanomoles per millilitre of cobalt chloride for 48 hours. Figure 5—Fibroblasts incubated with 200 nanomoles per millilitre of cobalt chloride for 72 hours. Note the morphological changes such as the rounding of the cells and pyknosis. Figure 6—Cells exposed to 100 nanomoles per millilitre of nickel chloride for 14 days.

each metal at least 50 cultures were classed into these groups and graphs drawn to show the zones for each class (Figs 1 and 2). Any cultures with a combination of incubation time and metal concentration below the lower line would result in no morphological changes to the cells. Similarly, cultures with a combination which lies between the two lines would be classed as type B changes, and a combination occurring above the upper line would cause gross necrotic changes.

The photomicrographs in Figures 3 to 6 show cultures of cells after various treatments and illustrate the above classification. The cells of a stock culture of fibroblasts maintained for 120 days in medium free from added metal were healthy and well spread on the substratum (Fig. 3). They had prominent nucleoli and normal cytoplasmic and nuclear staining. A mitotic figure was clearly visible in this field of actively growing cells. Cells treated with 200 nanomoles per millilitre of cobalt chloride for 48 hours are shown in Figure 4. At this stage the cells maintained a normal morphology and staining pattern although with slightly darker staining nucleoli. These cells were classed as type A. However, after incubation for 72 hours in 200 nanomoles per

millilitre of cobalt chloride many cells exhibited morphological changes such as the withdrawal of cytoplasmic processes and pyknosis, but other cells remained relatively unaffected. The appearance of these cells was classed as type B (Fig. 5). Fibroblasts treated with nickel chloride at 100 nanomoles per millilitre for 14 days are shown in Figure 6. Here the cells maintained their normal morphological appearance and staining pattern and were accordingly classed as type A.

The diagrams in Figures 1 and 2 show that the thresholds of toxicity for nickel chloride and for cobalt chloride are about 200 and 50 nanomoles per millilitre respectively for long incubation times (18 days). It is important to note that there is no linear relationship between metal concentration and incubation time. Cells in culture can withstand a high concentration of metal for a short time, so a short-term *in vitro* toxicity study would lead to an erroneously high threshold of toxicity for the metal under investigation.

The results from the experiments in which the release of lactate dehydrogenase was used as a marker of cell injury are summarised in Table I. At both

concentrations tested cobalt was the only metal to produce a significant release of lactate dehydrogenase after two days of incubation. The amount of lactate dehydrogenase was 1.7 and 5.5 times greater respectively than the control for 50 and for 500 nanomoles per millilitre of cobalt. Similarly, after seven days of incubation, these two concentrations of cobalt produced a release of lactate dehydrogenase of 2.1 and 2.6 more than the control. The apparent decrease in the release of the enzyme, when expressed as a proportion of the control, was due to a slow release of lactate dehydrogenase from the untreated cells.

Nickel did not produce a significant release of lactate dehydrogenase after incubation for two days. However, after seven days 50 nanomoles per millilitre of nickel resulted in a release 1.3 times greater than the control, whilst 500 nanomoles per millilitre resulted in 2.7 times as much lactate dehydrogenase being released. It is therefore evident that cobalt is more toxic to the cells under these conditions.

Results with the other soluble metal compounds showed that the release of lactate dehydrogenase from chromium-treated cells did not differ significantly from the untreated control cells. The cells treated with molybdenum only released a significant amount of enzyme after incubation for seven days at 500 nanomoles per millilitre. It is difficult to judge whether this is an effect of the metal since it was present in the anionic form in ammonium molybdate. Ferric chloride produced no morphological changes after 18 days of incubation.

In addition to testing the toxicity of soluble metal compounds some cultures were incubated with pure particulate metals. At various times during the incubation the amount of lactate dehydrogenase released by the cells, and the soluble metal which dissolved from the particulate material were measured (Fig. 7).

The cells which were treated with particulate cobalt or vanadium released much more lactate dehydrogenase into the supernatant fluid than the untreated control

cells. Exposure of the cells to particulate nickel, chromium, molybdenum, titanium or aluminium did not give any greater release of lactate dehydrogenase than the untreated cells. The concentration of soluble metal was determined at various times during the incubation period. The amounts of soluble metal were below the limits necessary for detection except for cobalt, vanadium and nickel. The concentration of cobalt and vanadium reached a maximum of about four and one micromoles per millilitre respectively after seven days of

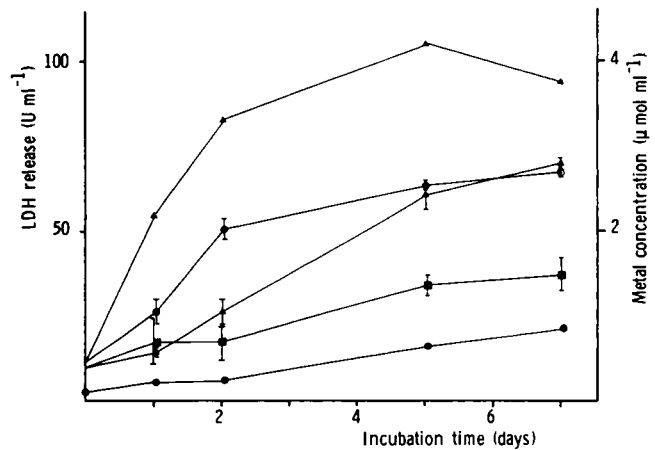


Fig. 7

Graph to show the release of lactate dehydrogenase from control cells (■); and cells exposed to particulate cobalt (○) and vanadium (△). The graph also shows the concentration of soluble cobalt (▲) and vanadium (●) which resulted from the dissolution of these particulate metals.

incubation. The concentration of soluble nickel produced was much lower and reached a maximum of three nanomoles per millilitre after seven days.

The appearance of cells after treatment with some particulate metals is shown in the photomicrographs (Figs 8 to 11) which were taken after exposure to the metal for nine days. Particles of chromium, molybdenum and titanium can be seen in intimate contact with the cells which retain their normal appearance and are

Table 1. Comparison of the relative toxicity of soluble metals for human synovial fibroblasts

Metal	Concentration (nmol ml ⁻¹)	Incubation for two days				Incubation for seven days			
		LDH release (units ml ⁻¹ ±SD)	Number	LDH release as proportion of control	P	LDH release (units ml ⁻¹ ±SD)	Number	LDH release as proportion of control	P
Control	—	16.6±5.5	12	1.0	—	48.7±16.6	24	1.0	—
Cobalt	50	28.0±6.0	6	1.7	<0.001	99.7±10.8	12	2.1	<0.001
Cobalt	500	90.5±4.3	6	5.5	<0.001	124.7±6.0	12	2.6	<0.001
Nickel	50	14.3±2.7	6	0.9	>0.1	63.6±20.5	12	1.3	<0.05
Nickel	500	20.2±5.1	6	1.2	>0.1	131.3±5.1	12	2.7	<0.001
Control	—	6.3±2.5	12	1.0	—	23.5±8.3	24	1.0	—
Chromium	50	5.0±0	6	0.8	>0.1	19.1±3.9	12	0.8	<0.1
Chromium	500	5.5±1.2	6	0.9	>0.1	25.5±8.2	12	1.1	>0.1
Molybdenum	50	4.3±1.4	6	0.7	>0.1	24.3±4.7	12	1.0	>0.1
Molybdenum	500	7.5±2.3	6	1.2	>0.1	40.9±3.4	12	1.7	<0.001

P=Probability value for Student's *t*-test comparing lactate dehydrogenase (LDH) release of metal-treated cells with the respective control cells.

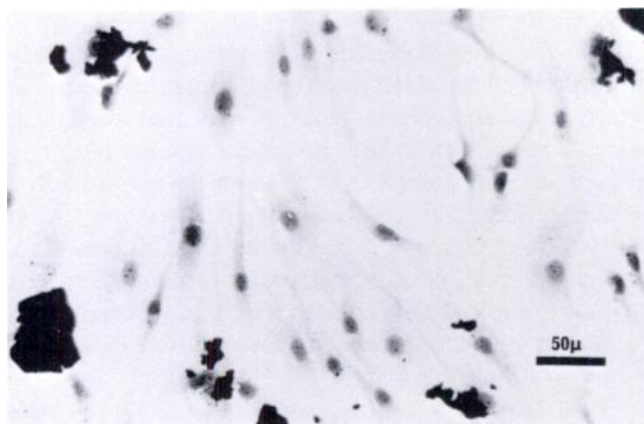


Fig. 8

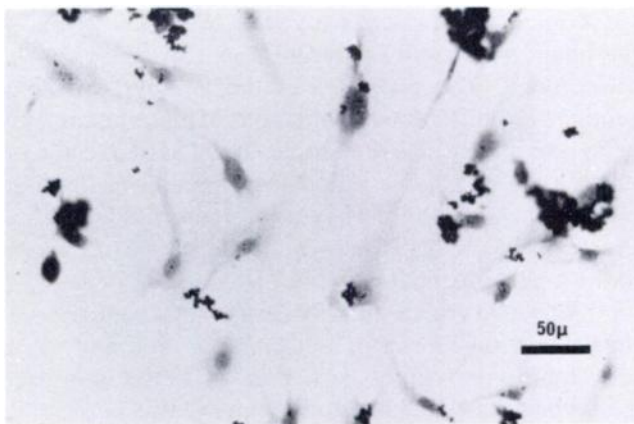


Fig. 9

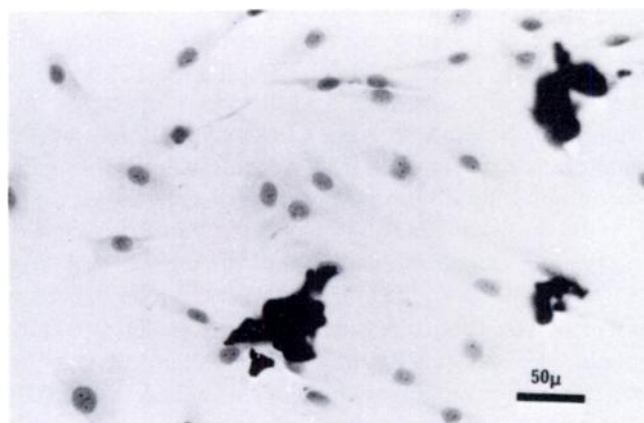


Fig. 10



Fig. 11

Photomicrographs taken after exposure of the fibroblasts to various metals for nine days. Stained with May-Grünwald and Giemsa stains. Figure 8—Fibroblasts in contact with particulate chromium are unaffected. Figure 9—Fibroblasts exposed to particulate molybdenum remain healthy and well spread. Figure 10—Cells in intimate contact with particles of titanium show no adverse effect. Figure 11—Particulate vanadium has caused gross necrosis and only cytoplasmic debris and pyknotic nuclei remain in contact with the metal.

apparently unaffected. The extremely toxic effect of vanadium can be seen in Figure 11; no recognisable cells remain and in many cases cellular debris surrounds the particulate metal.

DISCUSSION

The data presented here and elsewhere have established that some of the component metals used to manufacture the alloys for joint replacements are toxic. However, it is unknown whether sufficient quantities are released under clinical conditions to cause localised tissue necrosis.

The results indicated that the threshold of toxicity for nickel chloride under the conditions described was about 200 nanomoles per millilitre for 18 days' exposure. Morphological changes were used as an index of cell damage. However, 50 nanomoles per millilitre of nickel after seven days exposure had sufficiently damaged the cell membrane to cause lactate dehydrogenase to leak significantly into the supernatant fluid. Cobalt chloride was more toxic with morphological changes apparent at 50 nanomoles per millilitre after 18 days' exposure and with a significant release of lactate dehydrogenase after only two days at this concentration.

The levels of soluble metal produced by total hip prostheses are of the order of 0.85 nanomoles per millilitre of nickel for a stainless steel and high-density polyethylene total hip and 188.53 nanomoles per millilitre of cobalt for an all-cobalt-chromium alloy total hip (Rae 1979). On the basis of the investigations reported here, the nickel produced from a stainless steel hip would be about 235 times less than the toxic threshold *in vitro*, whilst cobalt from an all-metal hip would be about 3.8 times greater than the toxic threshold.

The other major components of stainless steel and cobalt-chromium alloy, including the soluble forms of chromium, molybdenum and iron, did not produce an abnormal cell morphology or an increase in the release of lactate dehydrogenase above the control values except in the cases of ammonium molybdate which caused an increase in release of the enzyme at the highest concentration tested after seven days of incubation. Caution must be exercised in the extrapolation of these results to the clinical situation since the soluble metal which occurs in the tissue fluids as a result of corrosion is unlikely to be present in the form of

simple metallic ions or oxy-anions. Little is known about the relative toxicity of different chemical species of the same metal. The estimates of the amounts of metal produced by different combinations of material can only be approximate since the simple model used to estimate the release of soluble metal from prostheses ignored factors such as the turnover of joint fluid and the excretion of metal. However, it is difficult to make a more reliable estimate.

Although it is technically easy to test the toxicity of the soluble ionic forms of metal *in vitro*, this may not be relevant to the clinical situation. A better approach would be to test a suspension of pure particulate metals in a tissue culture medium containing serum. Metals which are relatively soluble in biological fluids would produce complexes with the low molecular weight components, such as amino acids, and high molecular weight species, such as proteins. Such complexes would probably give more reliable results in tests for toxicity than those obtained from tests with simple ionic salts since the complexes are similar to those which form *in vivo*.

It was found that pure particulate chromium, molybdenum, titanium, aluminium and nickel tested in this way caused neither significant increases in the release of lactate dehydrogenase nor morphological changes. However, cobalt and vanadium produced a significant release of lactate dehydrogenase and significant morphological changes. This appears to be due to the high solubility of these two metals. The damaging effects of these metals may also be due to the particles, some of which have been shown to directly damage the cell membrane (Rae 1978), but this is probably not

important clinically.

In the study described here particulate nickel did not cause cellular damage although previous studies on murine macrophages *in vitro* showed that particulate nickel caused lactate dehydrogenase release and morphological changes (Rae 1975). This was probably a consequence of phagocytosis of the particles where even the low solubility of nickel was sufficient to poison the cells by a direct intracellular route. In contrast to this, the non-phagocytic fibroblasts used in the present study were not affected by the relatively low extracellular concentration of nickel.

To demonstrate the solubility of some pure metals, samples of 20 milligrams of particulate metal were incubated at 37 degrees Celsius in 20 millilitres of new-born calf serum under aerobic and aseptic conditions. The concentrations of metal after 105 and 210 days of incubation were as follows (micromoles per millilitre): cobalt 4.98 and 14.06; nickel 1.74 and 8.13; vanadium 6.19 and 28.6. No detectable amounts of chromium, aluminium or titanium dissolved.

In view of the high solubility and toxicity of particulate vanadium the effect of the wear debris from a titanium alloy (Ti-6Al-4V) and high-density polyethylene total hip was tested. After eight days of incubation with this material there was no evidence of cell damage as judged by the morphological appearance and release of lactate dehydrogenase. No detectable amounts of vanadium, titanium or aluminium dissolved from the alloy. It would appear therefore, that the vanadium component of the titanium alloy does not present a toxic hazard under the conditions described here.

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