ELECTRON-PROBE MICROANALYSIS OF TISSUE AND CELLS FROM IMPLANT AREAS

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The liberation into the tissues of the products of corrosion of various pure metals and of alloys used for surgical implants has been shown previously by spectrochemical analysis (Ferguson, Laing and Hodge 1960). The presence of these products has been thought to explain: 1) the persistence of infection in the presence of surgical implants; 2) the occurrence of an apparently sterile inflammatory response to a metallic implant (Ferguson *et al.* 1960); 3) persistent pain in relation to an implant (Scales, Winter and Shirley 1959).

To study further the influence of metal implants on these phenomena, the fate of the products of corrosion of implants at a cellular level has been investigated by electron-probe microanalysis. The concentration of the metallic corrosion products in cells cultured on metal grids *in vitro* and the distribution of metallic corrosion products in implant areas have been recorded.

EXPERIMENTAL MATERIALS AND METHODS

Preparation of metal grids—Figure 1 is a schematic diagram of cells growing on 0.63 centimetre metal grid of the type used for microanalysis. The grids* were made of nickel; or of nickel plated with gold, platinum or rhodium; or of stainless steel. In the last case the stainless steel grid was a square mesh of interlacing wire. The stainless steel was 18 per cent chromium-8 per cent nickel-3 per cent molybdenum steel complying with AISI 316, which approximates to the British Standard En 58J. Mesh of 52.3 lines per centimetre was used.

The grids were marked with a Reichert micro-hardness device to assist the identification of individual squares during microanalysis. Then the grids were degreased in acetone, rinsed and boiled for three minutes in 1 per cent 7×† solution, a non-toxic detergent, and washed in distilled water. The grids were hermetically sealed in sterilised chambers of glass and stainless steel (Weiss 1961). Some grids were placed in Perspex and glass culture chambers to check that the stainless steel in the chambers did not influence the results.

Preparation of cell cultures—Type "16C" cells (near-term foetal rat dermal fibroblasts of a "permanent" strain maintained at Strangeways Research Laboratory (Daniel, Dingle and Lucy 1961)) were dispersed from glass culture bottles by treating them with trypsin (0.25 per cent "Difco 1 : 250" in calcium-and-magnesium-free Tyrode's solution). They were washed and resuspended in 85 parts synthetic medium 199 (Morgan, Morton and Parker 1950) plus 15

FIG. 1

Schematic diagram of cells growing on a metal grid of the type used for microanalysis.

* The grids were kindly supplied by Messrs Smethurst High-Light Ltd., Sidcot Heaton, Bolton, Lancashire.
† Manufactured by Lubro Chemical Company Incorporated, New Haven, Connecticut, United States of America.

parts bovine serum. Drops of cell suspension were placed on the metallic grids contained in culture chambers which were then sealed and incubated overnight at 37 degrees Centigrade. After twenty-four hours the chambers were drained, refilled with fresh medium and incubated for a further forty-eight hours before microanalysis preparation.

Figure 2 is a phase contrast photomicrograph which shows cells growing adjacent to a nickel grid after three days in culture. Cell density was calculated on one-half of the grids: the number of cells per interstice ranged from 0 to 50, with an average cell count of about 10. The cells were dried satisfactorily by several methods, but rapid freezing or freeze drying in liquid nitrogen appeared to cause the least cellular damage. After two minutes in liquid nitrogen the grids were removed and photographed with bright field microscopy (Fig. 3). The grids were then placed in brass specimen capsules and analysed under vacuum in the electron-probe microanalyser. Two types of cell controls were used: either centrifuged cell deposits were placed on a carbon block and analysed immediately or cells prepared in the way previously described were analysed after culture on carbon blocks for three days. Microanalysis revealed no metallic contamination from either control. None of the solutions used for these experiments showed any metallic contamination by spectrochemical and qualitative analyses.

FIG. 2

FIG. 3

Figure 2—Phase contrast photomicrograph of living "16C" cells "on" pure nickel grid after three days' incubation. Bar width 53 microns. Figure 3—Photomicrograph of cells after rapid freezing in liquid nitrogen. Bar with 53 microns.

Preparation of tissue sections—Human tissues adjacent to implants of 18 per cent chromium-8 per cent nickel-3 per cent molybdenum stainless steel rods were removed with standard stainless steel instruments. Alternatively, tissues adjacent to acrylic discs buried in guinea-pigs were removed in the same way. Frozen sections 40 microns thick were prepared from formalin-fixed tissues using stainless steel microtome knives, mounted on nickel grids with Araldite and analysed by a technique resembling that of Mellors and Carroll (1961). The specimens were dehydrated and vacuum-coated with a carbon film at a pressure of 10^{-5} millimetres of mercury to improve electron conductivity (Bradley 1954), placed in brass specimen chambers and analysed on a Microscan* electron-probe microanalyser. Other specimens placed in the Cavendish Laboratory electron-probe microanalyser were dehydrated slowly by increasing the vacuum during twenty-four hours before microanalysis.

ELECTRON PROBE MICROANALYSIS

Principles of electron-probe microanalysis—The technique of electron-probe microanalysis uses a beam of electrons, accelerated to 5–50 kilovolts and brought to a fine focus on the surface of the specimen. If the energy of an electron beam is greater than the excitation energy of the elements present, characteristic x-rays are emitted. The x-rays can then be subjected to spectral analysis, giving, in the first instance, qualitative information about the chemical composition

* Mark II Microscan, Cambridge Instrument Company, Cambridge.

of the excited region. The x-ray intensity is measured by counters that record individual x-ray pulses. The concentration of an element in a specimen can be obtained by comparison of the intensity of the characteristic x-ray emission from the element in the specimen with that from a known standard, usually the pure element under identical experimental conditions (Castaing 1952). The observed intensity of an element in a sample differs from the actual concentration of the element unless certain corrections are made. When these corrections are made an accuracy of ± 0.1 to 1 per cent can usually be achieved. For biological specimens, however, the corrections are not generally made because drying of specimens causes gross damage including porosity and channelling; thus the quantitative results in this paper have been called intensities.

FIG. 4

FIG. 5

FIG. 6

FIG. 7

Figure 4—Electron image of "16C" cell on pure nickel grid. Bar width 53 microns. Figure 5—Enlargement of Figures 4 and 6 with line showing the position of x-ray trace across Figure 6. Figure 6—X-ray intensity scan line superimposed on analysed electron image of cell and grid shown in Figure 4. Starting from left, lowest flat portion of line represents nickel background. Slight rise of line beneath cell illustrates approximately 2 per cent nickel association with cell. Abrupt peak on right side of cell represents intensity of pure nickel grid, namely, 100 per cent. Figure 7—X-ray image of same cell. Intensity of x-ray images depends on photographic exposure time as well as on concentration of observed element.

The image due to the electron backscatter shows the surface topography and is very like a conventional optical image taken in oblique illumination. It also reveals variations in atomic number; the elements of higher atomic number backscatter more electrons than elements of lower atomic number and therefore appear lighter. This image can be used to locate the field of interest and to position the probe. The x-ray image shows the distribution and approximate concentration of the selected element over the area of the specimen being scanned. Analysis can be conducted on any selected region about 1 micron in diameter and 0.5 micron deep. Alternatively, average analyses can be obtained over any area up to about 0.5 millimetre square. The magnification of the image can be varied from $\times 250$ up to about $\times 5,000$. X-ray radiation of any element with an atomic number between 11 and 93 can be detected.

Figures 4 to 7 illustrate electron and x-ray images that are observed on the display screens. Figure 4 is a photograph of the backscattered electron images of a cell cultured on a nickel grid. Figure 5 is an enlargement of Figure 4. The line on Figure 5 represents a line of scan by the electron probe to measure the intensity of nickel across the cell. Figure 6 shows the actual scan, from left to right, across the interstice in the grid (0 per cent nickel), next across the cell (about 2 per cent nickel) and finally across a nickel grid bar (100 per cent nickel). Figure 7 shows the x-ray image of nickel for Figure 5. The white dots on the photograph represent x-ray radiation for nickel.

Experimental technique of electron-probe microanalysis—Some innovations have been made which improve established techniques of biological microanalysis (Cosslett and Nixon 1960,

FIG. 8

FIG. 9

FIG. 10

FIG. 11

Figure 8—Photomicrograph of formalin fixed and vacuum dried tissue section on supporting nickel grid recorded before microanalysis. Bar width 53 microns. Figure 9—Photomicrograph of specimen shown in Figure 8 after microanalysis. Specimen is rotated slightly counter clockwise. Analysed portion (*upper left*) appears darker because it is coated with decomposed molecules of pump oil. Figure 10—Electron image of tissue section before microanalysis. ($\times 450$.) Figure 11—Electron image of same specimen after microanalysis.

Boyd and Switzer 1962, Cosslett and Switzer 1962). When monolayers of cells are prepared, vacuum-deposited carbon or aluminium coatings should not be applied, because the suddenness of the evacuation produced by the vacuum pumps in the coating instrument ruptures cells from the metal surface. To limit the amount of cell rupture an hour or more should be taken to draw the vacuum of the electron-probe microanalyser. The minimal beam current to obtain an image should be used (10^{-7} to 10^{-8} amperes). Cells should be cultured in small square interstices of not more than 200×200 microns, so that as many cells as possible adhere initially to the metal and remain in contact with the metal after drying and evacuation in the microanalyser.

While tissue section specimens 8–10 microns thick may be analysed, specimens approaching a thickness of 40 microns will yield greater quantitative accuracy because there are fewer holes

through the dried tissue. If the tissue section is coated with carbon or preferably aluminium, the beam current will remain steadier during microanalysis. However, contrary to previous reports, it appears that electron-beam burning damage is not lowered by this procedure. Tissue burning is least with a slow scanning beam and without the use of a static probe. The tissue specimens should be slowly dried by the vacuum in the microanalyser. Again, an initial beam current of 10^{-8} amperes should be applied. During the first hour this value can be increased to 10^{-7} or 10^{-6} amperes until an adequate current is reached. Figures 8 to 11 show optical and electron images of a tissue sample taken before and after microanalysis. An innocuous dark deposit of molecules of decomposed pump oil coats the surface of the specimen where it has been exposed to the electron beam (Birks 1963). No tissue damage was found.

FIG. 12

FIG. 13

FIG. 14

Figure 12—Electron image of a "16C" cell on a nickel grid contaminated with copper. ($\times 800$.)
Figure 13—Nickel x-ray image of same cell showing nickel associated with the cell. Figure 14—Copper
image of same cell revealing copper associated with the cell.

FIG. 15

FIG. 16

FIG. 17

Figure 15—Electron image of cells on gold plated nickel grid where grid is scratched through gold plating. Grid interstice 162 microns per side. Figure 16—Nickel x-ray image of same cells revealing nickel associated with cells. Figure 17—Gold x-ray image of same cells showing no gold associated with cells.

EXPERIMENTAL RESULTS

Results for cells cultured on metal grids—Figures 4 to 7 show a typical cell cultured on a nickel grid. Microanalysis of cells grown on pure nickel grids revealed that nickel invariably became associated with cells. A line scan across a dried cell showed the nickel intensity to be about 1 to 2.5 per cent. If the nickel grids were contaminated with copper the electron-probe instrument revealed copper as well as nickel associated with the cells (Figs. 12 to 14). When cells were cultured on pure gold or platinum no gold or platinum x-radiation greater than the background radiation could be detected in the cells. When cells were cultured on pure rhodium, a trace of rhodium was observed in the portions of some cells that were adjacent to the metal surface. If the gold, platinum or rhodium platings on nickel grids were scratched until areas of bare nickel appeared, nickel was observed in the cells (Figs. 15 to 17). The nickel intensity

ranged from 1.5 per cent to 2 per cent nickel with gold and platinum platings, and 0.2 to 0.6 per cent nickel with rhodium platings. In these last experiments no gold or platinum was detected, although traces of rhodium were present in some cell regions adjacent to the metal surface. In all of these experiments with nickel grids, nickel ions could be detected in the culture medium with dimethylglyoxime reagent.

Figures 18 to 21 show the electron and x-ray images of a cell cultured on a stainless steel mesh. Iron, chromium and nickel radiation were detected by microanalysis. The iron intensity

FIG. 18

FIG. 19

FIG. 20

FIG. 21

Figure 18—Electron image of a cell attached to a stainless steel mesh (*bottom of micrograph*). Part of cell is obscured due to shadowing effects and low depth of focus of reflected electron image. ($\times 800$.) Figure 19—Iron x-ray image of same cell shows association of the element with the cell. ($\times 800$.) Figure 20—Chromium x-ray image of same cell. ($\times 800$.) Figure 21—Nickel x-ray image of same cell. ($\times 800$.) The x-ray images were photographed in the order Figures 21, 19 and 20 progressively and show incineration of the cell.

relative to the nickel and chromium intensities in the cell was roughly one-half that observed for 18 per cent chromium-8 per cent nickel-3 per cent molybdenum stainless steel implants. Because 18 per cent chromium-8 per cent nickel stainless steels invariably contain minute amounts of copper, the cells were examined for this element. Under a static probe a copper intensity up to 2 per cent per cubic micron was observed on several specimens. The culture was examined by a standard rubeanic acid and diethyldithiocarbonate procedures. Copper was never detected in the medium.

Results for tissue sections—Tissue sections from 18 per cent chromium-8 per cent nickel-3 per cent molybdenum stainless steel implant areas revealed a nickel intensity of 1 to 2 per cent in every sample. The nickel concentration was consistently highest at the tissue edge adjacent to the implant (Figs. 22 to 31). An iron signal was always detected. Although the distribution followed no consistent pattern as with nickel, the iron concentration generally increased as

the scan moved away from the implant edge. The intensity of chromium radiation was rarely greater than the background by a statistically valid amount. Molybdenum was never detected.

A copper intensity was recorded on some specimens (Figs. 26 to 31). In the specimens where copper was detected, the copper was around 1 per cent of the weight of the dry specimen. Under microanalysis the "acrylic disc" tissue specimens were shown to contain nickel consistently under 1 per cent (Figs. 32 to 35). On two occasions a statistically valid trace of iron was detected. No chromium, molybdenum or copper was ever observed.

FIG. 22

FIG. 23

FIG. 24

FIG. 25

Figure 22—Electron image of tissue section on supporting nickel grid. Tissue section edge, across centre of picture adjacent to stainless steel implant. ($\times 310$.) Figure 23—Vertical line showing position of x-ray line scan. Figure 24—Nickel x-ray scan line across same tissue revealing highest nickel intensity at tissue edge nearest implant. Figure 25—Nickel background intensity of x-ray scan.

DISCUSSION

These experiments indicate that nickel implants, whether in the form of a pure metal, a mixture or a solid solution in at least one stainless steel, may associate with individual cells. The nickel concentrations in cells and tissues are the same, so it appears that nickel in the implant yields nickel corrosion products in the interstitial fluid, which in turn becomes associated with individual cells. When an acrylic disc is implanted or removed tissue from the implant region contains a minute quantity of nickel derived from the stainless steel surgical instruments used during the operation. Whereas cells grown on gold, platinum or rhodium plated surfaces contain no appreciable amount of these metals, the bare nickel areas in the metallic culture surface will cause nickel uptake by the cells. In all of these cases nickel was apparent in the culture medium.

When trace amounts of copper are present in nickel or in 18 per cent chromium-8 per cent nickel-3 per cent molybdenum stainless steel implants, the element is concentrated by a single layer of cells: but surprisingly it cannot be detected in significant amounts in the culture medium. Copper is also concentrated in tissue sections. In view of the well known toxicity of copper to

biological systems generally, the mechanism of its uptake must be considered. Electron-probe microanalysis does not possess sufficient resolution to show the mechanism of heavy metal toxicity (Passow, Rothstein and Clarkson 1961). Quantitative information of metallic concentrations can be interpreted only at the subcellular level.

The toxicity of heavy metals now used for implants appears to be associated with at least three main factors: inhibition of enzymes, prevention of diffusion through the cell membranes or at the periphery of the cell, and breakdown of lysosomes (Gurd and Wilcox 1956; Klotz and Curme 1948; Koenig 1963; Passow, Rothstein and Clarkson 1961). Weiss and Dingle (1964) have shown that heavy metals may activate lysosomes to release their acid hydrolases.

FIG. 26

FIG. 27

FIG. 28

FIG. 29

FIG. 30

FIG. 31

Correlation between nickel and copper intensity in a tissue section excised from an implant. Figure 26—Electron image of tissue section on supporting nickel grid. Figure 27—The line of scan across the tissue for the x-ray micrographs. Figure 28—An electron image of the tissue section after microanalysis. No damage is apparent. ($\times 310$.) Figure 29—The nickel x-ray line scan again revealing the highest nickel intensity at tissue edge nearest implant. Figure 30—The background radiation for nickel. Figure 31—The copper x-ray line scan, also for the line of scan shown in Figure 28. The copper concentration is greatest at the same region as that for nickel, at the tissue edge nearest the implant.

They have also shown that lysosomal enzymes may be activated by changes in the outer cell membranes. Furthermore, Fell and Weiss (1965) have demonstrated *in vitro* that such a release of lysosomal enzymes will cause enzymatic degradation of the intercellular matrix of embryonic bones. Also, Dingle (1962) has suggested that enzymes released from lysosomes may damage connective tissue. It seems possible that such lytic processes may occur around metallic implants used in orthopaedic surgery and may contribute to bone resorption.

In the presence of chloride-containing solutions such as body fluids, 18 per cent chromium-8 per cent nickel-3 per cent molybdenum stainless steel is liable to highly localised corrosion in the form of pits (Kolotyrkin 1963, Mears 1965). Corrosion products at the pit sites contain iron, chromium and copper. Because the copper impurity in stainless steels is of the order 0.1 per cent it would be difficult to measure the minute quantity of copper present in the corroding medium at large. It appears that the cells can actively concentrate nickel and more readily copper even when these elements are present in minute localised sources. Apparently

corrosion products of iron, chromium and molybdenum are not concentrated by cells in any appreciable quantity.

This investigation has shown that some metallic corrosion products from implants become associated with individual cells; it has not yet been correlated with the clinical side-effects described previously. In view of the toxicity of nickel and copper, however, it appears that ferrous alloys should probably be discarded in favour of more inert materials—such as cobalt-chromium alloys, pure titanium or age-hardened titanium-molybdenum, titanium niobium alloy or titanium-tantalum alloy—particularly for long-term implants.

FIG. 32

FIG. 33

FIG. 34

FIG. 35

Figure 32—Electron image of tissue section from acrylic disc implant surface on supporting nickel grid. ($\times 310$.) Figure 33—Line of x-ray scan shown in Figures 34 and 35. ($\times 930$.) Figure 34—Nickel x-ray scan line showing nickel associated with tissue section. ($\times 930$.) Figure 35—Nickel background for scan shown in Figure 34. ($\times 930$.)

SUMMARY

1. Electron-probe microanalysis shows that corrosion of an 18 per cent chromium-8 per cent nickel-3 per cent molybdenum stainless steel implant and of some pure metal implants may affect not only the surrounding tissues but also the individual cells.
2. Metallic contamination from surgical tools is confirmed.
3. Electron-probe microanalysis is shown to be a useful tool for studying individual biological cells.
4. The principles of electron-probe microanalysis are described.

This paper describes one facet of a basic research programme on the corrosion and passivation of inert high strength alloys for surgical implants. Because the project involves electrochemistry, cell physiology, electron-probe microanalysis and clinical orthopaedics I have had to rely on colleagues who are specialists in each of these fields. I am particularly indebted to Dr L. Weiss of Strangeways Research Laboratory for his generous provision of tissue culture equipment and biological supervision. I would also like to thank my microanalysts, Miss C. G. Williams of the Metallurgy Department and Dr T. Hall and Dr V. R. Switzer of the Cavendish Laboratory. Dr J. T. Scales and his staff at the Institute of Orthopaedics, Royal National Orthopaedic Hospital, Stanmore, Middlesex, have provided tissue sections from implant areas. I am grateful to the Medical Research Council for generous financial support during the investigation.

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