NEOPLASTIC TRANSFORMATION OF CELLS BY ORTHOPAEDIC METALS *IN VITRO*

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ABBREVIATIONS

3-MCA	3-methylcholanthrene
$AlCl_3$	Aluminium Chloride
CHO	Chinese hamster ovary cells
CO_2	Carbon Dioxide
$CoCl_2$	Cobalt Chloride
CoCr	Cobalt-chromium alloy
$CrCl_2$	Chromium Chloride
DMSO	Dimethyl Sulphoxide
DNA	Deoxyribonucleic Acid
EBME	Eagle's Basal Medium with Earle's salts
FCS	Foetal calf serum
$FeIICl_2$	Ferrous Chloride
HGPTR	Hypoxanthine-guanine phosphoribosyl transferase
HMEM	Minimum Essential Medium with Hank's salts
HSF	Human synovial fibroblasts
IARC	International Agency for Research on Cancer
MFH	Malignant fibrous histiocytoma
MNNG	N-methyl-N'-nitro-N-nitrosoguanidine
Na_2CrO_4	Sodium Chromate
Na_2MoO_4	Sodium Molybdate
$NiCl_2$	Nickel Chloride

PBS	Phosphate-buffered saline
RPE	Relative plating efficiency
SCE	Sister chromatid exchange
SHE	Syrian hamster embryo cells
THR	Total hip replacement
Ti6Al4V	Titanium-aluminium-vanadium alloy
$TiCl_3$	Titanium Chloride
TKR	Total knee replacement
$VOCl_2$	Vanadyl Chloride
#	Fracture

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Summary

The development of successful joint replacement prostheses and the widespread use of fracture fixation devices have revolutionised the practice of orthopaedic and trauma surgery over the last thirty years. The metal alloys used for such implants are chosen both for their mechanical strength and high degree of biocompatibility. However, over time, the processes of *in vivo* corrosion and wear liberate metals from all of the alloys in current use. Some metals are recognized carcinogens in the industrial environment and in recent years there have been growing numbers of reports of malignant tumours associated with metal implants in patients. In relation to the millions of patients who have benefitted from such operations, these reports remain small in number but have raised concern over the possible effects of long term exposure to metal from implants.

Recent developments in cell culture techniques have made it possible to study, *in vitro*, cellular mechanisms associated with carcinogenesis and to apply these methods as screening tools. This study therefore investigated and compared the ability of the commonly used implant metals to induce neoplastic transformation and toxicity in fibroblasts in cell culture. Eight metals, namely cobalt, chromium, nickel, iron, molybdenum, aluminium, vanadium and titanium, were tested both as soluble salts and as solid particles. Two cell lines were employed, C3H/10T½ mouse fibroblasts and diploid human fibroblasts.

There were marked differences between the various metals in terms of both toxicity and transforming ability. In the mouse fibroblast assays, significant increases in transformation incidence were seen with cobalt, chromium, nickel and molybdenum, but not with iron, aluminium, vanadium or titanium. For most of the metals, transforming ability was directly related to toxicity though this correlation was not seen with molybdenum and vanadium. The physical form of the metal was important in that transformation occurred only with soluble prepara-

tions, possibly reflecting differences in cellular uptake. In the human cell assays, reliable controls could not be produced and metal transformation studies could not be pursued. However, cytotoxicity assays gave results similar to those seen in the mouse cells.

The mechanisms involved in the release, distribution and biological interaction of corrosion and wear products are complex and only partially explained. At the present level of understanding, it is not possible to quantify the risk, if any, of malignant complications. The results of this study provide a way of grading the relative risks of orthopaedic implant metals in terms of toxicity and potential carcinogenicity. Of the three available alloys, stainless steel and cobalt-chromium contain significant amounts of those metals which caused toxicity and cell transformation, whereas none of the constituents of titanium alloy showed evidence of transforming ability. On this basis the safest alloy in current use would appear to be titanium alloy.

CHAPTER ONE

THE CARCINOGENIC RISK OF IMPLANTED ORTHOPAEDIC METALS

1.1 The evolution of modern orthopaedic alloys.

1.1.1 Early developments.

The use of metal implants in surgical practice has its origins in the late eighteenth and early nineteenth centuries with the use of wire cerclage in the treatment of fractures and pseudarthroses. A number of metals were employed including brass, gold, silver and platinum, but the ever present problems of infection limited their widespread application. Nevertheless, even in those early days, some important observations were made. Bell (1804) noted the occurrence of galvanic corrosion of steel tipped silver pins in body fluids - a lesson which has been relearned many times in the years since. In the earliest biocompatibility experiments, Levert (1824) tested gold, silver, lead and platinum ligatures in dogs and found platinum to be the least irritating. The advent of Listerian antiseptic methods, which made possible the great advances in surgery of the late nineteenth century, also led to the more adventurous use of foreign implants, and it is probably no coincidence that Lister's own early reports included accounts of wire fixation of fractures (Lister 1883).

The earliest plating system for the fixation of fractures was described in the German literature by Hansmann (1886) who used nickel plated sheet steel. The plate was bent at a right angle so that one end protruded through the skin, as did the screw heads, in order to facilitate removal. Whether this was further assisted by sepsis is not known! Innovations were also being made in Britain, notably by Lane (1893) who used steel screws to fix ununited fractures, and later developed the forerunner of the modern fracture plate, fashioned from

high carbon (stout) steel (Lane 1914). Although it enjoyed a degree of popularity, Lane's plate was inherently weak, partly because of the relative brittleness of the material, and partly from deficiencies in its mechanical design, and breakage was not uncommon. Sherman (1912) introduced a major improvement with a more robust design made from the recently available vanadium steel, though this in turn developed problems as the plate, while mechanically satisfactory, was liable to corrosion and loosening. Contributions were also made around this time by Hey-Groves (1913) and Lambotte (1913). They studied various methods of fracture treatment and also investigated the materials used for internal fixation. Both found that magnesium plates corroded rapidly in the tissues and Hey-Groves suggested that nickel-plated steel produced the least reaction.

1.1.2 Stainless steel.

Progress had thus been made in overcoming infection and improving mechanical performance but it was clear that a more biologically compatible material was required. In addition further development was stimulated by the need for better methods of treating fractures of the femoral neck. The earliest version of stainless steel, containing 18% chromium and 8% nickel, became available in the 1920's. It was used by Smith-Peterson for his femoral neck nail which so greatly improved the treatment of femoral neck fractures (Smith-Peterson *et al*, 1931). A side plate was later added by Thornton (1937) to form the prototype of the now familiar proximal femoral fixation device. This "18-8" steel was far from being corrosion-free *in vivo*, as pointed out by later studies (Speed 1935, Harris 1938). The incorporation of molybdenum 2-4% was found to greatly improve its corrosion resistance. Although this alloy was originally patented in 1926, it was many years before "18-8-SMo" steel, as it was called, became the steel of choice for implantation. In the years since then, modifications in its

composition and fabrication have been made to produce the 316L stainless steel in use today.

1.1.3 Cobalt-chromium alloy.

Cobalt based alloys had been investigated by Zierold as early as 1924 in the form of Stellite (58% cobalt, 35% chromium, 4% tungsten) which was found to excite very little tissue reaction. Vitallium, a close relation of Stellite, which in addition contained molybdenum, became available around 1930. It found early use as a dental alloy, but only came to orthopaedic attention later with the studies of Venable who found that it was highly resistant to corrosion and produced virtually no bone or tissue reaction (Venable *et al* 1937). After that time it came progressively more into use as an alternative to stainless steel.

Smith-Peterson again made use of the new material. Despite the improvements in the fixation of femoral neck fractures failure and secondary arthritis remained a problem. This naturally led to interest in methods of arthroplasty and Smith-Peterson developed the concept of cup arthroplasty in an attempt to resurface the femoral head. After experimenting with glass and celluloid, he found that Vitallium had the necessary mechanical and biological properties (Smith-Peterson, 1948). His own results were reasonably good but were not repeated by other surgeons. Better methods of joint replacement were pursued.

The first total hip replacements were performed by Wiles as early as 1938 on young patients with Still's disease, using stainless steel for both components (Wiles, 1957). The prosthetic acetabulum was fixed to the pelvis with screws, and the new femoral head was on the end of a bolt inserted along the femoral neck and fixed to the femoral shaft with a plate and screws. His results were not encouraging however, and further developments were directed towards replacement of the femoral head alone. Thompson (1954) and Moore (1959)

developed their metal prostheses with more success. Both were made from cobalt-chromium-molybdenum alloy and had stems for insertion into the femoral shaft. With the desired mechanical properties and tissue acceptability they came into regular use and remain so today along with a number of similar devices.

1.1.4 Titanium alloy.

After the second world war, the commercial production of titanium increased markedly. It was well known for its high degree of corrosion resistance and the metal soon came to medical attention. Early studies by Leventhal (1951) and Beder *et al* (1956) indicated a high degree of tissue tolerance to the metal and by the late 1950's, titanium implants were being marketed in the United Kingdom. Initially, commercially pure titanium was used but was found to lack the desired fatigue resistance in load-bearing components. In the early 1970's, titanium-aluminium-vanadium alloy (Ti6Al4V) was introduced, having superior mechanical qualities but similar tissue acceptability to the pure metal.

The compositions of the three alloy systems used in orthopaedic implants are listed in Table 1.

1.1.5 Recent developments.

Further developments in implant surgery, particularly in the field of joint replacement have been aimed towards specific surgical and biomechanical problems. Femoral head replacements caused long term problems with acetabular erosion and were ineffective in treating established arthritis. The need for a satisfactory total hip replacement was clear. McKee (1951, 1966, 1970) and Charnley (1960, 1964, 1972) developed their prostheses concurrently but along different lines. McKee chose Vitallium for both components and produced a reasonably

successful prosthesis. Charnley used stainless steel but made his major advances in the use of polymethylmethacrylate cement for anchorage of the components, and the development of the "low friction" joint using polyethylene for the acetabular component. The great success of Charnley's design led to the introduction of countless variations of the original using all the standard orthopaedic alloys.

Mechanical loosening remains the major, and perhaps inevitable cause of joint replacement failure, particularly in younger more active patients. Revision surgery is often difficult due to loss of bone stock and the presence of retained cement. Ring (1974, 1983) had persisted with uncemented methods, relying on an interference fit for the stability of his components. Initially using an all-metal cobalt-chromium prosthesis he later changed to the now standard metal-plastic articulation. His results prompted many surgeons to adopt uncemented methods in an attempt to avoid the problems of revision surgery and in recent years increasing numbers of uncemented joint replacements have been inserted into relatively young patients.

The most recent development in the field of implant metals has been the introduction of porous coated implants, using either cobalt-chromium alloy (Welsh *et al* 1971) or titanium (Galante *et al* 1971). The aim is to encourage bone and soft tissue growth into the surface pores of such components to achieve "biological fixation" of the implant. Numerous devices are now available with certain parts of their surfaces covered with porous layers made from sintered cobalt alloy spheres, sintered pure titanium spheres or titanium fibres. The long term advantages of these devices remains to be proven however.

Table 1. Principal constituents of orthopaedic implant alloys.

Stainless Steel	
Iron	59-70%
Chromium	17-20%
Nickel	10-17%
Molybdenum	2-4%

Cobalt-chromium alloy (Vitallium)	
Cobalt	57-65%
Chromium	27-30%
Molybdenum	5-7%

Titanium alloy (Ti6Al4V)	
Titanium	88-92%
Aluminium	5-6%
Vanadium	3-4%

1.2 Corrosion and wear of modern implant alloys.

Until the last thirty years or so, the gradual refinements in alloy composition and design discussed in the previous section were made largely on the basis of rather crude assessment methods. Implanted metals, whether in animal models or in human patients, were inspected for gross signs of corrosion or mechanical failure. Implant sites were examined at surgery and radiographically for evidence of bone or soft tissue reaction, occasionally with the addition of histological study. In recent years improved analytical techniques have shown that modern implant alloys, though highly resistant to corrosion in the industrial environment or under simple test conditions, can still release appreciable amounts of corrosion products *in vivo*. This was clearly demonstrated by Ferguson *et al* (1960, 1962). In a series of experiments on rabbits, using a number of metals and alloys, they found increased levels of metal in the tissues surrounding static implants and in other organs distant from the site of implantation.

1.2.1 Rates of corrosion.

A full account of the mechanisms of *in vivo* corrosion of metal implants is beyond the scope of this work and may be found in other sources (Williams and Roaf 1973, Black 1988a). All metals are liable to develop a corrosion current in an aggressive electrolyte solution such as the interstitial fluid. This is known as uniform attack. During manufacture the corrosion resistance of metal implants is increased by acid treatment. This process, known as passivation, creates a surface layer of metal oxides and hydroxides which acts as an insulator. Steinemann (1980) has estimated the corrosion current to be around 10^{-2} to 10^{-3} $\mu A/cm^2$ for a cobalt-chromium implant with an intact passivating layer. This would be equivalent to a release of approximately 30-60 μg metal/day (11-22 mg/year) for the average femoral component of a total hip

replacement (Black 1988b). This calculation was based on a hypothetical femoral component with a total surface area of 200 cm^{-2}.

Other factors however may increase this basic rate. *In vitro* experiments indicate that the presence of serum proteins shifts the equilibrium towards more metal release. In the clinical situation, a well fixed cement mantle may afford some protection by reducing the exposed surface area though this is not entirely certain. Conversely, the use of uncemented components exposes a greater surface area of metal to the tissue fluids, and with the introduction of porous coated implants this increased surface area effect may be further enhanced.

Other forms of corrosion may add to or accelerate corrosion in metal implants. Galvanic corrosion occurs when two metals of differing electrochemical potential lie in contact in the electrolyte solution. The baser of the two metals corrodes preferentially while the other is protected. In clinical practice this situation only occurs with combinations of stainless steel and other alloys and is therefore avoided. Cobalt-chromium and titanium alloy have similar electrochemical potentials and can usually be combined without difficulty.

Crevice corrosion, as the name suggests, occurs in cracks or recesses such as between screw heads and plates. It appears to be related to oxygen depletion within the crevice relative to the surface of the device. It is probably of most concern for its possible weakening effect in fixation devices though it has been suggested that crevice corrosion may occur in the gap between metal and cement in joint replacements.

In joint replacements the components are not static but articulate with each other causing wear or fretting of the surface of the components. In metal/plastic articulations in normal conditions the wear on the metal side is not likely to be great, though there may be damage to the passivating layer. This process may be increased further by the presence of bone or cement debris

scratching the articulating surfaces and causing "third-body" wear. Mechanical loosening of the prosthesis may also increase relative movement of the components and consequently increase wear and metal release.

1.2.2 Metal release from all-metal joints.

Heavy tissue staining with metal wear debris or "metallosis" was a common finding with metal-on-metal articulations. The use of all-metal joint replacements has now been abandoned in favour of the mechanically superior metal-on-plastic articulation. Coleman, Harrington and Scales (1973), in one of the earliest studies, showed the importance of wear in all-metal hip joints. All nine patients with metal-on-metal joints had significantly raised blood and urine metal concentrations when compared with three patients with metal-on-plastic hips who had no measurable increases in blood or urine. Vernon-Roberts and Freeman (1977) examined the tissues adjacent to hip replacements at revision procedures. They found great differences in the levels of cobalt, chromium and nickel between all-metal CoCr joints, which gave very high levels, and CoCr-plastic hips which had lower levels. In addition, they found intermediate levels next to CoCr hemi-arthroplasties articulating with bone. Smethurst and Waterhouse (1978) examined all-metal hips requiring revision and recorded very high levels of cobalt and chromium in the tissues adjacent to the loose prostheses. Dobbs and Minski (1980) in a post mortem study also found high local levels but reported levels in distant organs including lungs, liver and spleen to be up to 50 times the expected values.

1.2.3 Metal release from metal-on-plastic joints: stainless steel and cobalt-chromium alloy.

More recent work has shown that measurable amounts of metal can be released by metal-on-plastic joints. Studies carried out on patients some years after joint replacement indicate local and systemic increases in metal concentrations in the presence of prosthetic loosening. Pazzaglia *et al* (1983) found increased blood and urine levels of chromium, nickel and manganese in patients with stainless steel-polyethylene total hip replacements. A further study showed increases in plasma and urine of cobalt and chromium in patients with CoCr-polyethylene joints (Pazzaglia *et al* 1986). Most of the patients in these studies had radiological evidence of loosening of the femoral component. Patients undergoing revision surgery for aseptic loosening of CoCr-polyethylene hip replacements also have increased concentrations of cobalt, chromium and nickel in the tissues surrounding the prosthesis (Michel *et al* 1984, Lewis *et al* 1991). Most recently, a post mortem study of a patient with a loose Charnley stainless steel-polyethylene hip replacement found evidence of dissemination of metallic debris to distant sites including lymph nodes, liver and spleen (Langkamer *et al* 1992).

Fewer investigations have been carried out on patients with well-fixed joint replacements and results are somewhat conflicting. Some studies on patients before and after CoCr-polyethylene total hip replacement revealed transient increases in serum chromium and nickel concentrations in the post-operative months (Black *et al* 1983, Bartolozzi and Black 1985). Others show post-operative increases in serum cobalt but not in chromium or nickel (Michel *et al* 1991). Linden *et al* (1985) found no evidence of increased blood nickel levels in 12 out of 13 patients with well-fixed stainless steel-polyethylene hip replacements nine to fifteen years after implantation. The one patient with hypernickelaemia also had renal insufficiency which may have been a contributory factor.

1.2.4 Wear of titanium alloy implants.

Of the materials currently in use, titanium and its alloys are the most recent on the scene with reputedly the greatest degree of corrosion resistance but, paradoxically, growing evidence of the greatest susceptibility to wear. Metallosis, formerly a common feature of all-metal joint replacements, has not been a problem with stainless steel or cobalt-chromium articulating with polyethylene. However, in recent years a number of papers have reported this finding in titanium alloy-polyethylene joints. Agins *et al* (1988) noted dark staining of the tissues around titanium alloy implants at revision procedures. On measuring the content of titanium, aluminium and vanadium in the tissues adjacent to loose femoral components they found alarmingly high levels in many cases. Similar tissue staining at revision procedures was described by Witt and Swann (1991). Black *et al* (1990) reported a case of metallosis in a well fixed Ti6Al4V-polyethylene hip replacement in which severe wear of the femoral head appeared to be the cause. These reports have prompted suggestions for alterations to the present titanium alloy in order to improve its wear resistance (Scales 1991).

The problem is illustrated in figures 1.1 and 1.2 which show sections of pseudocapsule removed at revision of a titanium alloy-polyethylene hip replacement. Revision was carried out for aseptic loosening three years after implantation. At operation the femoral component was loose within the femoral canal and the surrounding tissues were darkly stained with wear debris.

1.2.5 Metal release from uncemented and porous coated implants.

Studies of patients with uncemented or porous coated prostheses have yet to show clearly whether such devices liberate proportionately greater quantities of metal. Dorr *et al* (1990), in an ambitious study, examined the tissues of 46 patients with CoCr-polyethylene and Ti6Al4V-polyethylene hip replace-

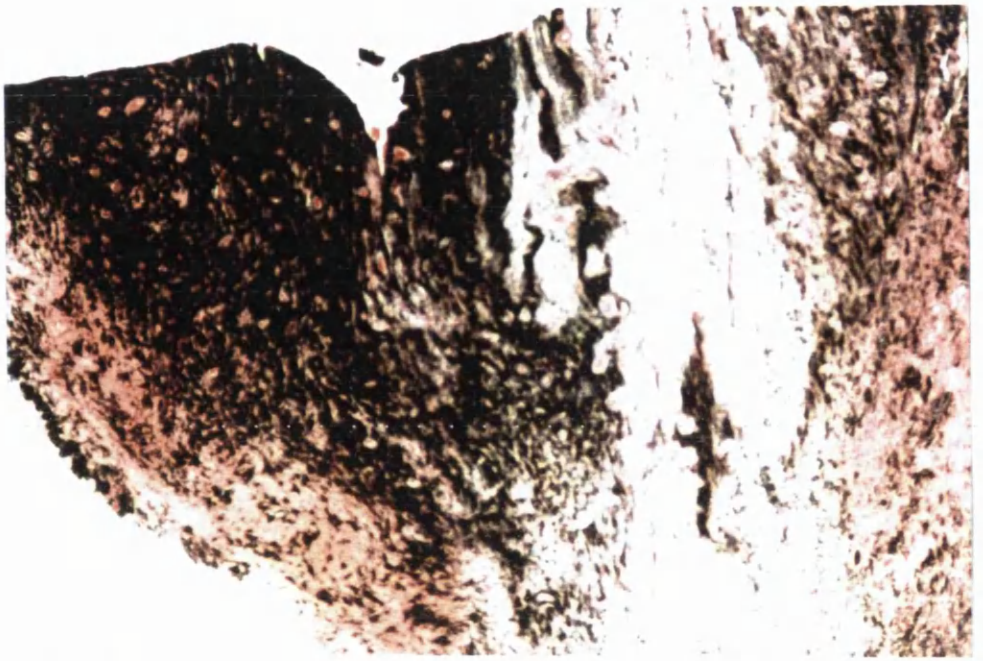


Fig. 1.1 Pseudocapsule removed at revision of Ti6Al4V femoral component. Low power view showing blackening of tissue with metal wear debris. (H&E, x25).

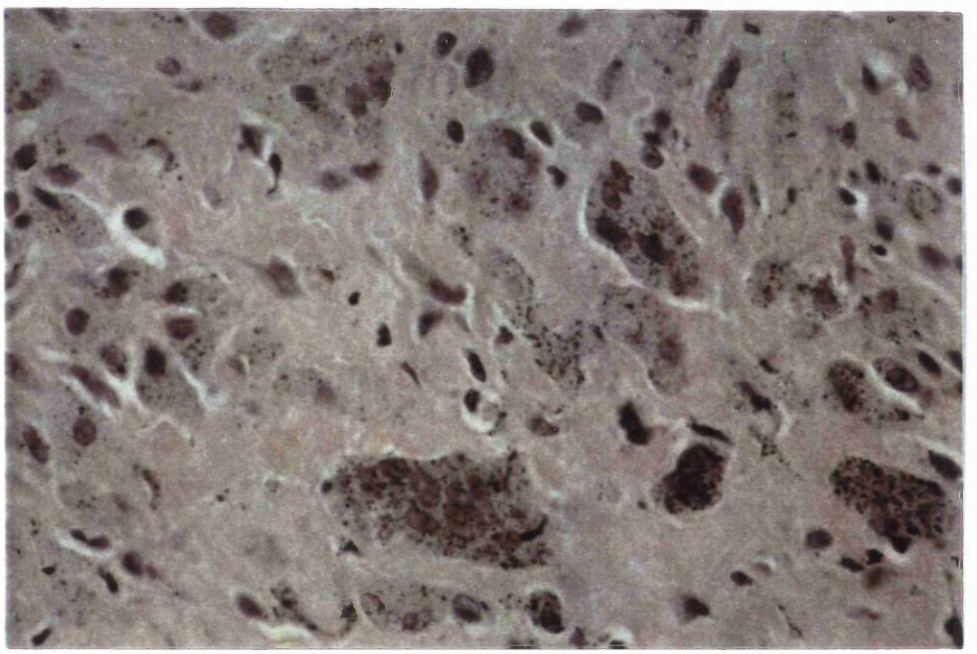


Fig. 1.2 High power view of less densely blackened area shows tissue macrophages and multinucleate giant cells containing particulate debris. (H&E, x400).

ments. There were cemented and uncemented varieties of each and they further subdivided them into loose and well-fixed prostheses. The final numbers in each subgroup were consequently small but there appeared to be a slightly increased level of metal ion release in the uncemented joints. Overall, the highest levels were associated with loose implants of both cemented and uncemented types.

Braun *et al* (1986) measured urinary cobalt and chromium in patients with a CoCr porous implant estimated to increase the surface area by about a factor of three. They found significant increases in chromium but not cobalt compared with controls. Output of both metals was far below levels found in patients with McKee-Farrar all-metal joints included in the same study. Conversely, Jorgensen *et al* (1983) compared patients with porous and non-porous CoCr hemi-arthroplasties and found a 69% increase in urinary cobalt in the porous group, though the numbers included were small and there was marked overlap between the groups. Sunderman *et al* (1989) examined a group of patients with porous-coated knee and hip replacements and found small but significant increases in serum and urine cobalt in those with knee replacements but not those with hip replacements.

At present there is therefore no substantive evidence that the increased surface area of porous-coated implants causes significant increases in metal release. However, concern has been expressed that loosening of sintered coatings may lead to accelerated fretting corrosion in some circumstances (Buchert *et al* 1986, Hamblen and Paul 1988).

Previous studies of metal release from implants have therefore produced varying and sometimes conflicting results. This reflects the many difficulties inherent in such work which include variations in implant designs and surgical techniques, differences in tissue selection and sampling methods, and

different methods and standards of analysis. As a result the actual values recorded from patients and controls may vary greatly from one study to another. Nevertheless the weight of evidence indicates that all joint replacements liberate some of their constituent metals and that the amounts are greatly increased by loosening and wear.

1.3 The adverse effects of implanted metals.

The general toxicity of many metals, including some of those used in orthopaedic implants, is well established and there is an extensive literature on the subject (Williams 1981). The overall effect of any implant on the recipient is the result of the balance between the properties of the implant materials and the host response to those materials. These responses may result in local effects around the implant or systemic effects elsewhere in the body.

1.3.1 Local effects.

All solid implants provoke a non-specific local reaction in the form of the normal healing response followed by a foreign body response. The latter is seen as an attempt to either break down or isolate the foreign material and results in the implant being enveloped in a fibrous capsule. This may be of little consequence in the case of static devices such as fracture plates or may be the beginnings of mechanical loosening in the mobile components of a joint replacement.

With the release of corrosion and wear products other tissue responses are provoked. Williams and Meachim (1974) studied the tissue changes around nonarticulating stainless steel implants and demonstrated varying degrees of inflammatory cell infiltration associated with areas of corrosion. In most cases this was not clinically significant but in some was severe enough to cause symptoms requiring removal of the device. Rae (1981) investigated the effects of

various metals at low concentrations on cultured human fibroblasts using enzyme release and morphological changes as indicators of toxicity. Toxic effects were observed with cobalt and nickel but, based on estimates of metal release from other experiments, he suggested that in clinical conditions toxic levels of metal were only likely with metal-on-metal cobalt-chromium hip replacements where excessive wear could produce high levels of cobalt. The evidence for direct cellular toxicity from metal in clinical circumstances is less clear cut. Cases of extensive aseptic tissue necrosis in association with high levels of metallic wear debris from cobalt-chromium joint replacements are well documented (Evans *et al* 1974, Jones *et al* 1975). However, the histological findings suggested that the severe tissue damage resulted from hypersensitivity to the metals involved rather than a direct toxic effect.

More recently metallic wear debris from titanium alloy hip replacements has been shown to produce severe granulomatous reactions leading to osteolysis and failure of the prostheses (Lombardi *et al* 1989). This reaction is not unique to metallic debris and is well known in association with polyethylene wear particles.

1.3.2 Systemic effects.

Many metals play essential roles in normal metabolic processes. Indeed, all of the major constituents of implant alloys with the exceptions of aluminium and titanium have been identified as essential trace elements (Mertz 1981). Chromium is required for the normal action of insulin in sugar metabolism and clinical effects from chromium deficiency have been described in patients on parenteral nutrition. Cobalt is an essential constituent of vitamin B12 and was formerly used in the treatment of anaemia. However, all trace elements can cause toxicity when the normal range of exposure is exceeded. Defects in

iron metabolism lead to haemochromatosis. The use of cobalt as a stabiliser in beer led to an outbreak of cardiomyopathy in heavy drinkers (Alexander 1969). Most recently aluminium has been implicated in the aetiology of Alzheimer's disease (Perl and Brody 1980). The effect of corrosion products on metabolic processes is not known. However, trace element analysis studies of periprosthetic tissues have shown changes, not only in the tissue levels of those metals derived from implants but also in the levels of other metals, suggesting some alteration in metabolic mechanisms (Michel *et al* 1984).

Metals by themselves do not cause allergies as such but, in combination with proteins, they are capable of provoking an immune response. Metal sensitivity is well recognised with items of jewellery and there is evidence that it occurs in patients with implants. Cramers and Lucht (1977) described dermatitis associated with stainless steel fracture plates in patients with positive skin tests for chromium and nickel. More recently it has been suggested that sensitivity to titanium may occur in some cases (Lalor *et al* 1991). The number of patients with overt metal sensitivity related to implants appears to be relatively small but studies using more sensitive cell migration inhibition tests have demonstrated sensitivity in over 50% of patients with implants (Merritt and Brown 1985).

The extent to which metal implants exert adverse or unwanted effects depends on a great number of factors. In the great majority of patients they do not appear to cause clinically significant problems but it is clear from this brief review that such effects may occur in a number of ways. One other possible risk of metal implantation remains - the risk of malignant change.

1.4 Metal-associated cancer.

Of all the possible biological effects of implanted metals, by far the most worrying is the risk of malignant change. A number of metals have received attention as potential carcinogens in the industrial environment and many have been investigated experimentally. The general field of metal carcinogenesis is reviewed extensively by Sunderman (1978,1984). Our knowledge of metal carcinogenesis comes from a number of sources. These include epidemiological studies of environmental and industrial exposure, animal experiments, cell culture studies, and, of particular interest in orthopaedics, reports of malignant tumours in patients with metal implants.

1.4.1 Epidemiological studies of industrial exposure.

Of the metals commonly used in orthopaedics, chromium and nickel are strongly implicated by epidemiological studies. Workers exposed to chromate dust have increased rates of lung cancer mortality (Alderson *et al* 1981, Satoh *et al* 1981, Hayes *et al* 1979), and nickel refinery workers have increased mortality from lung or nasal sinus malignancies (Chovil *et al* 1981, Magnus *et al* 1982, Enterline and Marsh 1980). The site of these tumours in the respiratory tract is clearly related to the route of exposure i.e. inhalation. How this relates to metal exposure from orthopaedic implants is less clear. Such studies have also shown that, as with other human carcinogens, there is usually a long latent period between exposure to the metals and tumour manifestation.

1.4.2 Carcinogenesis bioassays in animals.

A large number of reports have now established the ability of various metals to produce malignant tumours in experimental animals. These metals include nickel, chromium, cobalt and iron, in addition to a number of other metals

not normally used for orthopaedic purposes. These studies are too numerous to mention in detail, but are comprehensively reviewed by Sunderman (1978,1984) and Pedley *et al* (1981). Some are of particular interest as they studied orthopaedic alloys.

Oppenheimer *et al* (1956), implanted discs of metal foil subcutaneously in rats. A number of alloys were studied including stainless steel and vitallium, both of which were associated with tumour formation. Heath *et al* (1971) and later Swanson *et al* (1973), reported on the occurrence of sarcomata in 22 out of 80 rats injected with wear particles from cobalt-chromium alloy joint prostheses. The particles were generated in laboratory simulators and suspended in horse serum before injection. The presence of the horse serum may have had some influence on the outcome. Conversely, Meachim *et al* (1982) implanted cobalt-chromium particles in the dry state and in two size ranges into a large number of rats and guinea pigs and found no evidence of sarcogenicity. Investigating the effects of solid implants, Gaechter *et al* (1977) studied several orthopaedic alloys including stainless steel, wrought and cast cobalt-chromium alloy, pure titanium and Ti6AlV4. These were implanted as solid rods in the rat gluteal muscle. No local malignancies arose and there was no significant difference in generalised tumour rates between implanted rats and controls. Memoli *et al* (1986) studied a number of alloys implanted intraosseously as solids, powders or fibres. They noted a slight increase in implant site malignancies with powders and fibres of cobalt-chromium-nickel alloys but not with solid implants.

It is also worth noting that a number of other animal studies looking principally at the toxicity of orthopaedic alloys have also lacked evidence of tumour formation. Thus, although there is strong evidence of the carcinogenicity of individual metals in animals, the evidence from studies looking specifically at orthopaedic alloys in animals under experimental conditions is conflicting.

There are however, a number of reports in the veterinary literature of osteosarcoma associated with bone plates inserted for the treatment of fractures in dogs (Stevenson *et al*, 1982).

1.4.3 Implant-associated malignancy in patients: a case report.

Much of the recent interest in the carcinogenic risks of orthopaedic materials has arisen from the ever increasing number of reports of malignancies associated with metal implants in clinical practice. The following case is reported by kind permission of Mr. A.L. Gunn F.R.C.S.

In September 1975, a sixty-two year old man was admitted as an emergency following a road traffic accident in which he suffered multiple injuries including fractures of the right humerus and olecranon, multiple rib fractures, fracture of the neck of the right femur and a compound fracture of the shaft of the right femur.

After a prolonged period of in-patient treatment, the soft tissues healed and the fractured shaft of femur was fixed with a Kuntscher nail and bone grafting. As with his other fractures, the femoral shaft fracture then went on to unite uneventfully. Thereafter he appeared to do well despite malunion of the femoral neck fracture.

He was not seen again until July 1990 when he presented with a swelling in relation to the upper end of his right femur. Radiographs revealed a lytic lesion in the proximal femur (Fig.1.3). The swelling was explored and found to consist of a large mass of necrotic material arising from the femur and almost surrounding the intramedullary nail. Tissue sent for histological examination showed the features of an osteosarcoma (Fig.1.4).

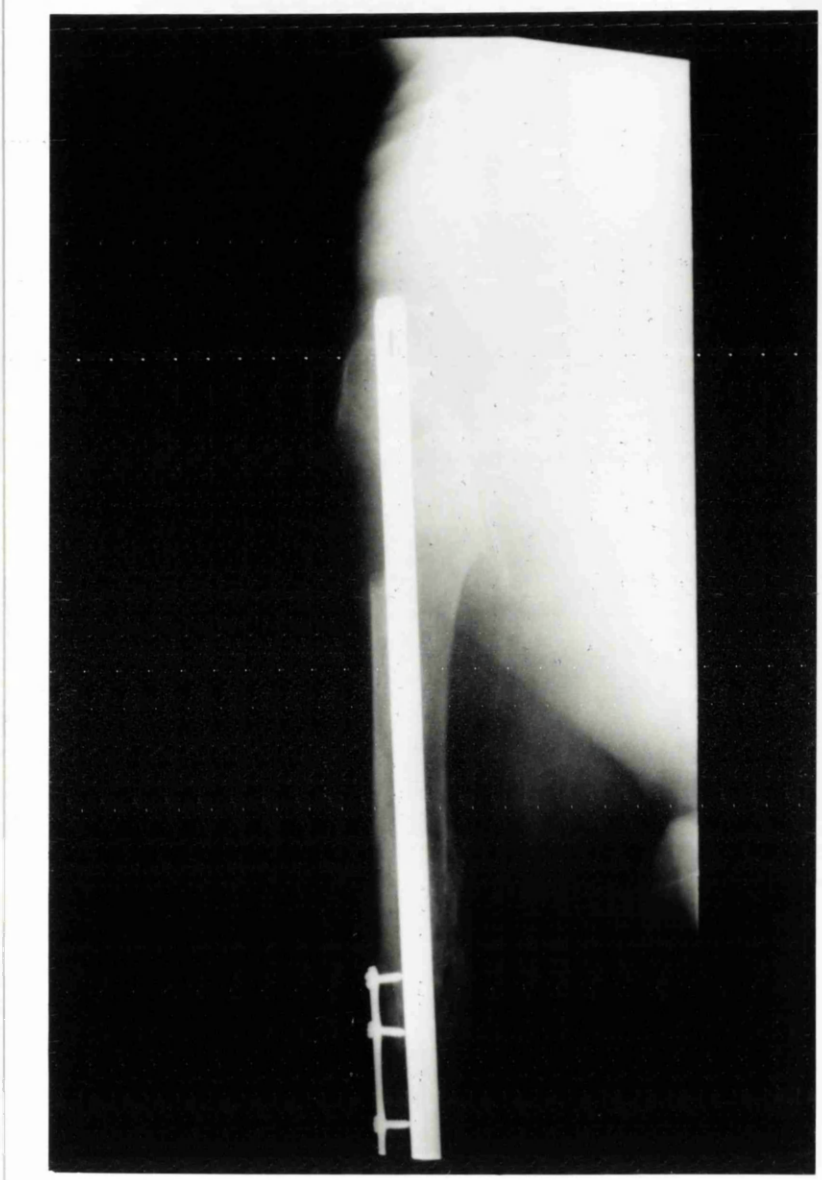


Fig. 1.3 Radiograph of right hip and femur 15 years after Kuntscher nailing. There is a lytic lesion eroding the cortex of the proximal femur in close proximity to the nail.

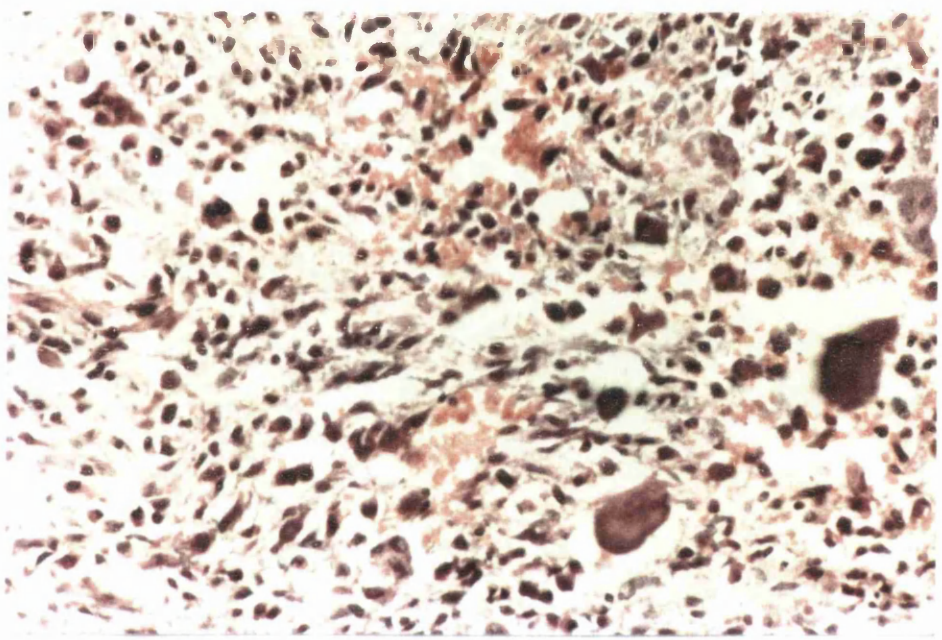


Fig. 1.4 Histological specimen from patient in Fig.1.3 The cells are pleomorphic with hyperchromatic nuclei and eosinophilic cytoplasm. There are spindle cells, other bizarre forms and osteoclast-like giant cells. The features are those of an osteosarcoma. (H&E, x250).

1.4.4 Implant-associated malignancy in patients: review of the literature.

In the past there were sporadic reports of carcinoma associated with retained metallic foreign bodies in the lung (Siddons and McArthur, 1952). Before 1980 there were only four reports of tumour associated with orthopaedic implants. In the last decade however, a number of new cases have emerged. These are summarised in Tables 2 and 3. Also included are four otherwise unpublished cases recently reported by the Journal of Bone and Joint Surgery (Goodfellow 1992). To date there appears to have been 30 such cases in total, including the case reported in this thesis. Twenty have involved joint replacements and ten have involved fixation devices.

Three reports cited in the literature are not included. The case mentioned by Arden and Bywaters (1978) is almost certainly the same as that reported by Swann (1984). The case described by Kolstad and Hogstorp (1990) was a secondary deposit from a primary gastric carcinoma. Although the surgical trauma of joint replacement may have predisposed to the site of the metastasis, it clearly cannot be contemplated as "implant-associated" in the same way as the other reported primary tumours. Rushforth (1974) reported on a patient who developed an osteosarcoma of the pelvis six months after total hip replacement who had also had radiotherapy for carcinoma of the cervix nineteen years previously. This was most probably a case of post-irradiation malignancy.

With respect to the nature of the tumours, there is clearly no uniformity of tumour histology. The majority are sarcomata, but there are also two cases of lymphoma. The single most common tumour type is malignant fibrous histiocytoma, which is of particular interest as it has been postulated that this tumour may arise from a chronic reparative process (Mirra *et al* 1974, Dunham and Wilborn 1979).

The types of implants are also diverse and the alloy composition is

Table 2: Reports of malignant tumours associated with joint replacement.

Report	Implant (Metal if known)	Operation	Age at diagnosis	Latent period	Histology
Castleman & McNeely 1965	Nail plate + Austin-Moore	Hemiarthroplasty # neck of femur	56	3 yrs	Giant cell-rich sarcoma
Bago *et al* 1984	Charnley-Muller THR	Arthroplasty for osteoarthritis	77	2 yrs	Malignant fibrous histiocytoma (MFH)
Swann 1984	McKee-Farrar THR (Vitallium)	Arthroplasty for osteoarthritis	63	4 yrs	Malignant fibrous histiocytoma (MFH)
Penman & Ring 1984	Uncemented THR (Vitallium)	Arthroplasty for osteoarthritis	80	5 yrs	Osteosarcoma
Weber 1986	TKR (Cobalt-chromium)	Arthroplasty for osteoarthritis	81	4½ yrs	Epithelioid sarcoma (probably MFH)
Ryu *et al* 1987	Uncemented THR (Co-Cr/ceramic)	Arthroplasty for osteoarthritis	53	1 yr	Undifferentiated sarcoma
Martin *et al* 1988	Charnley-Muller THR (Cobalt-chromium)	Arthroplasty for osteoarthritis	76	10 yrs	Osteosarcoma

Table 2: Reports of malignant tumours associated with joint replacement (continued).

Report	Implant (Metal if known)	Operation	Age at diagnosis	Latent period	Histology
Tait *et al*, 1988	Charnley-Muller THR	Arthroplasty for osteoarthritis	56	11 yrs	Malignant fibrous sarcoma
van der List *et al*, 1988	Charnley-Muller THR (Cobalt-chromium)	Arthroplasty for osteoarthritis	83	11 yrs	Malignant epithelioid haemangioendothelioma
Lamovec *et al*, 1988	Charnley-Muller THR 2 patients	Arthroplasty for osteoarthritis	62 65	12 yrs 10 yrs	Synovial sarcoma Spindle cell sarcoma
Haag & Adler, 1989	Weber-Huggler THR (Vitallium)	Arthroplasty for osteoarthritis	79	10 yrs	Malignant fibrous histiocytoma (MFH)
Brien *et al*, 1990	Charnley THR (Stainless steel)	Arthroplasty for osteoarthritis	69	8 yrs	Osteogenic sarcoma
Nelson & Phillips, 1990	Muller THR (Cobalt-chromium)	Arthroplasty for osteoarthritis	72	10 yrs	Malignant fibrous histiocytoma
Troop *et al*, 1990	Charnley-Muller THR Turner-Aufranc THR	Multiple revision procedures	54	15 yrs	Malignant fibrous histiocytoma

Table 2:Reports of malignant tumours associated with joint replacements (continued).

Report	Implant (Metal if known)	Operation	Age at diagnosis	Latent period	Histology
Jacobs *et al*, 1992	AML THR (Cobalt-chromium)	Arthroplasty for osteoarthritis	65	6 mths	Malignant fibrous histiocytoma
Harris (Can) *	Charnley (Stainless steel)	Arthroplasty for osteoarthritis	65	1 yr	Chondrosarcoma
Surin (Swe) *	Christiansen	Arthroplasty for osteoarthritis	59	9 yrs	Pleomorphic rhabdomyosarcoma
Lightowler (UK) *	Charnley (Stainless steel)	Arthroplasty for osteoarthritis	64	10 yrs	Poorly differentiated spindle cell sarcoma
Rees *et al* (UK) *	Thompson	Hemiarthroplasty # neck of femur	88	3 yrs	Malignant fibrous histiocytoma

* Not published in full: see Goodfellow 1992.

Table 3. Reports of malignant tumours associated with fixation devices.

Report	Implant (Metal if known)	Operation	Age at diagnosis	Latent period	Histology
McDougall 1956	Plate and screws (Stainless steel)	Fixation of # humerus	42	30 yrs	Malignant tumour uncertain histogenesis
Delgado 1958	Egger's plate and screws	Fixation of # tibia	40	4 yrs	Osteosarcoma
Dube & Fisher 1972	Plate and screws (Stainless steel)	Fixation non-union of tibia	84	36 yrs	Haemangioendothelioma
Tayton 1980	Plate and screws (Vitallium)	Fixation of osteotomy	11	7½ yrs	Ewing's sarcoma
McDonald 1981	Plate and screws (Vitallium)	Fixation of # tibia	48	17 yrs	Non-Hodgkin's lymphoma
Dodion *et al* 1982	Plate and screws (Vitallium)	Fixation of # neck of femur	50	14 mths	Non-Hodgkin's lymphoma

Table 3. Reports of malignant tumours associated with fixation devices (continued).

Report	Implant (Metal if known)	Operation	Age at diagnosis	Latent period	Histology
Lee *et al* 1984	Plate and screws	Fixation of # shaft of femur	59	15 yrs	Malignant fibrous histiocytoma
Hughes *et al* 1987	Single screw (Cobalt-chromium)	Fixation slipped upper femoral epiphysis	42	29 yrs	Malignant fibrous histiocytoma
Ward *et al* 1990	Smith-Peterson nail (Stainless steel)	Fixation of # neck of femur	65	9 yrs	Osteogenic sarcoma

not clear in some cases. The majority were made from cobalt-chromium alloy or stainless steel. One had aluminium oxide ceramic components. Only one case definitely had a titanium alloy component (Troop *et al* 1990). However this was the last in a series of prostheses inserted over many years the previous ones being cobalt-chromium alloy. Of the cases where the alloy composition is not stated, judging from the dates and types of implant, it is unlikely that they were made from titanium.

Two of the reports made note of gross corrosion of the implants (McDougall 1956, Dube and Fisher 1972), and in the cases of Bauer *et al* (1987) and Ward *et al* (1990), the tumour tissue was found to have excess levels of metal on spectroscopic examination.

It is generally accepted that the induction of malignant change by chemical carcinogens in humans usually involves a long latent period between exposure and the manifestation of the malignancy. In 15 of the reported cases the latent period was ten years or more and in the case of Ryu *et al* (1987) fixation screws had been in place for 12 years before hip replacement. In several of the others the interval was exceedingly short, casting doubt on the role of the metal in these patients.

Some cases had additional factors which may have contributed to the risk of malignancy. In Weber's patient there was a bone infarct present preoperatively in the distal femur, a condition known to have an association with malignant fibrous histiocytoma (Mirra *et al*, 1974). The patient reported by Dodion *et al* had previously had an infected fixation device at the same site. The risk of squamous carcinoma in osteomyelitis sinuses is well known and sarcoma has also been reported in association with bone infection (Akbarnia *et al*, 1976). The patient described by Troop *et al* had multiple revision procedures over many years, a prosthetic infection and a previously diagnosed bone infarct at the site of .pathe tumour.

Review of the known cases therefore reveals no definite correlations between implants and local malignancies. The number of reports, although increasing, remains very small in relation to the large numbers of implants in use.

1.4.5 Epidemiological studies in patients with joint replacements.

As pointed out by Black (1986), two additional factors should be considered. Primary bone tumours are very rare, accounting for only 0.25% of all primary tumours, which is equivalent to an incidence of around one per million of the adult population. Any increase in risk would therefore have to very large to be noticeable.

Alternatively, it may be that metal implants could increase the risk of tumour development in other more sensitive tissues. Investigating this possibility, Gillespie *et al* (1988) conducted the first epidemiological study of the incidence of all cancers following total hip replacement. In a series of 1358 patients treated between 1966 and 1973, the incidence of cancers of the lymphatic and haemopoietic systems was significantly greater than expected in the ten years following hip replacement, while over the same period there was a significant reduction in the rates of other cancers such as those of the breast, colon and rectum. While rightly advising caution in the interpretation of these results, the authors tentatively suggested a mechanism of "chronic stimulation of the immune system, encouraging the emergence of lymphoreticular malignancies but, by increasing immune surveillance, inhibiting the overt development of some epithelial cancers." A second study (Visuri and Koskanvuo 1991) produced remarkably similar results. In 433 patients followed for a mean of 10 years after McKee-Farrar all-metal total hip replacement, the risk of developing leukaemia and lymphoma increased while that of breast carcinoma decreased.

These studies suggest that patients with total hip replacements may have a different susceptibility to cancer from the general population. Alternatively, it is possible that patients with arthritis have a different predisposition. A number of possible variables must be considered in the interpretation of epidemiological studies but the results of these early studies are sufficiently intriguing to justify more investigations of this type.

1.5 Aims of this study.

The foregoing sections have attempted to summarise the developments and present knowledge of the biocompatibility of metal orthopaedic implants with particular reference to the risk of implant-induced cancer. Recent interest in this subject is reflected in three editorials published by the Journal of Bone and Joint Surgery. In the first, which followed three reports of tumours arising in the vicinity of total hip replacements, Hamblen and Carter (1984) stated that "the number of reported cases of sarcoma is so minute, compared with the vast number of replaced hips, that no surgeon or patient should feel undue concern on this account...". In the second, entitled "Malignancy and joint replacement: the tip of an iceberg?", Apley (1989) called for all surgeons to report incidences of tumours associated with joint replacements and said "Our aim is to discover if the association between replacement and local malignancy is purely coincidental and if not, to estimate the incidence of malignancy...". The most recent (Goodfellow 1992) publicised the result of this request which amounted to five new cases of malignancy. It was concluded that "no large iceberg has been revealed..." but that "it may be wise to suggest a verdict of 'not proven' rather than 'not guilty'...".

The ultimate answer to this question will come from the collective efforts and experience of orthopaedic surgeons and scientists in the fields of

biocompatibilty and cancer research. In the meantime a method of grading the relative risks of the various metals in current use would be of help in the rational choice of the safest implant materials.

This project therefore set out to examine the potential toxicity and carcinogenicity of the commonly used orthopaedic metals. It used cell culture systems designed to assess the ability of chemicals to induce changes in cell morphology and growth characteristics which are thought to be of importance in the carcinogenic process. Both human and animal cell systems were used, and the aims of the study were:

1. To demonstrate if certain cellular mechanisms associated with carcinogenesis could be induced by any, some, or all of the metals in question.
2. To establish, if possible, the relative risks of the individual metals and alloys in terms of toxicity and carcinogenicity.
3. To study the effect of the physical form in which the metals were presented.
4. To investigate the relationship, if any, between toxicity and carcinogenicity for each metal.
5. To compare the results in the human and animal cell systems in order to assess the value of each in considering the clinical situation.

CHAPTER TWO

THE USE OF SHORT TERM *IN VITRO* CARCINOGENICITY ASSAYS

2.1 Methods of detecting carcinogenic risk: the rationale of cell culture techniques.

Many of the substances now recognised as carcinogenic in humans were originally identified by the careful correlation of clinical observations and epidemiological studies of populations at risk. These methods remain of foremost importance in cancer research but require painstaking efforts in their construction and interpretation, and of course involve continued exposure of people to potentially dangerous materials. Even when the clinical and epidemiological evidence is incontrovertible as with tobacco, the precise identity and mechanism of action of the responsible agent or agents may remain uncertain. Other methods of investigation are needed.

Long term bioassays of carcinogenicity in experimental animals involve prolonged observation of animals over a substantial part of their lifetimes after or during continued exposure to suspected agents. Such studies allow the testing of a wide variety of agents with more precise manipulation of the methods of exposure. However they are time consuming, expensive and have extensive requirements for their construction and implementation, both for scientific and legal reasons. The great majority of such experiments are carried out on rodents, selected because of their availability, short lifespans and cheapness. Much has been learned from these studies, but the interpretation of their results must allow for all the possible interactions in a complex organism. Larger mammals such as non-human primates have none of the advantages of rodents and there is no indication that they would provide results more relevant to the human population.

For these reasons, great efforts have been made in recent years in

developing short term *in vitro* methods of carcinogenicity testing, both to study the cellular mechanisms of carcinogenesis and to allow rapid screening of chemicals and materials for carcinogenic risk.

Tissue culture originated in the early part of this century with the use of relatively crude methods to study growth from tissue explants (Harrison 1907). Since then it has evolved into a highly specialised field with sophisticated techniques for the study of basic cellular mechanisms. The major advantage of cell culture is the control it permits over the physicochemical and physiological environment of the cell. In addition, the development of specialised cell lines with homogenous characteristics minimises variability in response. Finally, the facility to use materials and reagents in small quantities affords advantages of economy. The main disadvantage of cell culture is the expertise required to avoid infection and maintain suitable environmental conditions. Despite this, there has been a massive explosion in the use of cell culture methods in the last 30 years, particularly in the field of cancer research. To quote Vasiliev and Gelfand (1981), "It would hardly be an exaggeration to say that the main achievements of cancer cell biology during the last two decades have been associated with the use of cell cultures."

2.2 The effect of metals in short term *in vitro* carcinogenesis assays.

Over the last 20 years or so, a number of *in vitro* methods have been developed to study the actions and mechanisms of carcinogens at a cellular level. These have allowed the identification of specific endpoints which can be used as indicators of carcinogenic potential. They include studies of bacterial mutagenesis, mammalian cell mutagenesis, chromosomal damage and DNA damage. A large number of metals have been studied using these systems but the following review will be restricted to metals of orthopaedic interest.

2.2.1 Bacterial mutagenicity assays.

Bacterial mutagenicity assays involve the manipulation of the growth requirements of the organism. Certain strains may need a particular amino acid in the growth medium for survival and in its absence no colonies will form. However, some strains have the capacity to change their growth requirements by mutation and when treated with a mutagenic agent will be able to form colonies in the deficient medium. The "Ames test" (Ames *et al* 1973a, 1973b) has been the most popular of these assays and measures reverse mutation in histidine dependent strains of Salmonella typhimurium. A similar assay utilises tryptophan dependent strains of Escherichia coli. Chromium in its hexavalent, but not in its trivalent form, has been found to be mutagenic in both the S.typhimurium and E.coli assays (DeFlora *et al* 1980, Venier *et al* 1982). However, nickel compounds known to be carcinogenic from other studies are not mutagenic in either assay (Sunderman 1981). Bacterial systems appear to be more reliable in detecting organic carcinogens than inorganic carcinogens (Costa 1980), and therefore have been of limited value in the study of metal carcinogenesis.

2.2.2 Mammalian cell mutagenicity assays.

The inherent differences between prokaryotic and eukaryotic cells place obvious limitations on the use of bacterial mutagenicity assays as screening methods for human carcinogens. The observation that various genetic changes, including gene mutation, occur in human tumour cells provides a rational basis for the use of assays to detect such changes in mammalian cell systems. Various metals have been studied in the V79 Chinese hamster cell system. This tests for mutagenicity at the hypoxanthine-guanine phosphoribosyl transferase (HGPTR) locus by selecting for 8-azaguanine resistance. Normal cells with HGPTR activity convert 8-azaguanine to toxic metabolites which cause cell death. Mutant cells

lose HGPTR activity and therefore survive. Miyaki *et al* (1979) found that beryllium caused a six-fold increase in resistance and that cobalt and nickel caused a two to four-fold increase. Hsie *et al* (1980) tested the mutagenicity of metals in Chinese hamster ovary (CHO) cells and found that among others, iron and nickel were mutagenic while titanium was not. Tests for induction of trifluorothymidine resistance at the thymidine kinase locus in mouse lymphoma cells have given positive results with nickel and hexavalent chromium and negative results with cobalt and aluminium (Amacher and Paillet 1980, Oberly *et al* 1982).

2.2.3 Chromosomal aberrations.

As with gene mutation, chromosomal aberrations such as translocations and deletions have been observed in tumour cells. Chromosomal alterations often lead to cell death but some such alterations allow cell survival and may be involved in the process of neoplastic change. Chemicals that induce structural chromosomal abnormalities are known as clastogens and various systems have been developed to detect such clastogenic effects. These are classified into two main groups - deletions and rearrangements of chromosomes, or sister chromatid exchanges (symmetrical exchanges of segments within a single chromosome). The clastogenic effects of metals have been demonstrated in a number of studies. Umeda and Nishimura (1979), using a mouse mammary carcinoma cell line found that nickel and hexavalent chromium compounds induced chromosomal aberrations while trivalent chromium did not. Larramendy *et al* (1981) observed that nickel caused chromosomal damage and increased frequencies of SCE in both Syrian hamster embryo cells and human lymphocytes. Ohno *et al* (1982) found that nickel and chromium compounds produced increased numbers of sister chromatid exchanges in Chinese hamster cells whereas titanium and iron did not.

2.2.4 DNA damage.

On the basis that carcinogenic agents exert their primary effects at the level of the DNA molecule, a great many methods of measuring the interactions of chemicals with DNA have been developed. These include measures of DNA binding, DNA strandbreaks and crosslinks, transcription infidelity and DNA repair mechanisms. A full account of these techniques is outwith the scope of this work but the results of studies of metals in these systems is reviewed by Sunderman (1984). Of the metals used in orthopaedic implants, chromium, nickel and cobalt have been shown to have damaging effects on DNA.

In summary then, these studies demonstrate that some of the metals used in orthopaedic practice are capable of causing various types of genetic damage indicative of carcinogenic potential.

2.3 *In vitro* cell transformation.

While many advances have been made through the use of the methods discussed in the previous section, they are largely based on the theoretical correlation between the measured endpoints and the carcinogenic process. This does not detract from their usefulness in studying cellular mechanisms, but does place limitations on their use as screening tests. For example, it can be said that all carcinogens are mutagens but it cannot be assumed that the reverse is true.

It is now generally accepted that carcinogenesis is a multistage process. This is borne out by a large amount of experimental evidence and is reflected in clinical observations such as the long latent period between exposure to carcinogens and tumour development, and the existence of various premalignant conditions which precede human and experimental cancers. These considerations indicated the need for test systems in which the measured endpoints had a

direct bearing on the natural history of cancer development *in vivo*, and led to the development of cell transformation assays.

Tumour growth in an animal is the result of a breakdown in the normal regulation of cell proliferation in the involved tissue. This leads to the development of recognisable alterations in behaviour such as abnormal differentiation, loss of normal histology and, in the case of malignant tumours, invasion of surrounding tissues and spread to secondary sites. Cell transformation assays attempt to mimic such changes within the confines of cell culture conditions. Cell transformation can therefore be defined as the induction in cultured cells of certain phenotypic alterations that are related to the carcinogenic process. They include morphological transformation, focus formation on cell monolayers, aquisition of indefinite lifespan, growth in semisolid media and altered growth requirements. These changes have been shown to be associated with neoplastic conversion of certain cell types by virtue of their ability to produce tumours in experimental animals.

The first demonstration of *in vitro* transformaton was reported by Berwald and Sachs (1963, 1965). They exposed primary and secondary cultures of Syrian hamster embryo cells to two carcinogenic aromatic hydrocarbons, benzo[a]pyrene and 3-methylcholanthrene, and continued to subculture the cells. Untreated cells exhibited a limited lifespan and underwent senescence after about four passages, whereas treated cells, after prolonged culture, developed foci of rapidly dividing cells capable of continuous proliferation. Injection of these transformed cells into hamsters produced tumours with the histological appearances of sarcomas.

Many of the properties of transformed cells are the result of cell surface modifications which lead to changes in their growth characteristics. Normal untransformed cells require a solid substrate on which to grow (anchorage

dependence). When the available surface has been covered, cell division stops (contact inhibition or density limitation of growth). Transformed cells, possibly as a result of alterations in cell surface proteins, may lose these inhibiting features and develop the ability to grow in semisolid media such as soft agar (anchorage independent growth), or form foci of continued cell growth on confluent monolayers. These features form the basis of many of the cell transformation assays in current use.

2.4 The C3H/10T½ mouse embryo fibroblast cell transformation assay.

In 1973, Reznikoff *et al* (1973a) reported on the establishment and characterisation of a permanent mouse fibroblast cell line derived from C3H mouse embryo cells, termed C3H/10T½ clone 8, so called because the line was established using a ten-day transfer schedule with an inoculum of 0.5×10^5 cells per dish. These cells are near tetraploid, have a low saturation density, and are highly sensitive to post confluence inhibition of division. A second paper (Reznikoff *et al* 1973b) reported on the neoplastic transformation of these cells by polycyclic aromatic hydrocarbon carcinogens. Treatment of the cells with benzo[a]pyrene, 3-methylcholanthrene or 7,12-dimethylbenz[a]anthracene induced foci of deeply staining cells which stood out against the faintly stained background monolayer when the cells were stained with Giemsa. Three types of foci were identified.

Type I foci show an increase in cell density and darker staining, but there is little or no multi-layering. The edge is poorly defined and blends into the background monolayer (Fig. 2.1).

Type II is a focus of piled up deeply staining cells with a well defined edge which stands out against the background (Fig. 2.2).

Type III is again a multi-layered deeply basophilic focus, but the

peripheral cells are spindle shaped, randomly orientated and appear to invade the monolayer (Fig. 2.3).

Cells from each type of focus were inoculated into irradiated syngeneic mice to test for tumour formation. Cells from Type I foci did not produce tumours, therefore Type I foci are not counted as transformed in the assay. Approximately 50% of Type II foci and 80% of Type III foci produced sarcomas in inoculated mice, therefore both Type II and Type III foci are counted as malignantly transformed.

The C3H/10T½ transformation assay was investigated by a working group commissioned by the International Agency for Research on Cancer (IARC 1985). It reviewed the collective experience of this assay and made recommendations regarding the experimental protocol in performing the assay. These recommendations were followed in the course of this study and are described in detail in the materials and methods section. As with all techniques, the assay has strengths and weaknesses and these are considered in the discussion of the results.

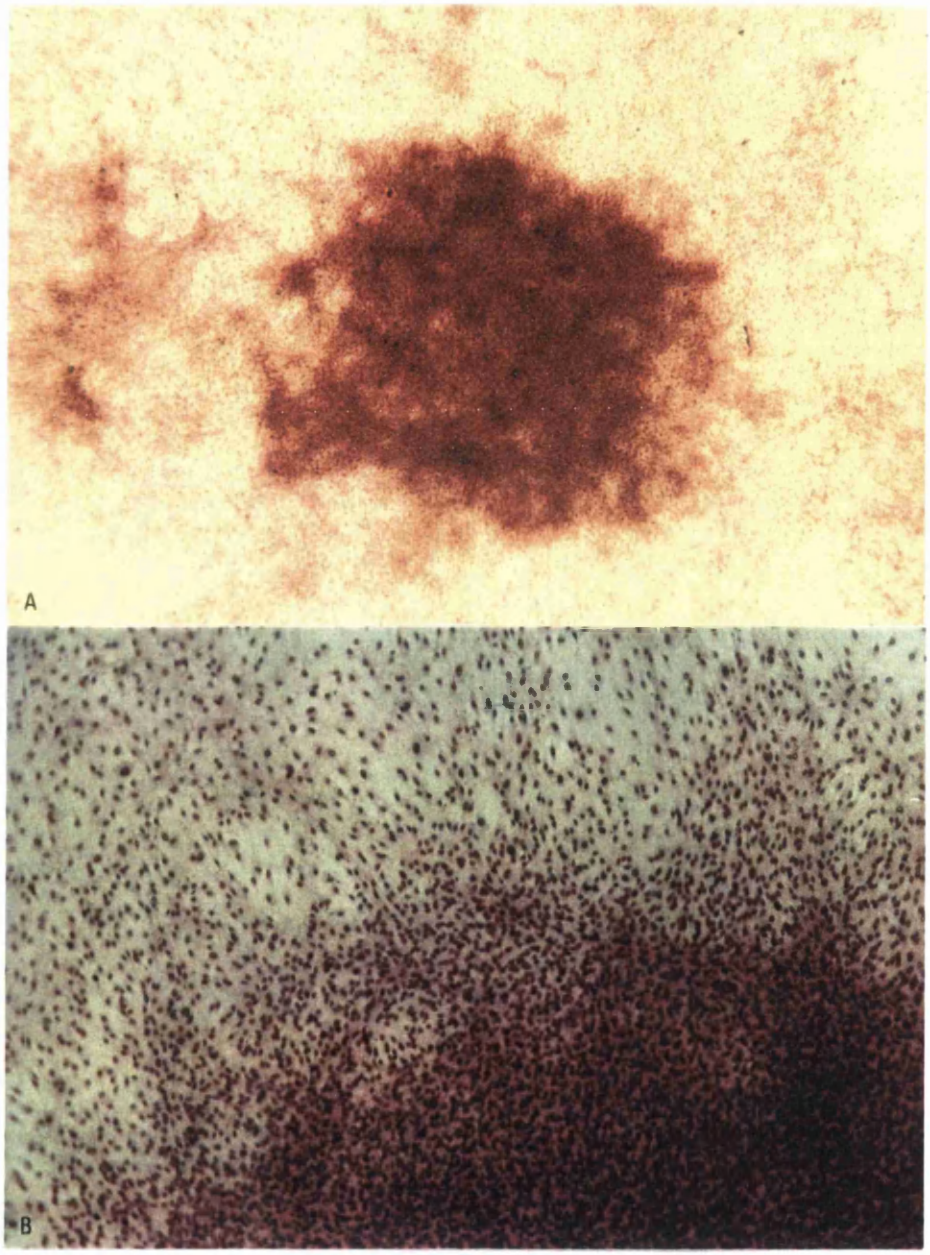


Fig. 2.1 Type I focus.

a) increased cell density but no multi-layering.

b) poorly defined edge blends with monolayer.

(Giemsa, a) x40 b) x200).

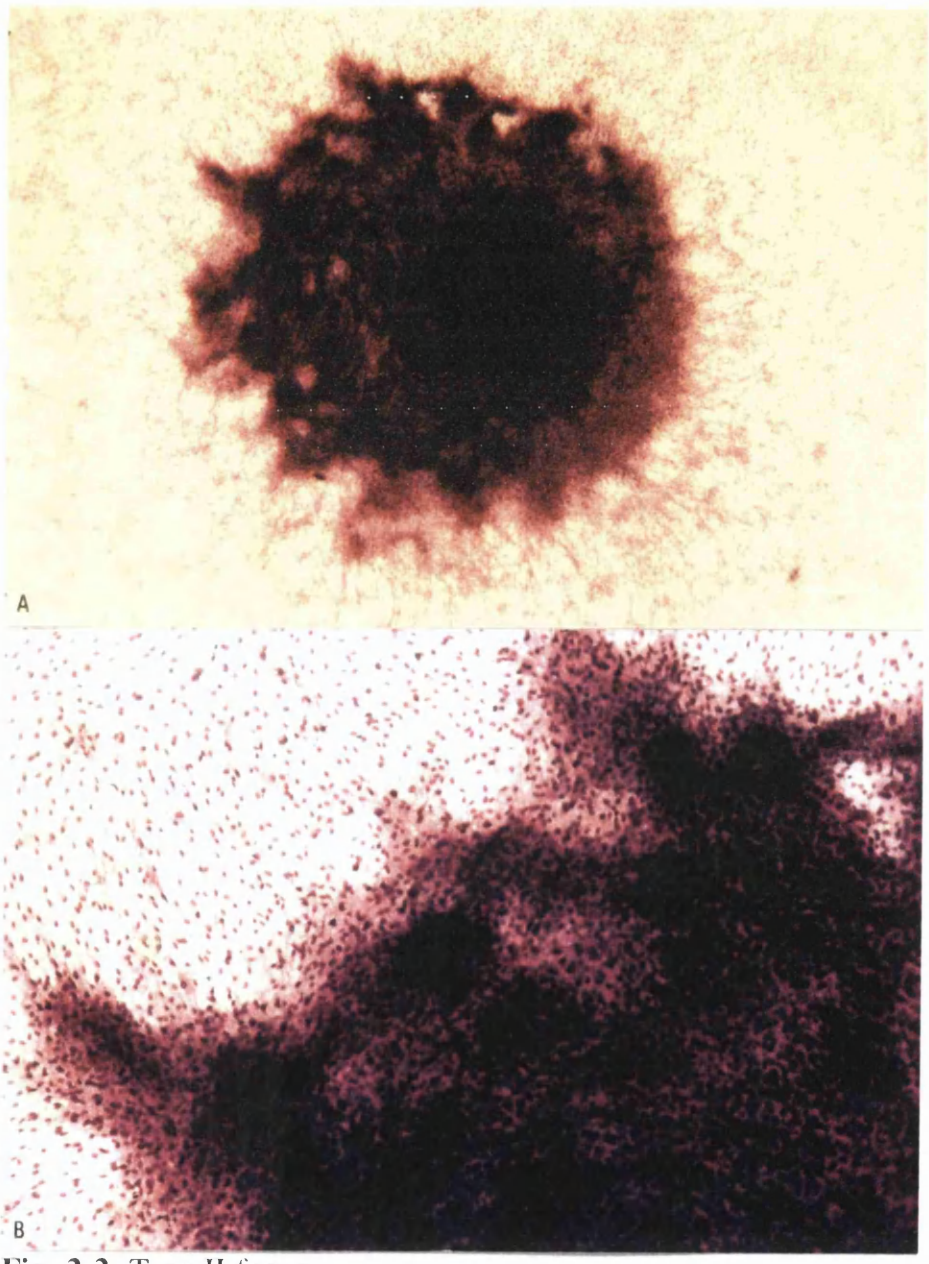


Fig. 2.2 Type II focus.

a) multi-layering and dark staining of cells.

b) well defined edge.

(Giemsa, a) x40 b) x200).

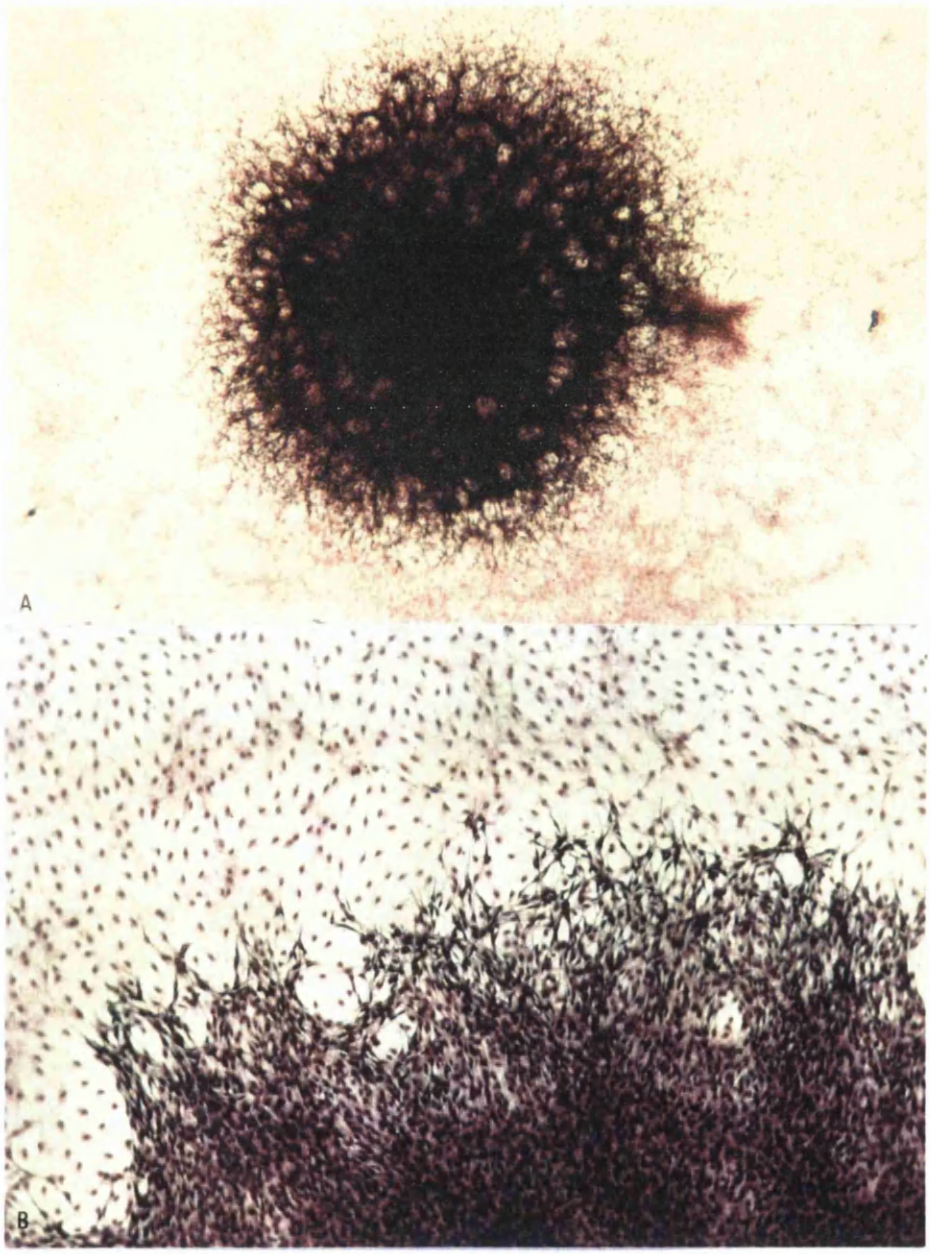


Fig. 2.3 Type III focus.

a) multi-layered deeply basophilic cells.

b) peripheral cells are spindle shaped and criss-cross randomly.

(Giemsa, a) x40 b) x200).

2.5 Induction of anchorage independent growth in diploid human fibroblasts.

A central problem in cancer research is the extrapolation of the knowledge obtained from laboratory and animal experiments to humans. *In vitro* models using human tissues and cells provide an obvious avenue towards solving this problem. Techniques for culturing many human cell types are widely available, but for the purposes of carcinogenesis research, the inherent genetic stability of human cells in culture systems has, so far, delayed the development of human cell transformation systems. The reasons for this are discussed in more detail in a later section. However, human fibroblasts have been "transformed" in culture by a variety of methods to produce growth characteristics similar to those seen in rodent cell systems. These changes in human cells have not been clearly shown to be representative of carcinogenic change, not least because of the moral difficulties in testing them in the natural host, and at present are considered to indicate stages in the carcinogenic process (Harris 1987).

In 1987, Biedermann and Landolph reported the induction of anchorage independent growth in human fibroblasts by metal salts. Cells treated with compounds of chromium, nickel and arsenic developed the ability to form colonies in semisolid agar medium whereas control cells did not. This was the first study to show that transformation-like changes can be induced in human cells by metals and it was therefore felt that this technique would be a valuable addition to this study.

CHAPTER THREE

MATERIALS AND METHODS

3.1 The C3H/10T½ cell transformation assay: maintenance of cells, staining method and preparation of test metals.

3.1.1 Routine maintenance of C3H/10T½ cell stocks.

C3H/10T½ clone 8 cells, grown and maintained for the purpose of transformation studies, were obtained at passage 8 from the American Tissue Culture Collection via the Public Health Laboratory Service, Porton Down. On receipt of the cells, stocks were rapidly expanded and stored frozen in liquid nitrogen at -196°C. Cells were then resurrected as required for performance of cytotoxicity and transformation studies. Stock cultures were grown in 75cm^2 tissue culture flasks, in Eagle's Basal Medium with Earle's salts (EBME), supplemented with 10% foetal calf serum (FCS) and L-glutamine 292.3 μg/ml, without added antibiotics. Cells were grown in a humidified incubator at 37°C in a 5% CO_2 atmosphere.

During routine maintenance, cells were seeded at 5 x 10^4 cells per 75 cm^2 flask and maintained in the exponential growth phase by reseeding every seven days, or as necessary before reaching confluence. Stocks were discarded at passage 15 to avoid possible increases in spontaneous transformation rates. In practice, due to restrictions on incubator space and the practicalities of handling large numbers of cultures at once, cells were seldom used beyond P13. All manipulations of cells were undertaken in class II vertical laminar flow cabinets. Cells were stained with Hoechst 33258(S) and found to be mycoplasma free.

3.1.2 Cell transfer and counting.

Culture medium was removed and the cells washed with phosphate buffered saline. 10ml. 0.25% trypsin in phosphate buffered saline (PBS) was added for 30 seconds then all but 0.5 ml. was removed and the cells incubated at 37° for a further two minutes. Detachment was confirmed microscopically and the cells then doused with 9.5 ml. of complete medium to neutralise the trypsin. Cells were then evenly suspended and a small amount taken for counting in a haemocytometer.

3.1.3 Freezing and storage of cells.

Cells were harvested as above and counted. The suspension was then centrifuged at 2000 rpm for 10 minutes at 4°C to form a pellet at the bottom of the centrifuge tube. The supernatant was removed and the cells resuspended in 1ml. of freezing medium, i.e. complete medium containing 10% Dimethyl Sulphoxide (DMSO). The suspension was transferred to a plastic cryotube which was then packed in a polystyrene box and frozen slowly over 24 hours in a -70°C freezer. Tubes were then transferred to a liquid nitrogen container for permanent storage. For resurrection, tubes were removed from liquid nitrogen, rapidly thawed in a water bath at 37°C, and transferred to culture flasks with added medium. Medium was changed 24 hours later to remove excess DMSO.

3.1.4 Determination of cell growth characteristics.

Plating efficiency was determined by seeding cells at 200 cells per 60mm well in four-well plates. Cells were then grown for 10 days changing the medium once, after which they were fixed in methanol and stained with Giemsa. Using a magnifying lens, colonies of over 50 cells were counted and the percentage plating efficiency derived by dividing the number of colonies formed by the

number of cells seeded, multiplied by 100. The colony count was the average of the four wells.

Growth curves were constructed as follows. Cells were seeded at $5x10^3$ cells per $2cm^2$ well in 24-well plates. At 24 hour intervals thereafter, 3 wells were trypsinised and the number of cells in each well counted. The average cell count was then converted to $\log_{10}$ and plotted against time on a semi-logarithmic graph.

3.1.5 Staining method.

Staining of cells was carried out by the method recommended by Freshney (1987). Growth medium was removed and the monolayer rinsed with PBS. The PBS was removed and replaced with PBS and methanol in a 1:1 mixture. The PBS/methanol was removed and replaced with fresh methanol which was left for 10 minutes. The methanol was discarded and the monolayer rinsed with fresh anhydrous methanol. Neat Giemsa stain was then added to cover the monolayer and left for two minutes after which it was diluted 1:5 with water and left for a further two minutes. Wells were then washed in running tap water until all scum and precipitate was removed and finally, rinsed with deionized water.

3.1.6 Preparation of metals.

The major components of the three commonly used orthopaedic alloys were tested, comprising cobalt, chromium, molybdenum, iron, nickel, titanium, aluminium, and vanadium. Each was tested in two forms, firstly as a soluble salt of either sodium or chloride, and secondly as the pure particulate metal. Chromium was tested in two soluble forms, the hexavalent (chromate) and the trivalent (chromium) form. In addition each alloy, cobalt-chromium-molyb-

denum, stainless steel, and Ti6Al4V was tested in particulate form.

Cobalt chloride ($CoCl_2$), chromium chloride ($CrCl_3$), nickel chloride ($NiCl_2$), vanadyl chloride ($VOCl_2$), and ferrous chloride ($FeIICl_2$) were purchased from BDH Ltd. Sodium molybdate (Na_2MoO_4) and aluminium chloride ($AlCl_3$) were purchased from Sigma Ltd. Titanium chloride ($TiCl_3$), sodium chromate (Na_2CrO_4), molybdenum particles (size 4-8μm), vanadium particles (325 mesh) and nickel particles (size $<3\mu$m) were purchased from Aldrich Chemical Co. Chromium particles (size $<2\mu$m), titanium particles (size $<150\mu$m), iron particles (size $<60\mu$m), stainless steel particles (AISI 316, pre-alloyed, size $<45\mu$m) and Ti6Al4V particles (pre-alloyed, size $<45\mu$m) were purchased from Goodfellow Ltd. Cobalt particles (size 1μm) and aluminium particles (size $<25\mu$m) were purchased from Kochlite Ltd. CoCrMo alloy particles (F75 standard, pre-alloyed, mesh 150-325) were a kind gift from Howmedica Ltd. All compounds and powders were of over 99% purity.

Test solutions and suspensions were prepared immediately prior to use. Metal salts were carefully weighed out on an analytic scale, calibrated to 0.0001g accuracy, and dissolved in double distilled water. In the case of $TiCl_3$ which is highly volatile in air, aqueous solutions were made in a nitrogen atmosphere cabinet. Stock solutions were sterilised by filtration through millipore filters. The desired test concentration was achieved by serial dilution, the final dilution being made in complete medium.

It has previously been shown that C3H/10T½ cells are capable of internalising particles by phagocytosis (Costa and Mollenhauer 1980, Patierno et al 1988). It was therefore decided to also test metal particles of 5μm or less. Attempts to filter metal particles through millipore filters with pore size 5μm were unsuccesful due to tearing of the filters by large particles. Suspensions of particles of the desired size were therefore prepared by sonication of the metal pow-

ders in acetone and allowing the larger particles to sink to the bottom of the test tube. Smaller particles remaining in suspension in the upper fraction were then removed with the acetone and the acetone evaporated off. Particle size was confirmed by microscopy using an eyepiece graticule and the process repeated as often as necessary to provide a sufficient amount. Particles were then heat sterilised and the final suspension made in double distilled water.

3.2 The C3H/10T½ cell transformation assay: experimental design.

The C3H/10T½ cell transformation assay was carried out following the recommendations of the IARC working group (IARC 1985). The assay is performed by exposing C3H/10T½ cells to the test compounds as well as positive and negative controls for 48 hours. The cells are then cultured and examined for cytotoxicity and induction of phenotypic transformation.

3.2.1 Selection of dose levels.

The dose levels for the test metals were selected following preliminary cytotoxicity tests based on estimation of plating efficiency using the method described above. 200 cells per 60mm well in four replicates were exposed to solvent alone and to increasing concentrations of metals for 48 hours. Cell survival was determined 10 days later and plating efficiency in the treated groups was expressed as a percentage of that in the solvent controls (relative plating efficiency RPE). When possible, the highest test dose was selected to give 80-90% toxicity and the lowest was the maximal nontoxic dose, with 2 to 3 intermediate doses added.

3.2.2 Controls.

For the test metals the solvent control was double distilled water. The positive control was 3-Methylcholanthrene (3-MCA) at a final concentration of 2.5 μg/ml. For treatment of cultures 3-MCA was dissolved in DMSO (final concentration 0.5%).

3.2.3 Preparation of target cells.

Each assay consisted of two limbs which were carried out concurrently. For ease of handling of the large number of cultures required for the study and optimal use of incubator space, plates comprising four 60mm wells were used instead of individual 60mm dishes.

Exponentially growing C3H/10T½ cells were seeded at 200 cells per 60mm well in four replicates for cytotoxicity studies and at 2000 cells per 60mm well in 20 replicates for transformation studies. Seeding was carried out the day before treatment to allow settling and adherence of the cells. As with the stock cultures, cells were grown in EBME, supplemented with 10% FCS without added antibiotics in a humidified atmosphere of 5% CO_2 in air at 37°C.

3.2.4 Treatment of target cells.

Concentrations of test compounds were prepared immediately prior to use in the appropriate solvent. 3-MCA was dissolved in DMSO and 50 μl of the solution were delivered to the cultures by micropipette to give a final concentration of 2.5 μg/ml. In the case of metals, the last dilution (or suspension of particles) was made in complete medium, 1 ml of which was added to cultures to give the desired final concentration in 5 mls of medium. The day of treatment was designated Day 0. Cells were exposed to test compounds or controls for 48 hours following which treatment media were removed and replaced with 5 mls of

complete medium. After this the medium was changed at least once a week for the duration of the assay.

3.2.5 Estimation of cytotoxicity.

Cells treated for cytotoxicity studies were grown for 10 days after which they were fixed and stained and the number of colonies counted. The degree of toxicity of each treatment was measured as the relative plating efficiency as compared with the control as described above. A typical cytotoxicity study is shown in Fig. 3.1.

3.2.6 Expression of transformed phenotype.

Cells treated for transformation studies were maintained in culture for six weeks. After this period the wells were fixed with methanol, stained with Giemsa and scored for morphologically transformed Type II and Type III foci using the method and criteria described below. Control transformation assays are shown in Fig. 3.2.

3.2.7 Validation of the assay.

It is known that C3H/10T½ cells require specific batches of foetal calf serum to attach properly and grow optimally in culture (Landolph 1985). In addition, some batches of FCS yield good plating efficiencies but do not support transformation with 3-MCA (Oshiro et al 1982). It is therefore necessary to pre-screen serum batches before use to ensure that they support both the required growth characteristics and morphological transformation. According to the IARC protocol, the plating efficiency of the solvent control should optimally be 30% and no less than 20%, and the population doubling time in the exponential phase should be 17-20 hours. In transformation studies, the negative control should not

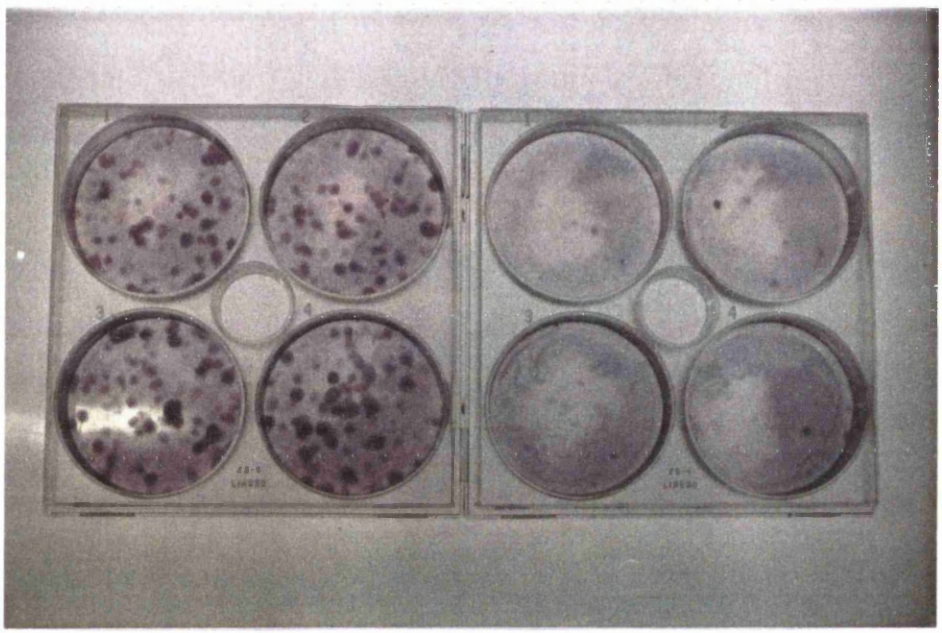


Fig. 3.1 Cytotoxicity as shown by relative plating efficiency. There are numerous colonies in the control wells (left). After treatment with $VOCl_2$ 1μg/ml (right) the surviving colony count is greatly reduced.

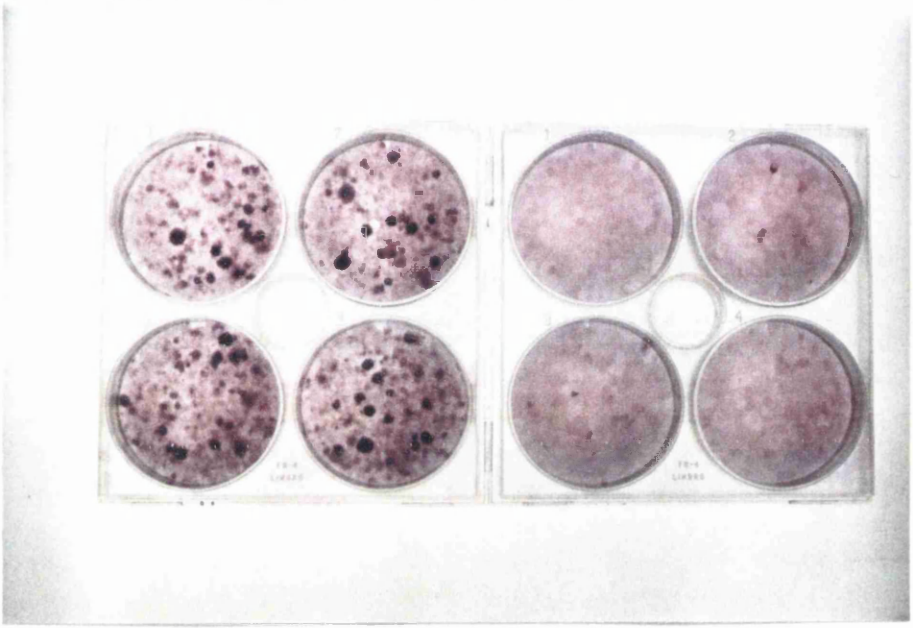


Fig. 3.2 Transformation assay controls.

Left: 3-MCA 2.5μg/ml. There are numerous transformed foci.

Right: DMSO 0.5%. The cells remain contact inhibited with no focus formation.

exhibit spontaneous transformation frequencies of more than 0.07 foci per dish, and the positive control should induce a significantly ($P < 0.05$) greater number of transformed foci than the negative control. In this study, only serum batches which met these criteria were used. For the batch screening assays the positive control was 3-MCA 2.5 μg/ml and the negative control was DMSO 0.5%.

3.2.8 Scoring of foci.

On completion of an assay, the plates were fixed, stained and stored for later scoring. When a sufficient number of assays had been completed, the plates were coded and scored blind for formation of type II and type III foci. Scoring was done by eye using a magnifying lens. Foci that appeared intermediate in character (I/II or II/III) were assigned to the less aggressive category. The total number of type II and type III foci for each treatment was recorded. The transformation frequency for each treatment was taken as the number of wells with foci divided by the total number of wells scored. The difference between treatments and controls was evaluated statistically using Fisher's exact probability test.

3.3 Induction of Anchorage Independent growth in Human Fibroblasts.

In 1987, Biedermann and Landolph described the induction of anchorage independent growth in human foreskin fibroblasts by metal salts. As part of the interest of this study was the possible carcinogenic effects of metals on periarticular tissues, it was decided to attempt to use this method with human synovial fibroblasts as well as human foreskin fibroblasts.

3.3.1 Source and preparation of cells.

Primary cultures of human synovial fibroblasts were grown from specimens of synovial tissue removed from patients in the course of surgical procedures. Synovium was removed under aseptic conditions and transported to the laboratory in Minimum Essential Medium with Hank's salts (HMEM). It was then immediately minced into small pieces with a scalpel. The explants were transferred to culture wells containing complete medium and incubated in a 5% CO_2 atmosphere at 37°C. Cultures were examined periodically to confirm cell outgrowth from the explants and were grown until the monolayer approached confluence. The explants were then removed and the monolayer was trypsinised. Cells were transferred to flasks at a 1:3 split and were harvested and frozen at passage 3. Primary cultures of human synovial fibroblasts were prepared in this way using synovium obtained from a 9 year old patient undergoing open reduction of a congenitally dislocated hip. Human foreskin fibroblasts were a kind gift from Dr. J. Wright (Rheumatology Research Unit, Addenbrooke's Hospital). These were originally grown from circumcised infant foreskins. Methods of cell transfer, freezing and resurrection, and routine maintenance were similar to those described previously for C3H/10T½ cells.

3.3.2 Experimental method.

Cytotoxicity assays were carried out using the same plating efficiency method as described for C3H/10T½ cells. Metal salts and particles were also prepared by the same method as described previously.

Assays for induction of anchorage independent growth were carried out by seeding cells in semi-liquid agar medium onto a base of solid agar medium, after exposure to test chemicals, as follows. 10^5 cells were seeded into 75cm^2 flasks and 24h later were treated with test chemicals for 48h. The medium was then removed and replaced with fresh medium. Cells were subcultured as necessary to maintain exponential growth and at 12 days, the cells were trypsinised and suspended in 3 ml of 0.3% agar noble mixed with double strength growth medium in a 1:1 mix. Growth medium consisted of HMEM supplemented with non-essential fatty acids, hydrocortisone (50 μg/ml), insulin (0.15 units/ml), 10% foetal calf serum, and 10% tryptose phosphate broth. The cell suspension was placed on top of 5 ml of a 0.5% agar base layer containing growth medium in 60mm wells in four replicates and incubated in a 5% CO_2 atmosphere at 37°C. Cells were fed every 6 days with 0.5 ml of HMEM containing 15% FCS. Four weeks later, cells were stained with 1 mg/ml iodonitrotetrazolium violet in PBS and examined microscopically for colonies greater than 0.1 mm in diameter. The positive control for the assay was the known carcinogen N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) dissolved in acetone (final concentration of acetone 0.5%) and the negative control was acetone 0.5%.

CHAPTER FOUR

RESULTS

4.1 Validation of serum batches in C3H/10T½ cell cultures.

Four batches of foetal calf serum were used in the course of the study, namely NBL batch 19827, Imperial batch 960585, Gibco batch 06G9982Y and SeraLab batch 801053.

4.1.1 Cell growth characteristics.

During batch testing, they yielded plating efficiencies of 50.4%, 50.4%, 43.7% and 51.9% respectively. Growth curves were similar for each batch with population doubling times of 12-14 hours.

4.1.2 Cell transformation.

Transformation assays confirmed that all four batches supported 3-methylcholanthrene-induced transformation with transformation incidences of 12/20, 14/20, 14/20 and 18/20 respectively. In dimethyl sulphoxide controls, none of the batches produced spontaneous transformation frequencies in excess of the recommended limit of 0.07 foci per well.

The results of these validation studies are shown in more detail in Appendix A.

4.2 Results of metal salts in the C3H/10T½ transformation assay. Cytotoxicity and transformation incidence.

In the following account, the results are given for each metal salt individually. As explained in the methods section, each assay is double-limbed, consisting of cytotoxicity and transformation assays carried out concurrently for each concentration. The result given for each salt is the combination of two such double-limbed assays and is shown both as a table of values and a graph. In the table, the relative plating efficiency for each concentration is the average of two results and the transformation incidence is the sum of two results. In the graph the transformation incidence is expressed as a percentage value. The separate results for each cytotoxicity and transformation assay can be found in appendices B and C respectively. Metals were considered to be transforming if the transformation incidence reached the 0.05 level of significance at any concentration level compared with the negative control (Fisher's exact probability test, one-tailed method).

In such a system measuring two outcomes for each test, four types of result are possible for the substances being tested. They may be toxic and transforming, non-toxic and transforming, toxic and non-transforming or non-toxic and non-transforming. Metal salts fell into each of these categories and are presented in that order.

4.2.1 Metals which were toxic and transforming.

Cobalt Chloride (Fig.4.1).

$CoCl_2$ was tested at concentrations of 0.1, 1, 5, 10 and 20 μg/ml, and demonstrated increasing toxicity and transformation in a dose related fashion. Even at the lowest concentration of 0.1 μg/ml, there was noticeable toxicity with a relative plating efficiency of 78.8%. Although the R.P.E. at the highest concentration of 20 μg/ml was only 0.2%, enough cells survived the exposure period to form a confluent monolayer during the six week course of the transformation assay. The incidence of transformation became significant at 5 μg/ml ($p=0.001$) and became more so at higher concentrations, though it can be seen from the table and the graph that there is an increasing trend throughout the test range. It will be noted that all the transformed foci were of type II morphology.

Nickel Chloride (Fig.4.2).

$NiCl_2$ was only slightly less toxic than $CoCl_2$, showing minimal toxicity at 1 μg/ml and maximal decrease in plating efficiency at 40 μg/ml, with intermediate effects at 10 and 20 μg/ml. A further increase to 60 μg/ml produced similar effects as the 40 μg/ml level. Again at the maximally toxic levels, enough cells survived to form monolayers in the transformation assay. Significant increases in transformation were seen at 10 ($p=0.014$) and 20 μg/ml ($p=0.003$), but just failed to reach significance at 40 and 60 μg/ml ($p=0.054$). As with $CoCl_2$, all the transformed foci were type II.

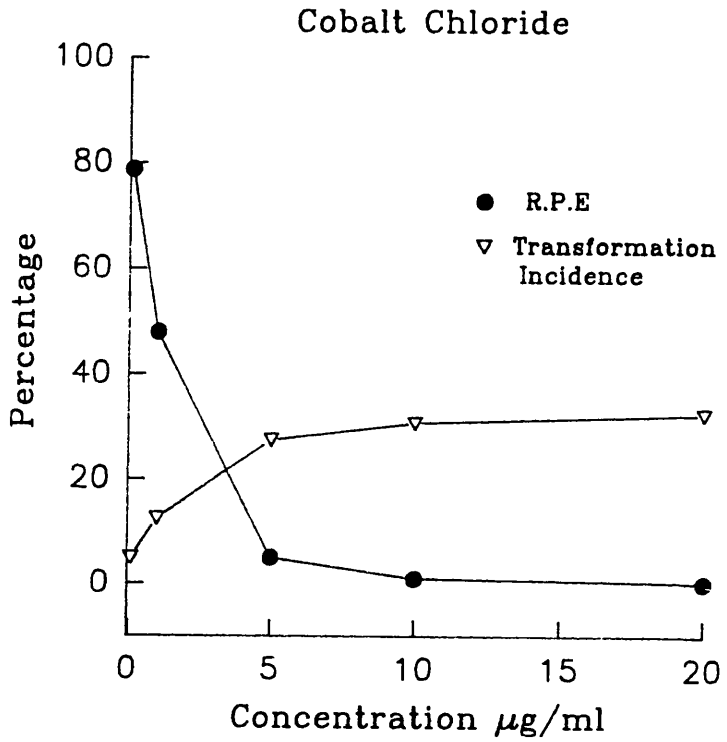
Chromium Chloride (Fig.4.3).

$CrCl_3$ was tolerated in much higher concentrations with no toxicity at 300 μg/ml, but increasing cell death at 600 and 900 μg/ml. At 1200 μg/ml however, $CrCl_3$ precipitated out of solution accompanied by the appearance of

denatured aggregates in the culture medium to varying degrees. This may account for the marked discrepancy between the two cytotoxicity assays done at this concentration (see appendix B.). In addition there was clearly a delayed effect on cell growth in the transformation assay at 1200 μg/ml. At 300, 600 and 900 μg/ml the monolayers were normal in appearance with the occurence of some type II foci though not enough to reach significance. However, at 1200 μg/ml extensive cell death and detachment of the monolayer from its surface occurred in the late stages of the assay, such that only 10 wells were available for scoring (appendix C.). Despite this the transformation incidence reached significance ($p=0.014$) and two of the foci were of type III morphology. Whether this result was due to the $CrCl_3$ alone or to some additional effect from the change in the culture medium during the exposure period is difficult to know.

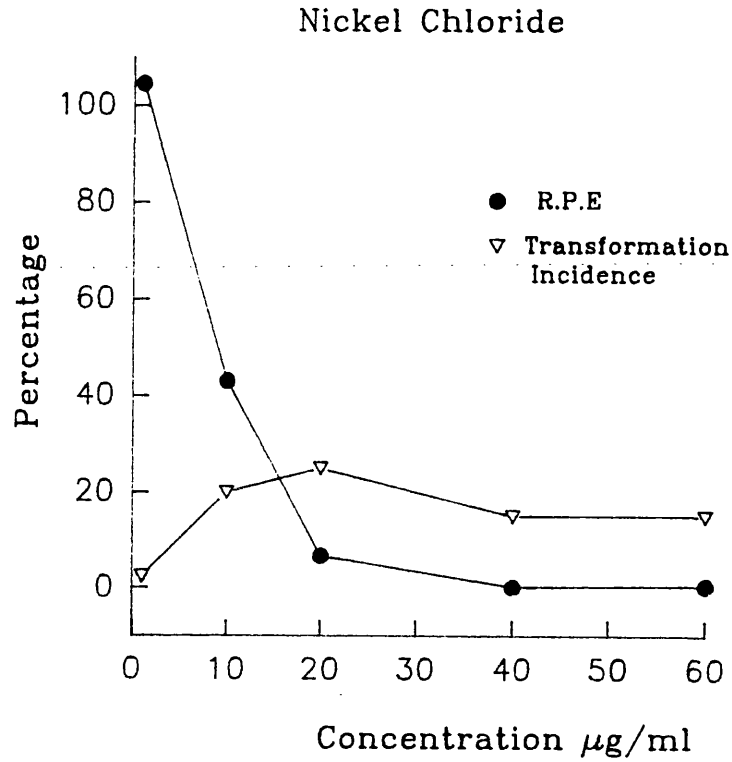
Sodium Chromate (Fig.4.4).

Na_2CrO_4 was clearly the most toxic of all the metal salts tested with maximal toxicity at only 0.5 μg/ml, minimal toxicity at 0.01 μg/ml, and intermediate values at 0.05 and 0.1 μg/ml. Significant transformation occurred only at the most toxic dose, though here it was quite marked with 10 out of 36 wells having foci ($p=0.002$). As can be seen from the table, there was quite a jump down in plating efficiency between the 0.1 and 0.5 μg/ml concentrations and it could be fairly argued that an intermediate dose would have been helpful in demonstrating any dose related increase in transformation.



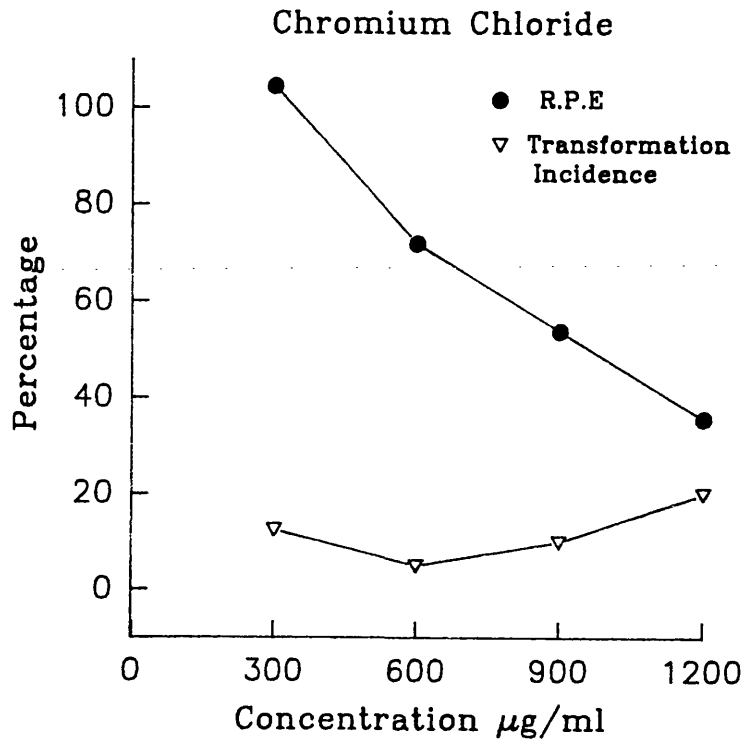
$CoCl_2$ μg/ml	R.P.E.(%)	Focus type II	Focus type III	Transformation Incidence
0.1	78.8	2	-	2/40
1.0	47.9	5	-	5/40 p=0.1
5.0	5.0	11	-	11/40 p=0.0016
10.0	1.0	13	-	12/39 p=0.0006
20.0	0.2	17	-	13/40 p=0.0003
Water	100.0	1	-	1/40
3-MCA	78.0	80	25	34/40

Fig. 4.1 Results of cobalt chloride in the C3H/10T½ assay.



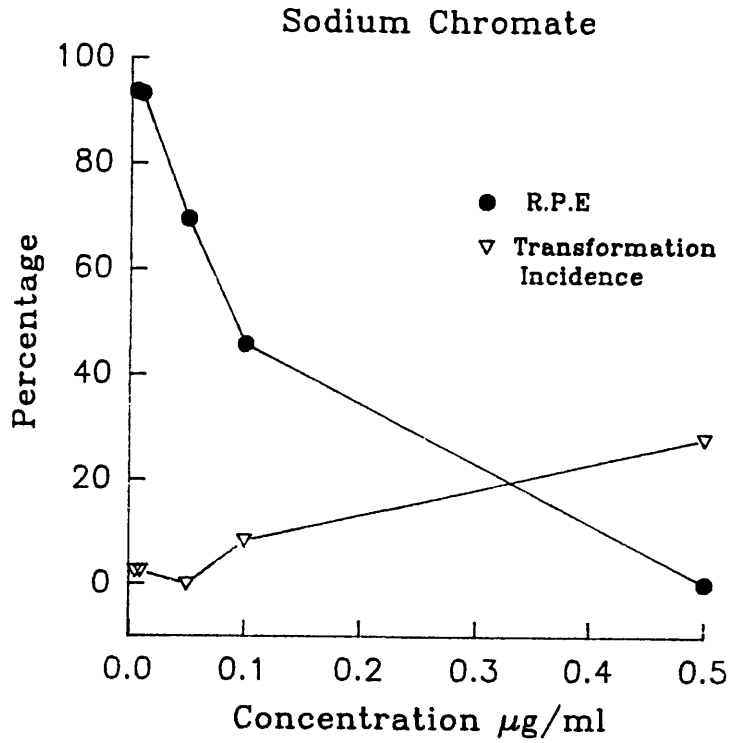
$NiCl_2$ μg/ml	R.P.E.(%)	Focus type II	Focus type III	Transformation Incidence
1.0	104.5	1	-	1/40
10.0	43.0	9	-	8/40 p=0.014
20.0	6.7	15	-	10/40 p=0.003
40.0	0.2	8	-	6/40 p=0.054
60.0	0.5	8	-	6/40
Water	100.0	1	-	1/40
3-MCA	78.0	80	25	34/40

Fig. 4.2 Results of nickel chloride in the C3H/10T½ assay.



$CrCl_3$ μg/ml	R.P.E.(%)	Focus type II	Focus type III	Transformation Incidence
300.0	104.5	5	-	5/40
600.0	71.9	2	-	2/40
900.0	53.8	4	-	4/40
1200.0	35.6	13	2	8/40 p=0.014
Water	100.0	1	-	1/40
3-MCA	78.0	80	25	34/40

Fig. 4.3 Results of chromium chloride in the C3H/10T½ assay.



Na_2CrO_4 μg/ml	R.P.E.(%)	Focus type II	Focus type III	Transformation Incidence
0.005	93.6	-	1	1/40
0.01	93.2	2	-	1/40
0.05	69.4	-	-	0/40
0.1	45.7	3	-	3/36
0.5	0.2	12	-	10/36 p=0.002
Water	100.0	1	-	1/40
3-MCA	78.0	80	25	34/40

Fig. 4.4 Results of sodium chromate in the C3H/10T½ assay.

4.2.2 Metals which were non-toxic and transforming.

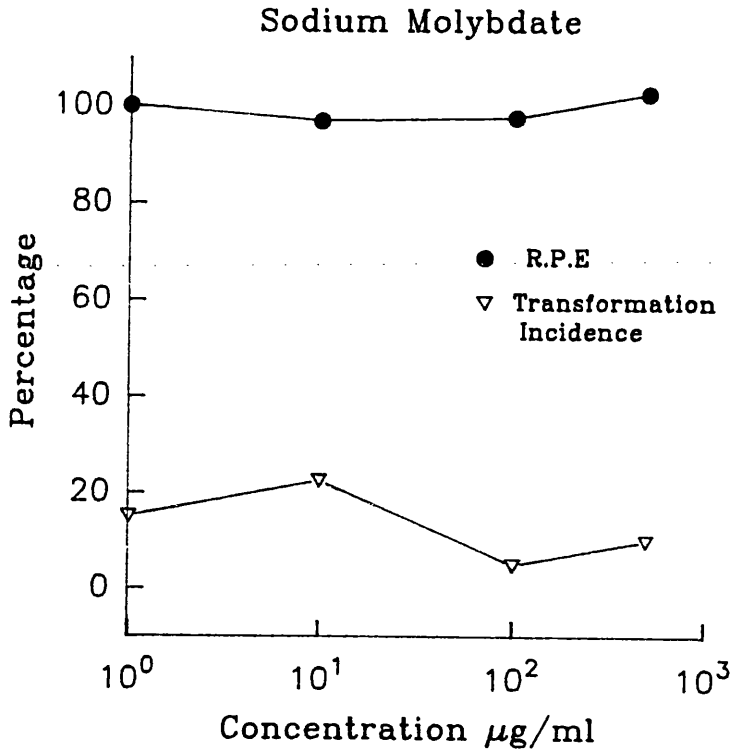
Sodium Molybdate (Fig.4.5).

Only Na_2MoO_4 came into this category. It was tolerated in high concentrations up to 500 μg/ml with virtually no sign of toxicity. A significant transformation incidence was reached at 10 μg/ml with 9/40 wells producing foci ($p=0.007$). Foci also occurred at the other concentrations but not in enough numbers to reach significance, and there was no trend to suggest that the effect was dose related. Once again most of the foci were type II but at 10 μg/ml, three were type III in nature.

4.2.3 Metals which were toxic and non-transforming.

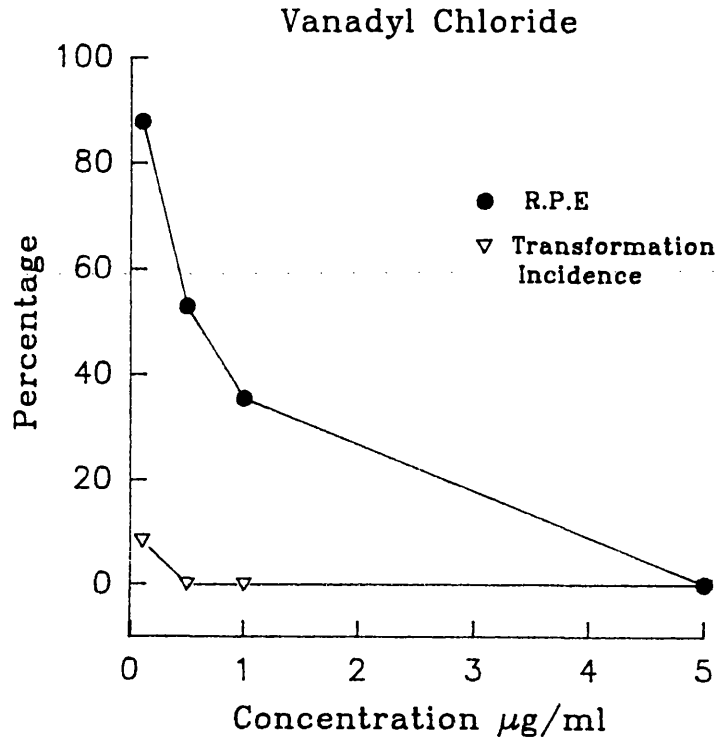
Vanadyl Chloride (Fig.4.6).

$VOCl_2$ was only slightly less toxic than Na_2CrO_4 showing increasing toxicity at 0.1, 0.5 and 1 μg/ml, with maximum toxicity at 5 μg/ml. Although there was some focus formation at 0.1 μg/ml, two of which were type III, the transformation incidence did not reach significance, and there was no focus formation at the other concentrations. $VOCl_2$ was so toxic at 5 μg/ml that the monolayer was only just complete in some wells at the end of the transformation assay, i.e. colonies were just merging. It is possible then that at this concentration there was not enough time for foci to form after completion of the monolayer.



Na_2MoO_4 μg/ml	R.P.E.(%)	Focus type II	Focus type III	Transformation Incidence
1.0	100.1	6	-	6/40 p=0.054
10.0	96.9	8	3	9/40 p=0.007
100.0	97.6	2	-	2/40
500.0	102.7	4	-	4/40
Water	100.0	1	-	1/40
3-MCA	81.8	44	27	28/39

Fig. 4.5 Results of sodium molybdate in the C3H/10T½ assay.



$VOCl_2$ μg/ml	R.P.E.(%)	Focus type II	Focus type III	Transformation Incidence
0.1	87.8	1	2	3/36
0.5	52.8	-	-	0/40
1.0	35.4	-	-	0/40
5.0	0.0	-	-	0/40
Water	100.0	2	-	2/40
3-MCA	83.9	52	16	26/39

Fig. 4.6 Results of vanadyl chloride in the C3H/10T½ assay.

4.2.4 Metals which were non-toxic and non-transforming.

Ferrous Chloride (Fig.4.7).

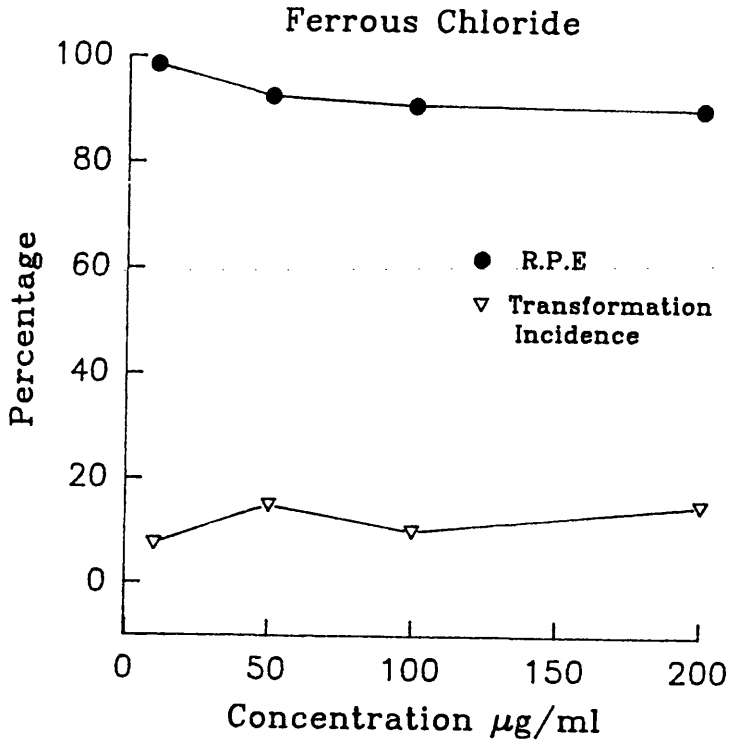
$FeIICl_2$ produced very little toxicity at concentrations of 10, 50, 100 and 200 μg/ml, the R.P.E. at the highest concentration remaining at 90.2%. At concentrations above 200 μg/ml, $FeIICl_2$ precipitated out and led to more extensive denaturing of the growth medium than that seen with $CrCl_3$, resulting in no cell growth. Transformation foci occurred at all concentrations, but although two foci were type III, the transformation incidence failed to reach significance at any dose level.

Aluminium Chloride (Fig.4.8).

Similarly, $AlCl_3$ displayed little toxicity over a range from 1 to 500 μg/ml, and again, although some foci appeared, the transformation incidence did not reach significance at any concentration.

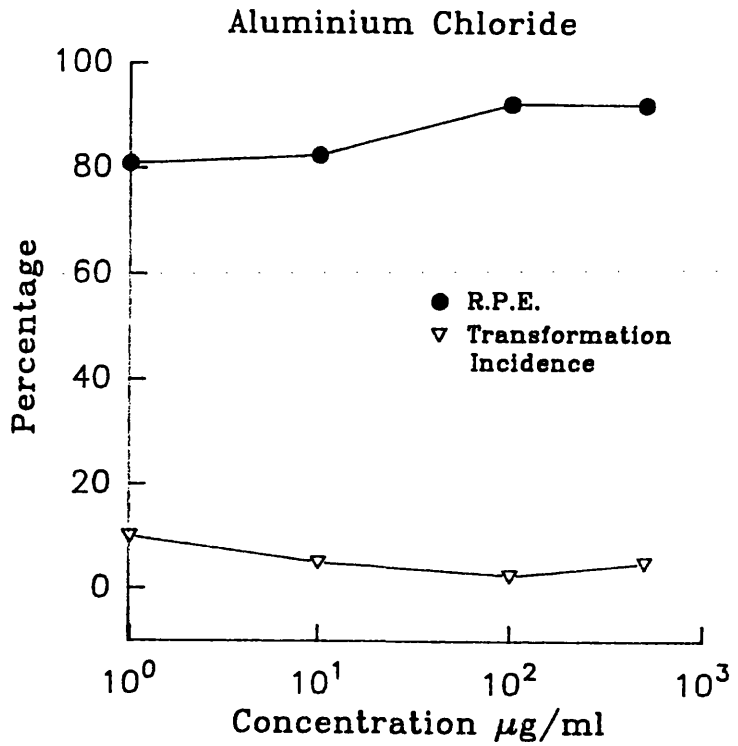
Titanium Chloride (Fig.4.9).

$TiCl_3$ was tested at 10, 50, 100 and 500 μg/ml and again demonstrated very little toxicity. It should be noted that the R.P.E. of 79.9% at 500 μg/ml is the result of only one cytotoxicity assay as the other was lost to infection. More importantly, there were no transformation foci at any concentration level. $TiCl_3$ was the only metal tested to demonstrate this effect.



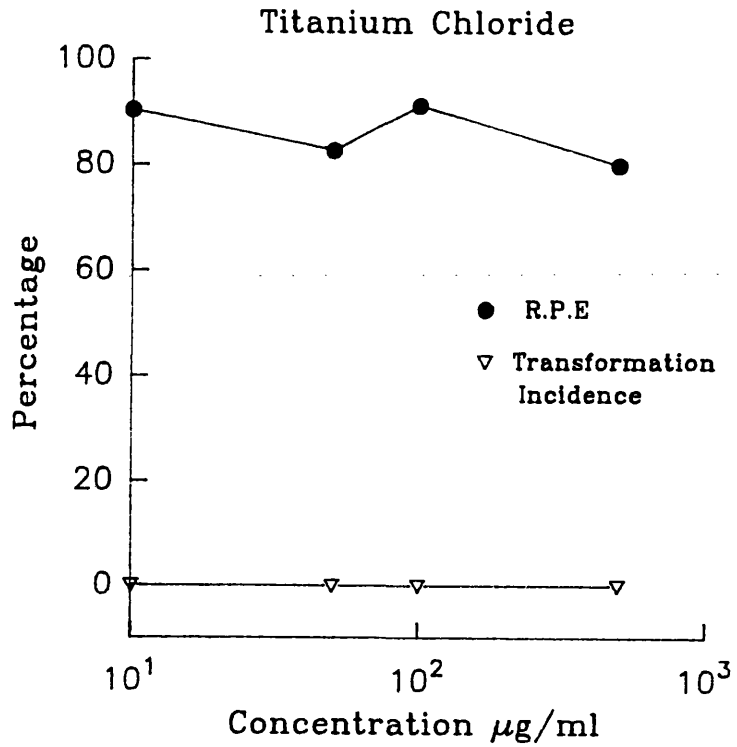
$FeIICl_2$ μg/ml	R.P.E.(%)	Focus type II	Focus type III	Transformation Incidence
10.0	98.4	4	1	3/40
50.0	92.5	6	-	6/40 p=0.13
100.0	90.8	4	-	4/40
200.0	90.2	6	1	6/40
Water	100.0	2	-	2/40
3-MCA	82.7	50	21	31/40

Fig. 4.7 Results of ferrous chloride in the C3H/10T½ assay.



$AlCl_3$ μg/ml	R.P.E.(%)	Focus type II	Focus type III	Transformation Incidence
1.0	80.9	6	-	4/40
10.0	82.5	2	-	2/40
100.0	92.3	1	-	1/40
500.0	92.1	2	-	2/40
Water	100.0	1	-	1/40
3-MCA	81.8	44	27	28/39

Fig. 4.8 Results of aluminium chloride in the C3H/10T½ assay.



$TiCl_3$ μg/ml	R.P.E.(%)	Focus type II	Focus type III	Transformation Incidence
10.0	90.3	-	-	0/40
50.0	82.6	-	-	0/40
100.0	91.2	-	-	0/40
500.0	79.9	-	-	0/40
Water	100.0	2	-	2/40
3-MCA	86.5	14	24	25/39

Fig. 4.9 Results of titanium chloride in the C3H/10T½ assay.

4.3 Results of metal particles in the C3H/10T½ assay.

In the metal particle tests, the assays were carried out with the same 48 hour exposure time as in the metal salt assays. In practice however, it proved impossible to remove substantial amounts of the particles after the exposure period as they tended to adhere to the cell surfaces despite repeated washing with saline (Fig.4.10). In effect then, these results reflect a period of continuous exposure of the cells to metal particles during both cytotoxicity and transformation assays, particularly at the higher concentration levels. Unfortunately electron microscopy facilities were not available to ascertain whether the retained particles were truly internalised or simply remained on the cell surface.

Details of the results of metal particles in the C3H/10T½ assay are shown in Appendix D. In each case the result is from one assay for each pure metal or alloy.

4.3.1 Cell transformation.

In contrast to the soluble metal salts, metal particles produced no significant increases in transformation incidence in any of the assays. In fact, in the majority of cases no foci were seen at all. This difference presumably reflects the physical form in which the metals were delivered.

4.3.2 Cytotoxicity.

There were, however, differences in toxicity between the various metal particles tested. Cobalt particles were most toxic over a range of 0.5 to 10 μg/ml. Nickel particles became markedly toxic at 5 μg/ml and vanadium at 50 μg/ml. Chromium particles became noticeably toxic at 100 μg/ml, but the remaining pure metals molybdenum, aluminium and titanium showed marked toxicity only at the highest concentration of 500 μg/ml. In the case of iron, the 500

μg/ml concentration was lost but it showed little toxicity at 100 μg/ml. As might be expected, the three alloys stainless steel, CoCr and Ti6Al4V showed patterns of toxicity which were intermediate between the most and least toxic pure metals. Perhaps more surprisingly, CoCr with its large cobalt component appeared somewhat less toxic than the other two alloys.

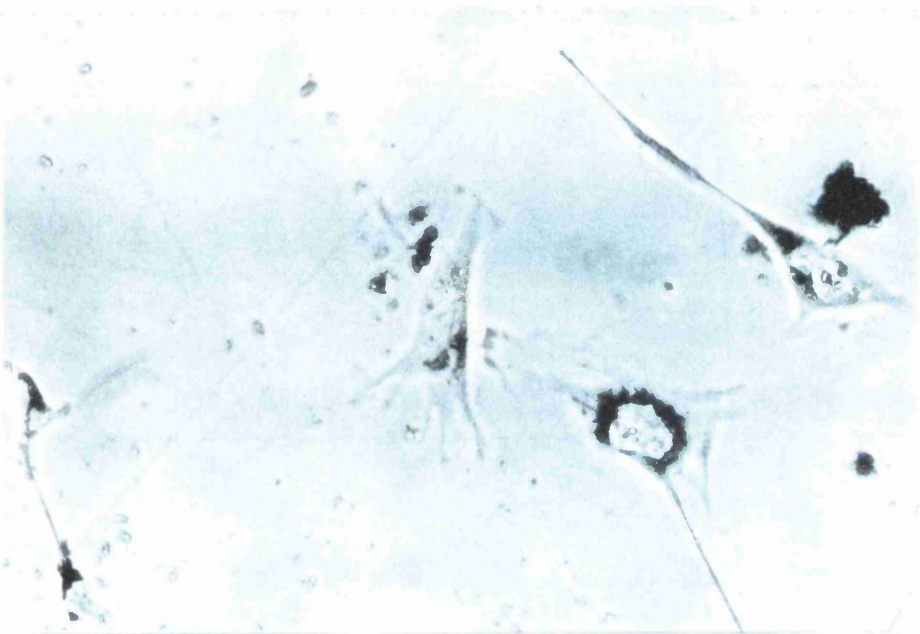


Fig. 4.10 C3H/10T½ cells after washing with PBS. Particles of titanium remain adherent to the cells (unstained, x400).

4.4 Results of human cell assays.

4.4.1 Anchorage independent growth.

Several attempts were made to induce the formation of colonies of human cells in semi-solid agar medium by known carcinogens. Both human synovial fibroblasts and human foreskin cells were used and were treated with N-methyl-N'-nitro-N-nitrosoguanidine and 3-methylcholanthrene in separate assays. However, although both cell lines grew well under normal growth conditions, no anchorage independent colonies arose in response to carcinogen treatment. In view of this failure it was not possible to proceed with studies of metal-induced transformation.

4.4.2 Cytotoxicity.

Cytotoxicity assays were carried out on human synovial fibroblasts (HSF). They were treated at the same concentration levels as C3H/10T½ cells for both metal salts and metal particles. Results of these assays are shown in Appendix E. In each case the result is of one assay.

In general there were no major differences between the results of the cytotoxicity tests on human and murine fibroblasts. Metal salts which were toxic to C3H/10T½ cells ($CoCl_2$, $NiCl_2$, Na_2CrO_4, $CrCl_3$ and $VOCl_2$) were equally toxic to HSF cells over the same concentration ranges. Salts which were non-toxic to C3H/10T½ cells ($FeIICl_2$, Na_2MoO_4, $AlCl_3$ and $TiCl_3$) were also non-toxic to HSF cells. The findings were similar with metal and alloy particles.

CHAPTER FIVE

DISCUSSION OF RESULTS

5.1 Study Design.

As stated in Chapter 1, the aims of the study were essentially comparative in nature. The C3H/10T½ assay was employed as a screening tool to compare the effects of the various metals used in orthopaedic prostheses rather than to investigate mechanisms of carcinogenicity or toxicity. Some of the the involved metals have been studied previously in a variety of *in vitro* systems, others have not. To date no previous studies have examined all of the principal constituents of orthopaedic implants in the same system under the same conditions. The IARC protocol was followed to allow valid comparison with other studies as far as possible.

Metals were tested in two physical forms which may arise from *in vivo* corrosion and wear i.e. as soluble ions and solid particles. In the case of chromium, numerous studies have demonstrated differences in activity between the trivalent and hexavalent forms (see Patierno *et al* 1988), therefore chromium was tested in both valence states.

In order to evaluate the consistency of the results it was intended to perform every assay twice. This was achieved with the metal salt preparations and the results appeared to be generally consistent for all the metals tested. Because of the time taken for each assay and the large number of replicates required it was not possible to repeat the metal particle experiments in the time available for the study. In view of the uniform lack of effect of the metal particles in the transformation studies, it seems unlikely that repeating the assays would have yielded further information.

As originally planned, the results of the transformation experiments

in the human cell assay would have been of great help in the interpretation and extrapolation of the mouse cell studies. With the failure of the human cell controls to give valid results, this was not possible. However cytotoxicity assays were carried out on human cells with all the metal preparations to allow comparison of the two cell lines in that respect.

5.2 Strengths and weaknesses of the C3H/10T½ transformation assay.

Before considering the results in more detail, some aspects of the C3H/10T½ transformation assay require clarification.

As a permanent cell line with well defined growth characteristics and requirements, C3H/10T½ cells are relatively easily maintained and manipulated. Once stained, transformed foci stand out clearly against the background monolayer making scoring relatively easy. Used below passage 15, the cells have a low spontaneous transformation frequency and the use of 3-methylcholanthrene allows clear validation that the assay is working. The assay is well established and appears to have reasonably good interlaboratory reproducibility (Dunkel *et al*, 1988). A number of other features such as the facility to carry out concomitant mutagenicity assays make the line suitable for mechanistic studies of *in vitro* transformation.

However, when using the assay as a screening tool as in this study, a number of potentially restricting factors need to be considered. As a permanent cell line C3H/10T½ cells are not normal but have already acquired some of the features of neoplastic transformation such as aneuploidy and immortalisation while retaining some features of untransformed cells such as contact inhibition and normal morphology. The *in vitro* transformation of C3H/10T½ cells is therefore a measure of the capacity to induce some of the steps in the multistage process of carcinogenesis. Secondly, as 50% of type II and 80% of type III foci produce

tumours in inoculated mice, the induction of foci is often but not always equivalent to the induction of tumorigenicity. Thirdly, a number of studies have shown that attempts to quantify transformation by measuring transformation frequency (number of foci per viable cell treated) give results that are highly dependent on the initial number of treated cells - transformation frequency tends to decrease with increasing numbers of treated cells (Kennedy *et al* 1980, Huband *et al* 1985). The reason for this is not fully understood at present and this makes it difficult to analyse the assay in a quantitative way. Transformation *frequency* is therefore not calculated and the result is given as the transformation *incidence*, derived from the number of foci-containing dishes per total number of treated dishes. The significance of the increase in transformation incidence over the negative control helps in the interpretation of the results but the assay remains to some extent a qualitative study.

Finally, it has been noted already that some batches of foetal calf serum do not support transformation by 3-methylcholanthrene. It seems possible then that batches which do support transformation may do so to different extents and thus bias the results. In this study, comparison of the results of the batch tests with the controls used in the actual assays showed no evidence that any serum batch was more likely to give positive results than the others. In addition, the two assays carried out for each metal salt were done using different serum batches. It is hoped then that any batch related effect was minimised.

5.3 Comparison with previous work.

In common with other work in the field of metal carcinogenesis, previous studies of *in vitro* transformation by metals have tended to concentrate on metals with widespread industrial and occupational exposure, particularly nickel and chromium, with somewhat less attention to the other metals of orthopaedic

interest. Metals have been tested in a number of cell systems.

5.3.1 Metal-induced transformation of C3H/10T½ cells.

A large number of substances have been tested in the C3H/10T½ system but relatively few studies have looked specifically at metals.

In an early study Saxholm *et al* (1981) found that particles of nickel subsulphide, a common constituent of nickel ores, induced the formation of type I, II and III foci but at a lower frequency than 3-methylcholanthrene.

Patierno *et al* (1988) investigated the effects of chromium compounds and found that insoluble particles of lead chromate produced a low but reproducible rate of type III transformation whereas soluble calcium chromate and potassium dichromate did not induce transformation. A second study from the same laboratory (Miura *et al*, 1989) found that insoluble nickel subsulphide, nickel monosulphide and nickel oxide produced transformation which was predominantly of type II morphology while soluble nickel chloride and nickel sulphate gave negative results. In the latter two studies the soluble nickel and chromate salts were tested in concentration ranges equivalent to those used in the present study and caused similar reductions in plating efficiencies. The results of the transformation studies of soluble and particulate metals in these reports are clearly different from those in the present study. The different results with the particles can be accounted for by the nature of the particles and is discussed in more detail in the next section. The differences in the soluble salt studies may reflect different standards in the scoring of transformed foci or may be related to differences in the serum used in the culture medium.

Most recently Kowalski *et al* (1992) reported that addition of sodium vanadate to bovine papillomavirus DNA-transfected C3H/10T½ cells increased the number of transformed foci up to 50-fold suggesting that vanadate may act as

a tumour promotor, though they did not specify the morphology of the foci produced.

5.3.2 Metal-induced transformation of BALB/3T3 cells.

The BALB/3T3 Cl.A31 transformation assay is similar to the C3H/10T½ system in that it uses as its endpoint the formation of transformed foci in a monolayer of contact-inhibited mouse embryo fibroblasts. Saffiotti and Bertolero (1989) reported that soluble potassium chromate induced dose dependent type III transformation of BALB/3T3 cells and that the transformed cells produced non-metastatic fibrosarcomata in nude mice. Sabbioni *et al* (1991) studied the effects of soluble vanadium compounds in the same system and found that pentavalent vanadium (ammonium vanadate) produced type III transformation whereas tetravalent vanadium (vanadyl sulphate) did not cause transformation.

5.3.3 Metal-induced transformation in other rodent cell systems.

Metals have been more extensively studied in the Syrian Hamster Embryo (SHE) transformation assay. This system differs from the C3H/10T½ assay in that individual colonies of exponentially growing cells are examined for morphological transformation after a shorter incubation period following treatment.

DiPaulo and Casto (1979) examined a number of metal compounds in the SHE system and obtained positive results with nickel sulphate, nickel subsulphide and sodium chromate while ferric oxide, titanium dioxide and aluminium chloride did not induce transformation. Costa *et al* (1978) also found that nickel subsulphide and nickel dust induced morphological transformation in SHE cells and that nickel subsulphide-transformed cells produced sarcomas in nude mice (Costa *et al* 1979). Costa and Mollenhauer (1980) then showed that the transfor-

mation of SHE cells by nickel subsulphide was proportional to the uptake of the particles by phagocytosis and found similar effects with particles of cobalt sulphide (Costa *et al* 1982).

Other studies have examined metals as enhancers or promoters of cell transformation. Casto *et al* (1979) tested a large number of metal compounds for their capacity to enhance viral transformation of SHE cells. Among those giving positive results were calcium chromate, cobalt acetate, nickel sulphate and ferrous chloride while aluminium chloride and titanium dioxide did not increase the rate of transformation. Similarly, Rivedal and Sanner (1981) demonstrated that nickel sulphate and potassium chromate promoted SHE cell transformation initiated by benzo[a]pyrene and more recently Rivedal *et al* (1990) found a similar effect with various vanadium compounds.

Baby Hamster Kidney (BHK21) cells have been used to study anchorage independent growth. Using this method Fradkin *et al* (1975) obtained positive results with calcium chromate. Similarly, Hansen and Stern (1982) induced transformation with a number of nickel preparations.

5.3.4 Metal-induced transformation in human cells.

Attempts to develop transformation assays in human cells based on focus formation as in the rodent cell assays have generally been unsuccessful. However Biedermann and Landolph (1987, 1990) developed an assay in which anchorage independent growth of diploid human fibroblasts was induced by a number of nickel and chromium salts including lead chromate, potassium dichromate, nickel subsulphide and nickel sulphate. No other studies of this type appear to have been done and in the present study the method was not successful.

5.3.5 Summary.

No previous investigations of *in vitro* transformation by metals have specifically examined metals of orthopaedic interest with the aim of comparing their relative risks. Although some of the metals, particularly nickel and chromium, have been tested a number of times in various studies, others have received little attention and direct comparisons are difficult to make owing to variations in the assay methods and the preparations used.

Only nickel and chromium have previously been tested in the standard C3H/10T½ assay. These studies produced conflicting results with the present study in that soluble nickel and chromate salts did not induce transformation. However insoluble nickel and chromate salts did produce positive results.

Soluble nickel and chromate salts have repeatedly yielded positive results in the other assay systems. Of the other metals which induced transformation in this study namely cobalt, trivalent chromium and molybdenum, only cobalt appears to have been tested previously, producing positive results in the SHE assay.

Titanium, aluminium, iron and tetravalent vanadium did not cause transformation in the present study. Of these titanium, aluminium and tetravalent vanadium have also produced negative results in other assays. One study suggests that ferrous chloride may enhance transformation of SHE cells and studies of vanadium suggest it has transforming potential in its pentavalent form.

The results in this study are therefore in broad agreement with previous work on metal-induced transformation, bearing in mind that with the exceptions of nickel and chromium, previous studies are somewhat limited in number.

5.4 Effect of the physical form of metals.

The importance of the physical form in which the metals are presented is clearly shown in the results of the metal particle experiments. Several of the metals tested as soluble salts produced transformation. When tested as metallic particles they did not do so but still showed evidence of toxicity. These differences may be related to a number of factors.

5.4.1 Phagocytosis and transformation.

Costa and Mollenhauer (1980) found that particles of nickel subsulphide were taken up by vacuolation in SHE cells and were more potent transforming agents than soluble nickel chloride. Further studies (Costa *et al* 1981, Costa *et al* 1982) indicated that the cellular uptake and consequent transforming potency of particulate nickel compounds was related to their physical structure. Particles of crystalline nickel subsulphide were actively phagocytised and induced transformation whereas particles of amorphous nickel sulphide and of metallic nickel were not taken up and did not cause transformation. More recently, Patierno *et al* (1988) related the transformation of C3H/10T½ cells by insoluble particles of lead chromate to internalisation of the lead chromate particles as shown by electron microscopy. The authors suggested that this may be the result of prolonged intracellular release of metals by slow dissolution or due to more complex and at present unrecognised intracellular mechanisms. These observations suggest that in the present study, although the metal particles were seen to be in close association with the cells, they were not truly internalised.

5.4.2 Solubility of metals.

The effects of differences in the solubilty of metal particles on cells in culture was demonstrated previously by Rae (1981). In a study examining the

toxicity of metals used in orthopaedic implants, he found that particles of cobalt and vanadium were toxic to cells in culture whereas particles of nickel, chromium, molybdenum, titanium and aluminium were not. Measurements of the levels of soluble metals in the culture medium during the incubation period revealed that only cobalt, vanadium and, to a lesser extent, nickel particles produced detectable amounts of soluble metal. It was concluded that the toxicity of the metal particles was related to their solubility. Similar effects were seen in the present study with cobalt, nickel and vanadium particles producing toxicity while the other metal particles were relatively non toxic.

5.4.3 Physical effects of metal particles.

It will be noted that in the cases of cobalt and nickel particles, toxicity occurred at around the same dose levels as with the soluble salts. As the metal particles did not completely dissolve in the culture medium - indeed they remained clearly visible throughout the incubation period - the actual concentration of soluble metal in the particle experiments would have been much lower than in the equivalent metal salt assays. Rae (1978) also showed that some metal particles, notably cobalt, nickel and Co-Cr alloy, caused direct damage to cell membranes. It therefore seems probable that the toxicity seen with some of the metal particles was a result of both the release of soluble metal ions and direct physical damage to the cells.

Equally, visible amounts of metal particles remained in the culture dishes during the transformation assays and presumably gave rise to soluble metal ions throughout the incubation period. However, the medium was changed at regular intervals so it is unlikely that there was any significant accumulation of soluble metal after the initial exposure period.

5.5 The relative risks of orthopaedic metals as measured by this study.

One of the primary aims of this study was to assess the relative risks of the main orthopaedic alloys and of their constituent metals in terms of potential carcinogenicity and toxicity. The design of the transformation assays allows both these aspects to be tested and also allows comparison with a known potent carcinogen. The relative plating efficiency gives a quantitative measure of cytotoxicity allowing direct comparison of the metals. As stated previously, the measurement of transformation incidence does does not give a truly quantitative value. However, a semi-quantitative comparison may be made from the dose levels at which transformation occurs and a further qualitative comparison may be made from the focus morphology.

5.5.1 Soluble metal salts.

Nine metal salts were tested in the C3H/10T½ cytotoxicity/transformation assay.

Cobalt chloride, nickel chloride, chromium chloride, and sodium chromate demonstrated both toxic and neoplastic effects. All four compounds demonstrated dose related toxic effects and a trend towards dose related neoplastic transformation. For these metals, there appeared to be a correlation between toxicity and transformation with transformation increasing with toxicity (Fig. 5.1). In terms of the concentrations at which toxicity and neoplastic transformation occurred, sodium chromate was the most potent agent and chromium chloride the least potent, with cobalt chloride and nickel chloride intermediate. None of these metals produced transformation to the same extent as 3-methylcholanthrene and all of the transformation foci were of the less "aggressive" type II morphology.

At the other extreme, three compounds, ferrous chloride, aluminium chloride and titanium chloride were neither toxic nor did they cause significant in-

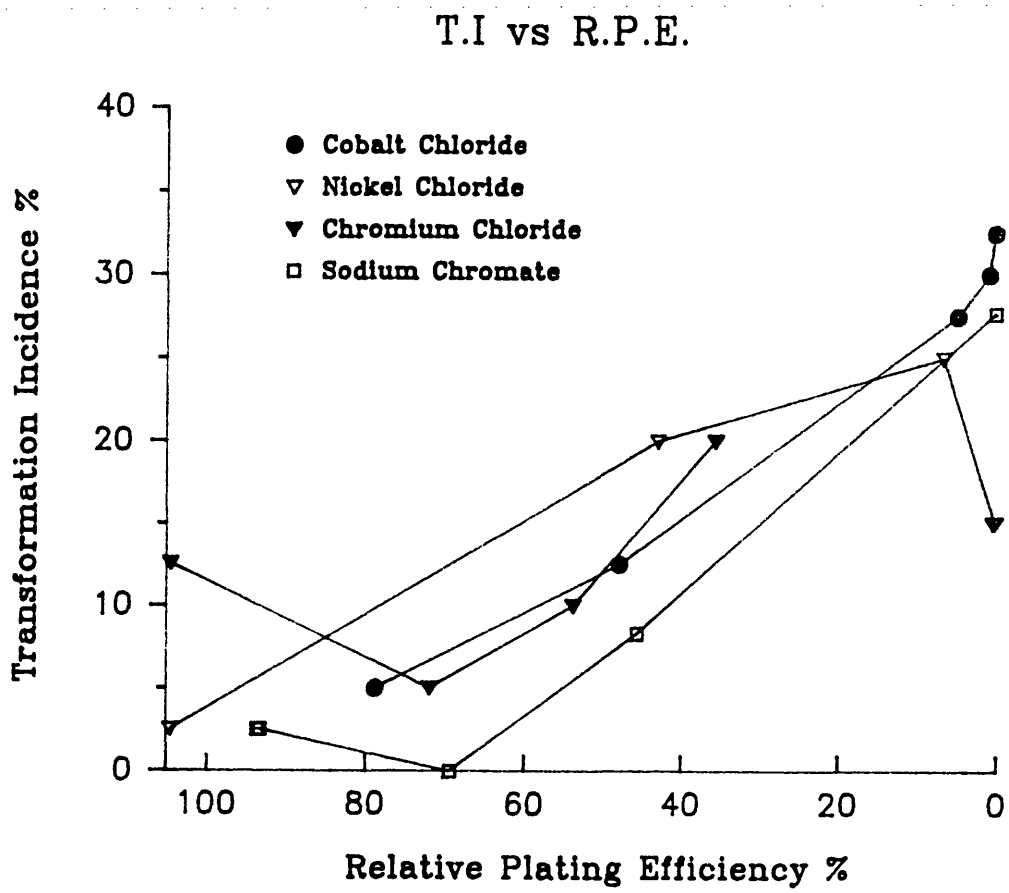


Fig. 5.1 Transformation incidence (TI) and relative plating efficiency (RPE) for those metal salts which were both toxic and transforming.

creases in transformation incidence. Titanium chloride produced no transformation foci at any concentration.

The remaining two compounds however, did not demonstrate this relationship between toxicity and transformation. Sodium molybdate showed very little evidence of toxicity but did produce significant transformation, though not in a dose related fashion, whereas vanadyl chloride was highly toxic but did not cause transformation.

5.5.2 Particulate metals and alloys.

Eight particulate metals and three particulate alloys were tested in the C3H/10T½ assay.

When tested in particulate form, none of the metals or alloys produced cellular transformation. Cobalt, nickel and vanadium were markedly toxic, while chromium, iron, molybdenum, aluminium and titanium were relatively non-toxic. The three alloys, stainless steel, CoCr and Ti6Al4V showed intermediate toxicity.

5.5.3 Summary.

This study has shown that some of the metals used in orthopaedic prostheses are capable of inducing cellular transformation *in vitro*. The metals were weaker transforming agents than the control, 3-methylcholanthrene, both in terms of the incidence of transformation and the degree to which the cells were transformed. Transformation occurred with soluble metal salts but not with metal particles, possibly reflecting differences in the intracellular availability of free metal ions. In general there appeared to be a relationship between toxicity and carcinogenicity in that most of the metals which were toxic produced transformation and those which were not toxic did not transform, though two metals did not

display this correlation.

It is clear that under the conditions of this study of the metals used in orthopaedic prostheses some are potentially more hazardous than others, depending to some extent on the physical form in which the metals are presented. The greatest risks, both for toxicity and carcinogenicity, are associated with cobalt, chromium and nickel and the least with iron, aluminium and titanium. Vanadium is potentially harmful because of its cytotoxicity and molybdenum showed evidence of possible carcinogenicity.

On this basis, the safest alloy in current use would appear to be titanium-aluminium-vanadium. None of the constituent metals caused transformation and only vanadium, a minor element of the alloy, was toxic. Both stainless steel and cobalt-chromium alloy contain appreciable amounts of those metals which showed evidence both of transforming ability and toxicity.

These observations are necessarily based on the results of experiments on rodent fibroblasts. The results of the human cell experiments indicate that these conclusions hold true for human cells as far as toxicity is concerned but it was not possible to confirm the transformation findings in human cells.

5.6 Reasons for the failure of the human cell transformation assay.

5.6.1 General difficulties with human cells in culture.

Although there are several well documented assays for the carcinogen-induced transformation of rodent cells in culture, *in vitro* transformation of normal human cells to malignant behaviour has proved to be more difficult. A number of authors have reviewed these difficulties in detail (DiPaulo 1983, Gabrielson and Harris 1985, Milo and Casto 1986, Harris 1987, McCormick and Maher 1988). All are agreed that the problems in transforming human cells stem from their inherent stability in culture. Rodent cell cultures often give rise to cell lines with infinite lifespans and abnormal karyotypes and it is in these abnormal populations that transformation to malignant behaviour may be induced. In contrast, the spontaneous appearance of such characteristics as infinite lifespan and karyotypic abnormalities rarely, if ever, occurs in cultures of diploid human cells. This difference is probably a reflection not only of the greater species susceptibility of the rodent cells, but also of the inbred nature of the strains of laboratory animals from which the cells are derived. Cultured human cells are of necessity much more heterogenous in origin.

Because of this difficulty, a reproducible assay for the malignant transformation of human cells has yet to be developed. There have been reports of the transformation of human cells (Kakunaga 1978), but this has not been reproduced in other laboratories. Indeed, a later study indicated that these results were due to contamination of the assay by fibrosarcoma cells and concluded that no one had yet succeeded in producing malignantly transformed cells by carcinogen treatment of normal human fibroblasts (McCormick and Maher 1988).

5.6.2 Factors relevant to this study.

Studies of carcinogenicity in human cell cultures have therefore concentrated on the induction of "transformation-like" changes which are thought to represent steps in the multistage process of malignant change (Harris 1987). Conversion to anchorage independent growth has been one of the most widely used endpoints and the study of Biedermann and Landolph (1987) was the first to report positive results with metals using this endpoint. In the present study, valid results could not be obtained and the following observations may help explain this.

The assay for anchorage independent growth is technically more difficult to perform than the more straightforward growth of monolayer cultures and it is possible that more experience with the technique would have yielded better results.

It was originally hoped to perform the assay using human synovial fibroblasts as it was thought they would be more relevant in a study related to orthopaedic implants. However, the great majority of previous studies on human fibroblasts have used neonatal foreskin cells and it seems likely that synovial cells from a nine year old donor are not suitable for this type of study.

Tejwani *et al* (1982) reported that only early passage neonatal foreskin fibroblasts can be successfully transformed to anchorage independence. The foreskin cells used in the present study were obtained at passage 9 and used at passage eleven onwards. Biedermann and Landolph (1987) whose protocol was followed do not state precisely at which passage number cells were used but as they were grown de novo from foreskin explants it is likely that they were earlier passage cells than those used in this study.

A second factor known to influence the assay is the use of cell cycle synchronisation prior to carcinogen exposure. Zimmerman and Little (1981) showed that pre-treatment synchronisation by use of amino acid deficient medium

increased the frequency of transformation compared with treatment of cells used at confluence. Similarly, Silinskas *et al* (1981) showed that the use of synchronised cells produced an increase in transformation of 25% over that obtained with randomly dividing cells.

It is possible that with greater experience of the assay and the use of early passage primary cultures combined with pre-treatment cell synchronisation, results in the present study could have been improved. Unfortunately restrictions on the time available for the study meant that these avenues could not be explored further.

CHAPTER SIX

GENERAL DISCUSSION

6.1 Overview

The history of implant surgery is one of continual development and refinement with the principles of biomechanics and biocompatibilty progressing hand in hand. As some problems are solved others emerge to be investigated and dealt with in the quest for the perfect material. Past successes mean that millions of patients worldwide now enjoy the undisputed benefits of metallic implants in fracture fixation and joint replacement surgery. However the question of implant-associated malignancy reminds us of the need for constant vigilance and ongoing assessment.

The background review of this thesis clearly demonstrates the multi-disciplinary nature of the study of biocompatibility. A comprehensive investigation of implant-related carcinogenesis would include many interrelated aspects and is beyond the scope of any single piece of work. This study confined itself to one of these aspects, the role of metals as chemical toxins and carcinogens. It examined and compared the principal constituents of the standard implant alloys in a single cell culture system designed to assess toxicity and carcinogenic potential. Some metals induced cell transformation and others did not. While this does not prove that metal implants cause cancer, the results add to our knowledge of metal carcinogenesis and provide a way of grading the relative risks of the currently used alloys. The greatest risks were associated with cobalt, chromium and nickel and the least with iron, aluminium and titanium. These findings are in broad agreement with the results of previous work including epidemiological studies of human exposure, experimental animal bioassays and *in vitro* cell transformation studies. On this basis the safest alloy in terms of toxicity and potential carcinoge-

nicity would appear to be Ti6Al4V. The relevance of this work to clinical practice requires consideration of a number of other factors.

6.2 Relevance to the *in vivo* situation.

Closed cell culture systems do not always reflect the complex mechanisms of the intact organism. Most of the metals released from implants play biological roles as trace elements and it is likely that homeostatic mechanisms exist for their uptake, distribution and excretion. However great increases over the normal trace levels may overwhelm these mechanisms and lead to sustained high systemic levels of metal as reflected in the high blood levels found in some studies. Similarly, the cells of the lymphoreticular system act as scavengers in removing particulate debris but it is clear that when wear is excessive there are limits to this process and that local levels remain high. Thus when regulatory mechanisms fail, the situation *in vivo* may be similar to that created *in vitro*.

Clearly, the results of observations on rodent cells must be interpreted with caution in considering the human recipient. It was not possible to conduct transformation experiments on human cells but the findings in the cytotoxicity tests indicate that, at least as general toxins, the tested metals behave similarly in human and mouse cells.

Studies on patients with joint replacements show conclusively that metals are released from all of the alloys in use. However, little is known of the precise physical form in which they are released or of their interactions with homeostatic mechanisms. It is generally accepted that metals are biologically most active in the soluble ionic form. Histological examination of periprosthetic tissue frequently shows the presence of particles but measurements of metal levels in such tissues give only a gross weight-for-weight figure with no indication of the concentration of soluble ions. Increased blood levels of metals are seen in cases

of prosthetic wear but it is not known whether the metals in these cases are predominantly freely dissolved or protein bound. Such considerations create difficulties in assessing the significance of metal release *in vivo*. However, it seems likely that in clinical situations where wear occurs, the bulk of released metal exists as solid particles which then give rise to the release of soluble ionic forms. When wear has not occurred soluble metals are released more slowly by pure corrosion from the surface of the implant.

In the present *in vitro* study cell transformation was only seen with soluble metals. The metal concentrations used in this study were far greater than those reported in body fluids from patients. This must be weighed against the considerably longer exposure time which may occur *in vivo*.

Particulate metals and alloys did not cause cell transformation in this study. In other cell culture studies the transforming potency of particles of metal compounds has been related to their cellular uptake. It is clear from histological studies that *in vivo*, large numbers of wear particles accumulate intracellularly. This may give rise to prolonged intracellular release of metal ions and it is therefore conceivable that metallic wear debris may actually be more dangerous *in vivo* than suggested by the cell culture system used in this study.

There is also uncertainty as to the nature of the metallic wear particles seen around implants. Although measurements clearly show that the particles contain metals it is possible, particularly in the case of titanium, that they arise from wear of the passivated surface of the prosthesis and therefore consist of metal oxides and hydroxides rather than pure metals. The significance of this difference in terms of potential carcinogenicity is not known.

6.3 Clinical implications.

The overall risks of implanted metals depends on the outcome of complex physical and biological processes, many of which are still poorly understood. At present the evidence for implant-induced cancer in patients remains scanty and somewhat controversial. Conversely the benefits to patients are indisputable. However there is a growing body of evidence that some metals are carcinogenic in certain circumstances. This study indicates that of the alloys used in orthopaedic prostheses, some are potentially more dangerous than others in this respect, the safest being titanium alloy. Given that it has so far proved impossible to completely prevent the release of metal from implants it would seem reasonable to direct future developments toward greater use of titanium alloys in permanent implants. Wear of the currently used Ti6Al4V alloy is a problem however and a more wear resistant titanium alloy is needed. The application of a wear resistant coating is an alternative approach to the problem. Early studies of a very hard diamond-like carbon polymer indicate satisfactory biocompatibility (Thomson *et al* 1991), but its wear resistance *in vivo* remains to be demonstrated. As long as wear and metal release continue to be a problem, efforts should be made to minimise tissue burdening by undertaking revision of loose prostheses as early as possible with due attention given to other clinical considerations.

Patients with permanent joint replacements tend to be from the older age groups with consequently shorter life expectancy. However many young patients receive fracture fixation devices which may then remain in place for several decades. While metal release from static devices is low, with the prospect of such prolonged exposure it would seem prudent to remove these devices in young patients after fracture healing has occurred. Metal removal is not always a straightforward procedure and should be weighed against the potential risks but should be possible in most cases. This policy has obvious cost implications in a

financially constrained health service. The development of biodegradable fixation devices may in future reduce the need for metal removal.

6.4 Recommendations for future work.

In vitro cell transformation studies have become valuable tools for the screening of potential carcinogens and in the study of carcinogenic mechanisms. They have a number of limitations but hopefully will become increasingly sophisticated as the understanding of cellular mechanisms improves. The development of reliable *in vitro* human cell transformation systems would greatly increase their value. Further attempts to produce metal-induced anchorage independent growth in human cells along the lines described in chapter 5 could produce very useful comparisons with the results already achieved and should be pursued. However, early passage neonatal foreskin cells are less easy to obtain in the United Kingdom than in the United States where the method was developed and where elective circumcision is more common practice. It is possible that a similar assay using umbilical cord cells may be more successful.

In recent years it has been discovered that in some tumours the process of carcinogenesis is associated with the activation of specific genes called oncogenes. Evidence of oncogene activation has been found in transformed C3H/10T½ cells (Billings *et al* 1987). It would be of great value to apply this technique to metal-transformed cells to aid in the confirmation and quantification of transformation.

The present study was limited to testing metal salts and metal or alloy particles. It seems likely that the particulate debris produced *in vivo* is at least partly composed of metal oxides and hydroxides. Since this and other studies show that the physical form of the metal is significant, it is important to conduct further studies on metal oxide/hydroxide complexes.

The study of molecular biology has made great strides in the under-

standing of carcinogenic mechanisms. Historically, the discovery of carcinogenic agents and the assessment of exposure risk owes much to epidemiological research. To date only two such studies have examined the question of metal implant-associated cancer and clearly more such studies are needed. Finally, it is important that orthopaedic surgeons remain aware of the possible biological effects of their treatment and continue to report occurrences of implant-associated tumours.

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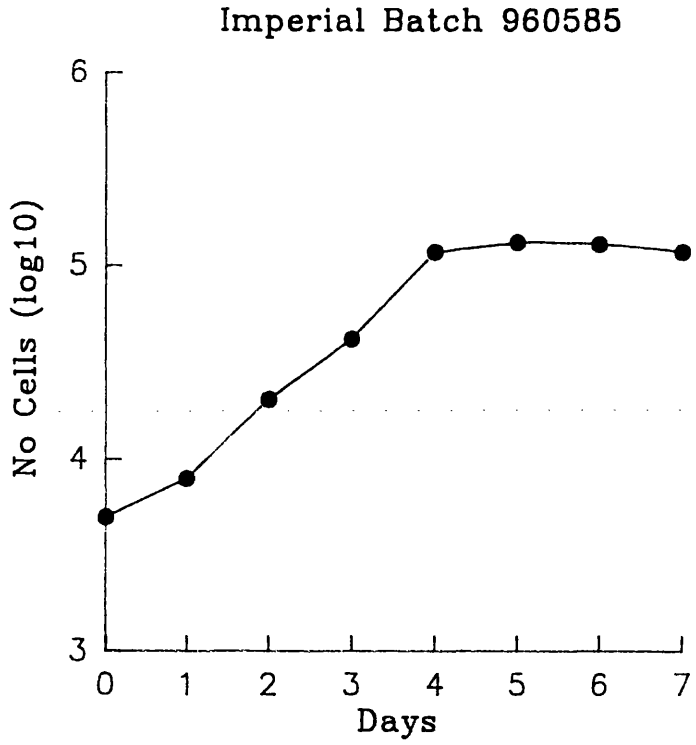
Appendix A. Batch testing and validation studies.

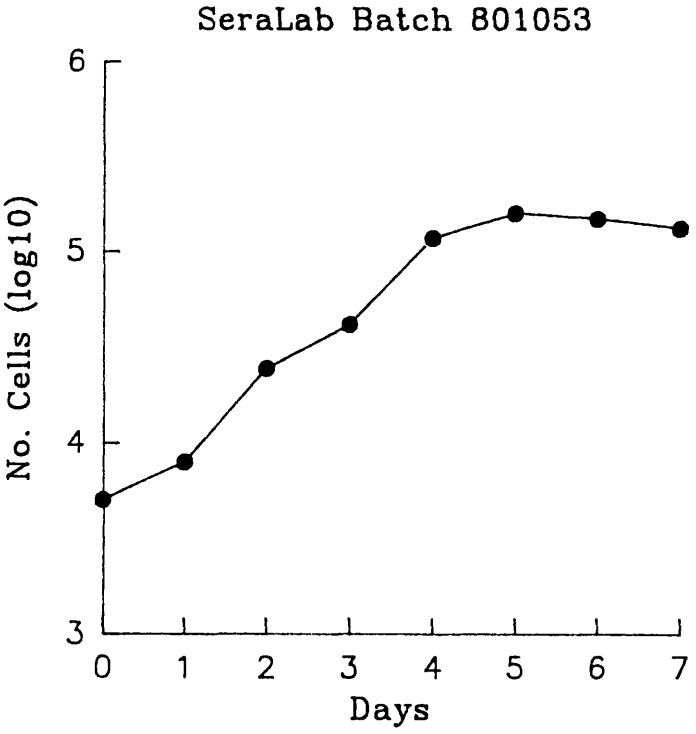
1. Plating efficiencies of serum batches.

Batch	Plating Efficiency	Population Doubling Time
NBL Batch 19827	50.4%	13 hrs
Imperial Batch 960585	50.4%	12 hrs
Gibco Batch 06G9982Y	43.7%	13 hrs
SeraLab Batch 801053	51.9%	14 hrs

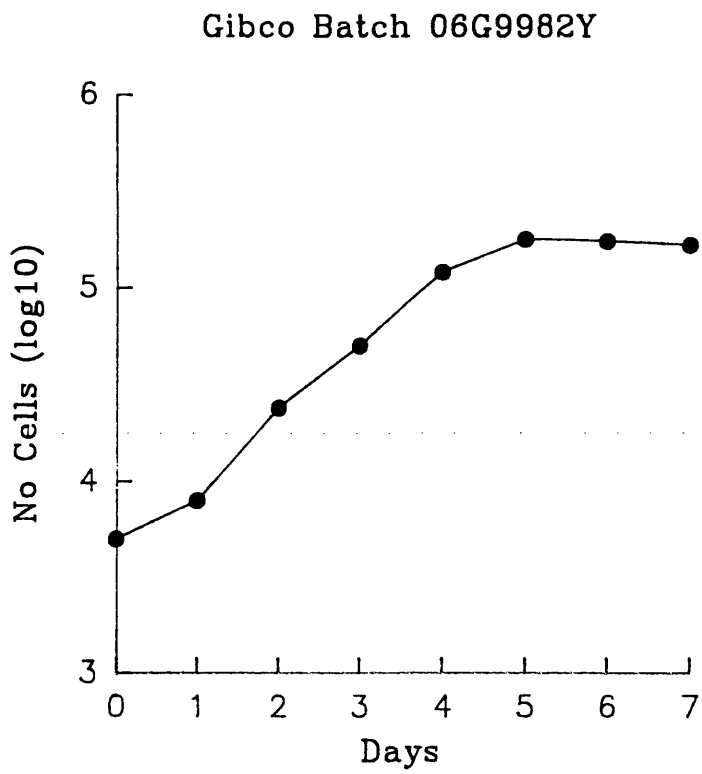
2. Batch transformation assays.

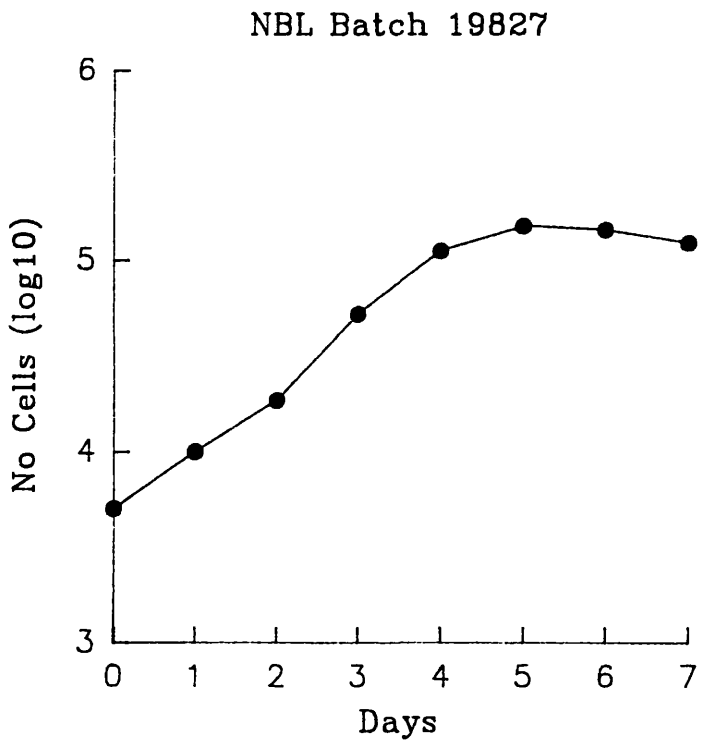
Batch	Focus type		Transformation Incidence
	II	III	
NBL 19827			
3-MCA 2.5 μg/ml	16	4	12/20
DMSO 0.5%	-	-	0/20
Imperial 960585			
3-MCA 2.5 μg/ml	10	8	14/20
DMSO 0.5%	-	-	0/20
Gibco 06G9982Y			
3-MCA 2.5 μg/ml	21	3	14/20
DMSO 0.5%	1	-	1/20
SeraLab 801053			
3-MCA 2.5 μg/ml	30	18	18/20
DMSO 0.5%	-	-	0/20





3. Growth curves for serum batches.





3. Growth curves for serum batches (continued).

Appendix B. Results of metal salt cytotoxicity assays.

Cobalt Chloride

Concentration (μg/ml)	Assay 1		Assay 2	
	P.E.(%)	R.P.E.(%)	P.E.(%)	R.P.E.(%)
0	20.8	100	19.9	100
0.1	19.2	92.3	13.0	65.3
1.0	12.4	59.6	7.2	36.2
5.0	0.6	2.9	1.4	7.0
10.0	0.4	1.9	0	0
20.0	0.1	0.5	0	0

Nickel Chloride

Concentration (μg/ml)	Assay 1		Assay 2	
	P.E.(%)	R.P.E.(%)	P.E.(%)	R.P.E.(%)
0	21.4	100	19.9	100
1.0	22.5	105	20.7	104
10.0	9.9	46.3	7.9	39.7
20.0	1.4	6.5	1.4	7.0
40.0	0.1	0.5	0	0
60.0	0	0	0.2	1

Chromium Chloride

Concentration (μg/ml)	Assay 1		Assay 2	
	P.E.(%)	R.P.E.(%)	P.E.(%)	R.P.E.(%)
0	22.2	100	19.9	100
300.0	24.2	109	19.9	100
600.0	21.7	97.7	9.2	46.2
900.0	13.2	59.5	9.6	48.2
1200.0	0	0	14.2	71.3

Sodium Chromate

Concentration (μg/ml)	Assay 1		Assay 2	
	P.E.(%)	R.P.E.(%)	P.E.(%)	R.P.E.(%)
0	18.9	100	19.9	100
0.005	18.7	98.9	17.6	88.4
0.01	15.2	80.4	21.1	106.0
0.05	16.1	85.2	10.7	53.7
0.1	9.2	48.7	8.5	42.7
0.5	0.1	0.5	0	0

Ferrous Chloride

Concentration (μg/ml)	Assay 1		Assay 2	
	P.E.(%)	R.P.E.(%)	P.E.(%)	R.P.E.(%)
0	23.0	100	34.9	100
10.0	21.9	95.2	35.5	101.7
50.0	23.6	102.0	29.0	83.1
100.0	21.2	92.2	31.2	89.5
200.0	20.6	89.6	31.7	90.8

Sodium Molybdate

Concentration (μg/ml)	Assay 1		Assay 2	
	P.E.(%)	R.P.E.(%)	P.E.(%)	R.P.E.(%)
0	33.5	100	36.5	100
1.0	35.5	106	34.4	94.2
10.0	32.7	97.6	35.1	96.2
100.0	32.7	97.6	35.6	97.6
500.0	31.9	95.2	39.9	109.2

Vanadyl Chloride

Concentration (μg/ml)	Assay 1		Assay 2	
	P.E.(%)	R.P.E.(%)	P.E.(%)	R.P.E.(%)
0	21.0	100	36.5	100
0.1	16.7	79.5	35.1	96.2
0.5	10.6	50.5	20.1	55.1
1.0	8.6	40.9	10.9	29.9
5.0	0	0	0	0

Aluminium Chloride

Concentration (μg/ml)	Assay 1		Assay 2	
	P.E.(%)	R.P.E.(%)	P.E.(%)	R.P.E.(%)
0	33.5	100	36.5	100
1.0	26.6	79.4	30.1	82.5
10.0	27.2	81.2	30.6	83.9
100.0	31.0	92.5	33.6	92.1
500.0	30.6	91.3	33.9	92.9

Titanium Chloride

Concentration (μg/ml)	Assay 1		Assay 2	
	P.E.(%)	R.P.E.(%)	P.E.(%)	R.P.E.(%)
0	36.5	100	34.9	100
10.0	28.9	79.2	35.4	101.4
50.0	27.9	76.4	31.0	88.8
100.0	30.9	84.7	34.1	97.8
500.0	*		27.9	79.9

* lost to infection

Appendix C. Results of metal salt transformation assays.

Cobalt Chloride Assay 1

Concentration (μg/ml)	Focus type II	III	Total foci	Transformation Incidence
0.1	1	-	1	1/20
1.0	5	-	5	5/20
5.0	5	-	5	5/20
10.0	7	-	7	6/20
20.0	11	-	11	7/20
water	1	-	1	1/20
3-3-MCA 2.5μg/ml	44	7	51	16/20

Cobalt Chloride Assay 2

Concentration (μg/ml)	Focus type II	III	Total foci	Transformation Incidence
0.1	1	-	1	1/20
1.0	-	-	-	0/20
5.0	6	-	6	6/20
10.0	6	-	6	6/19*
20.0	6	-	6	6/20
water	-	-	-	0/20
3-MCA 2.5μg/ml	36	18	54	18/20

* one well lost to infection

Nickel Chloride Assay 1

Concentration (μg/ml)	Focus type II	III	Total foci	Transformation Incidence
1.0	1	-	1	1/20
10.0	5	-	5	5/20
20.0	1	-	1	1/20
40.0	6	-	6	4/20
60.0	1	-	1	1/20
water	1	-	1	1/20
3-MCA 2.5μg/ml	44	7	51	16/20

Nickel Chloride Assay 2

Concentration (μg/ml)	Focus type II	III	Total foci	Transformation Incidence
1.0	-	-	-	0/20
10.0	4	-	4	3/20
20.0	14	-	14	9/20
40.0	2	-	2	2/20
60.0	7	-	7	5/20
water	-	-	-	0/20
3-MCA 2.5μg/ml	36	18	54	18/20

Chromium Chloride Assay 1

Concentration (μg/ml)	Focus type II	III	Total foci	Transformation Incidence
300.0	5	-	5	5/20
600.0	1	-	1	1/20
900.0	4	-	4	4/20
1200.0*	13	2	15	8/20
water	1	-	1	1/20
3-MCA 2.5μg/ml	44	7	51	16/20

* extensive detachment of monolayer in 10 wells,foci counted in remaining wells with intact monolayers.

Chromium Chloride Assay 2

Concentration (μg/ml)	Focus type II	III	Total foci	Transformation Incidence
300.0	-	-	-	0/20
600.0	1	-	1	1/20
900.0	-	-	-	0/20
1200.0*	-	-	-	0/20
water	-	-	-	0/20
3-MCA 2.5μg/ml	36	18	54	18/20

* extensive detachment of monolayer in all wells, patches of increased cell density in remaining areas but no true foci.

Sodium Chromate Assay 1

Concentration (μg/ml)	Focus type II	Focus type III	Total foci	Transformation Incidence
0.005	-	1	1	1/20
0.01	2	-	2	1/20
0.05	-	-	-	0/20
0.1	3	-	3	3/16*
0.5	5	-	5	4/16*
water	1	-	1	1/20
3-MCA 2.5μg/ml	44	7	51	16/20

* four wells lost to infection

Sodium Chromate Assay 2

Concentration (μg/ml)	Focus type II	Focus type III	Total foci	Transformation Incidence
0.005	-	-	-	0/20
0.01	-	-	-	0/20
0.05	-	-	-	0/20
0.1	-	-	-	0/20
0.5	7	-	7	6/20
water	-	-	-	0/20
3-MCA 2.5μg/ml	36	18	54	18/20

Ferrous Chloride Assay 1

Concentration (μg/ml)	Focus type II	Focus type III	Total foci	Transformation Incidence
10.0	4	1	5	3/20
50.0	-	-	-	0/20
100.0	2	-	2	2/20
200.0	-	1	1	1/20
water	1	-	1	1/20
3-MCA 2.5μg/ml	44	7	51	16/20

Ferrous Chloride Assay 2

Concentration (μg/ml)	Focus type II	III	Total foci	Transformation Incidence
10.0	-	-	-	0/20
50.0	6	-	6	6/20
100.0	2	-	2	2/20
200.0	6	-	6	5/20
water	1	-	1	1/20
3-MCA 2.5 μg/ml	6	15	21	15/20

Sodium Molybdate Assay 1

Concentration (μg/ml)	Focus type II	III	Total foci	Transformation Incidence
1.0	5	-	5	5/20
10.0	8	3	11	9/20
100.0	2	-	2	2/20
500.0	3	-	3	3/20
water	-	-	-	0/20
3-MCA 2.5μg/ml	36	18	54	18/20

Sodium Molybdate Assay 2

Concentration (μg/ml)	Focus type II	III	Total foci	Transformation Incidence
1.0	1	-	1	1/20
10.0	-	-	-	0/20
100.0	-	-	-	0/20
500.0	1	-	1	1/20
water	1	-	1	1/20
3-MCA 2.5 μg/ml	8	9	17	10/19*

* one well lost to infection

Vanadyl Chloride Assay 1

Concentration (μg/ml)	Focus type II	III	Total foci	Transformation Incidence
0.1	1	-	1	1/20
0.5	-	-	-	0/20
1.0	-	-	-	0/20
5.0	-	-	-	0/20
water	1	-	1	1/20
3-MCA 2.5μg/ml	44	7	51	16/20

Vanadyl Chloride Assay 2

Concentration (μg/ml)	Focus type II	III	Total foci	Transformation Incidence
0.1	-	2	2	2/16*
0.5	-	-	-	0/20
1.0	-	-	-	0/20
5.0	-	-	-	0/20
water	1	-	1	1/20
3-MCA 2.5 μg/ml	8	9	17	10/19**

* four wells lost to infection
** one well lost to infection

Aluminium Chloride Assay 1

Concentration (μg/ml)	Focus type II	III	Total foci	Transformation Incidence
1.0	6	-	6	4/20
10.0	1	-	1	1/20
100.0	1	-	1	1/20
500.0	2	-	2	2/20
water	-	-	-	0/20
3-MCA 2.5μg/ml	36	18	54	18/20

Aluminium Chloride Assay 2

Concentration (μg/ml)	Focus type II	III	Total foci	Transformation Incidence
1.0	-	-	-	0/20
10.0	1	-	1	1/20
100.0	-	-	-	0/20
500.0	-	-	-	0/20
water	1	-	1	1/20
3-MCA 2.5 μg/ml	8	9	17	10/19*

* one well lost to infection

Titanium Chloride Assay 1

Concentration (μg/ml)	Focus type II	III	Total foci	Transformation Incidence
10.0	-	-	-	0/20
50.0	-	-	-	0/20
100.0	-	-	-	0/20
500.0	-	-	-	0/20
water	1	-	1	1/20
3-MCA 2.5 μg/ml	8	9	17	10/19*

* one well lost to infection

Titanium Chloride Assay 2

Concentration (μg/ml)	Focus type II	III	Total foci	Transformation Incidence
10.0	-	-	-	0/20
50.0	-	-	-	0/20
100.0	-	-	-	0/20
500.0	-	-	-	0/20
water	1	-	1	1/20
3-MCA 2.5 μg/ml	6	15	21	15/20

Appendix D. Results of metal particle cytotoxicity/transformation assays.

Cobalt Particles

Concentration μg/ml	R.P.E.(%)	Focus type II	Focus type III	Transformation Incidence
0.5	34.8	-	-	0/20
1.0	29.3	-	-	0/20
5.0	2.5	2	-	2/20
10.0	0	-	-	0/20
water	100	1	-	1/20
3-MCA	-	6	15	15/20

Nickel Particles

Concentration μg/ml	R.P.E.(%)	Focus type II	Focus type III	Transformation Incidence
1.0	71.2	-	-	0/20
5.0	38.3	-	-	0/20
10.0	25.8	-	-	0/20
50.0	8.6	-	-	0/20
100.0	0.7	-	-	0/20
water	100	1	-	1/20
3-MCA	-	8	9	10/19*

* one well lost to infection

Chromium Particles

Concentration μg/ml	R.P.E.(%)	Focus type II	Focus type III	Transformation Incidence
5.0	78.7	-	-	0/20
10.0	88.0	-	-	0/16
50.0	89.2	-	-	0/16
100.0	38.8	-	-	0/20
500.0	17.8	-	-	0/20
water	100	1	-	1/20
3-MCA	-	8	9	10/19*

* one well lost to infection

Iron Particles

Concentration μg/ml	R.P.E.(%)	Focus type II	Focus type III	Transformation Incidence
5.0	99.9	1	-	1/20
10.0	98.8	1	-	1/20
50.0	89.9	-	-	0/20
100.0	93.8	-	-	0/20
water	100	1	-	1/20
3-MCA	-	8	9	10/19*

* one well lost to infection

Molybdenum Particles

Concentration μg/ml	R.P.E.(%)	Focus type II	Focus type III	Transformation Incidence
5.0	107.4	1	-	1/20
10.0	106.4	-	-	0/20
50.0	102.8	-	-	0/20
100.0	98.5	-	-	0/20
500.0	28.7	-	-	0/20
water	100	1	-	1/20
3-MCA	-	6	15	15/20

Titanium Particles

Concentration μg/ml	R.P.E.(%)	Focus type II	Focus type III	Transformation Incidence
5.0	95.3	1	-	1/20
10.0	97.8	-	-	0/20
50.0	59.4	-	1	1/20
100.0	60.9	-	-	0/20
500.0	26.5	-	-	0/17*
water	100	1	-	1/20
3-MCA	-	8	9	10/19**

* three wells lost to infection
** one well lost to infection

Vanadium Particles

Concentration μg/ml	R.P.E.(%)	Focus type II	Focus type III	Transformation Incidence
5.0	91.3	-	-	0/20
10.0	65.2	-	-	0/16
50.0	4.0	-	-	0/20*
100.0	0.0	-	-	0/20**
water	100	1	-	1/20
3-MCA	-	6	15	15/20

* incomplete monolayer at end of transformation assay
** no viable cells at end of transformation assay

Aluminium Particles

Concentration μg/ml	R.P.E.(%)	Focus type II	Focus type III	Transformation Incidence
5.0	93.7	-	-	0/20
10.0	88.0	-	-	0/20
50.0	69.0	-	-	O/20
100.0	76.0	-	-	0/20
500.0	0.0	-	-	0.20
water	100	1	-	1/20
3-MCA	-	6	15	15/20

Stainless Steel

Concentration μg/ml	R.P.E.(%)	Focus type II	Focus type III	Transformation Incidence
5.0	82.1	1	-	1/20
10.0	81.8	-	-	0/20
50.0	54.8	-	-	0/20
100.0	21.6	-	-	0/20
500.0	0.0	-	-	0/19*
water	100	1	-	1/20
3-MCA	-	8	9	10/19*

* one well lost to infection

Cobalt-chromium alloy

Concentration μg/ml	R.P.E.(%)	Focus type II	Focus type III	Transformation Incidence
5.0	79.2	-	-	0/20
10.0	93.0	-	-	0/20
50.0	79.6	-	-	0/20
100.0	63.4	-	-	0/20
500.0	10.6	-	-	0/20
water	100	1	-	1/20
3-MCA	-	6	15	15/20

Ti6Al4V Alloy

Concentration μg/ml	R.P.E.(%)	Focus type II	Focus type III	Transformation Incidence
5.0	97.2	-	-	0/19*
10.0	94.9	-	-	0/20
50.0	46.7	-	-	0/20
100.0	15.7	-	-	0/20
500.0	0.0	-	-	0/20
water	100	1	-	1/20
3-MCA	-	6	15	15/20

* one well lost to infection

Appendix E. Results of cytotoxicity assays with human synovial fibroblasts.

I. Metal salts

Cobalt Chloride

Concentration (μg/ml)	P.E.(%)	R.P.E.(%)
0.0	28.6	100
0.1	26.6	93.1
1.0	20.2	70.8
5.0	11.6	40.6
10.0	10.1	35.4
20.0	5.1	17.9

Nickel Chloride

Concentration (μg/ml)	P.E.(%)	R.P.E.(%)
0.0	26.8	100
1.0	28.6	106.8
10.0	16.0	59.7
20.0	4.0	14.9
40.0	0	0
60.0	0	0

Chromium Chloride

Concentration (μg/ml)	P.E.(%)	R.P.E.(%)
0.0	29.8	100
300.0	28.2	97.8
600.0	21.6	72.6
900.0	24.2	81.4
1200.0	16.1	54.1

Sodium Chromate

Concentration (μg/ml)	P.E.(%)	R.P.E.(%)
0.0	28.4	100
0.005	28.5	100
0.01	30.9	108.7
0.05	22.2	78.3
0.1	17.4	61.2
0.5	0	0

Ferrous Chloride

Concentration (μg/ml)	P.E.(%)	R.P.E.(%)
0.0	27.8	100
10.0	32.2	116
50.0	21.8	78.5
100.0	24.5	88.1
200.0	14.2	51.3

Sodium Molybdate

Concentration (μg/ml)	P.E.(%)	R.P.E.(%)
0.0	26.0	100
1.0	24.1	92.8
10.0	22.5	86.5
100.0	24.5	94.2
500.0	25.1	96.6

Vanadyl Chloride

Concentration (μg/ml)	P.E.(%)	R.P.E.(%)
0.0	22.8	100
0.1	25.3	110.7
0.5	25.5	111.8
1.0	20.9	80.3
5.0	0.7	3.3
10.0	0	0

Aluminium Chloride

Concentration (μg/ml)	P.E.(%)	R.P.E.(%)
0.0	15.4	100
1.0	14.4	93.3
10.0	15.8	102.3
100.0	12.4	80.4
500.0	10.5	68.2

Titanium Chloride

Concentration (μg/ml)	P.E.(%)	R.P.E.(%)
0.0	24.1	100
10.0	24.1	100
50.0	18.5	76.8
100.0	16.0	66.4
500.0	4.5	18.7

II. Metal particles

* denotes average of six controls (P.E. range 41.4 - 45.9)

Cobalt Particles

Concentration (μg/ml)	P.E.(%)	R.P.E.(%)
0.0	43.2	100 *
0.1	25.6	59.2
0.5	10.2	23.7
1.0	1.4	3.2
5.0	0	0
10.0	0	0

Nickel Particles

Concentration (μg/ml)	P.E.(%)	R.P.E.(%)
0.0	43.2	100 *
5.0	33.6	77.7
10.0	21.1	48.8
50.0	4.75	11.0
100.0	1.1	2.5

Chromium Particles

Concentration (μg/ml)	P.E.(%)	R.P.E.(%)
0.0	43.2	100 *
5.0	44.4	102.7
10.0	36.4	84.2
50.0	37.5	86.8
100.0	35.6	82.4
500.0	23.0	53.2

Iron Particles

Concentration (μg/ml)	P.E.(%)	R.P.E.(%)
0.0	43.2	100 *
5.0	40.7	94.3
10.0	41.1	95.1
50.0	39.6	91.6
100.0	42.0	97.2
500.0	25.6	59.2

Molybdenum Particles

Concentration (μg/ml)	P.E.(%)	R.P.E.(%)
0.0	43.2	100 *
5.0	38.4	88.9
10.0	41.2	95.5
50.0	29.2	67.7
100.0	30.0	69.4
500.0	9.1	21.1

Titanium Particles

Concentration (μg/ml)	P.E.(%)	R.P.E.(%)
0.0	43.2	100 *
5.0	38.5	89.1
10.0	34.0	78.7
50.0	25.4	58.8
100.0	18.6	43.1
500.0	8.7	20.2

Vanadium Particles

Concentration (μg/ml)	P.E.(%)	R.P.E.(%)
0.0	13.7	100
5.0	11.9	86.4
10.0	9.8	70.9
50.0	1.6	11.8
100.0	0	0

Aluminium Particles

Concentration (μg/ml)	P.E.(%)	R.P.E.(%)
0.0	43.2	100 *
5.0	33.7	78.1
10.0	34.7	80.4
50.0	29.0	67.1
100.0	32.5	75.2
500.0	13.2	30.7

Stainless Steel

Concentration (μg/ml)	P.E.(%)	R.P.E.(%)
0.0	13.8	100
5.0	11.1	80.6
10.0	10.5	76.0
50.0	4.0	29.0
100.0	2.2	16.3
500.0	0.1	1.0

Cobalt-chromium alloy

Concentration (μg/ml)	P.E.(%)	R.P.E.(%)
0.0	43.2	100 *
5.0	42.2	97.8
10.0	41.6	96.3
50.0	39.6	91.6
100.0	30.5	70.6
500.0	21.9	50.7

Ti6Al4V Alloy

Concentration (μg/ml)	P.E.(%)	R.P.E.(%)
0.0	14.9	100
5.0	13.4	96.9
10.0	7.5	54.3
50.0	3.0	21.7
100.0	1.1	8.1
500.0	0	0

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