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THE "IN VITRO" INTERACTION BETWEEN THE
SURFACES OF BIOCOMPATIBLE MATERIALS
AND HUMAN LEUCOCYTES

by

Christina Doyle

A Thesis submitted to the University of Surrey
for the Degree of Doctor of Philosophy.

April 1980
ABSTRACT

An in vitro study has been conducted in order to improve the understanding of the interaction between lymphocytes and implant materials. The uptake of cellular thymidine for DNA synthesis was used to monitor the cellular responses, in conjunction with microscopy. Material elutions stimulated the uptake of thymidine by cells, whereas solid material caused an inhibition of thymidine uptake. This inhibition was seen both in PHA-stimulated cultures and in cultures in the presence of materials only. Inhibition of thymidine uptake was found to be caused indirectly by contact with material surfaces and directly by an active cell secretion, produced in response to the materials. It was postulated that this active cell secretion was a lymphotoxin.
ACKNOWLEDGEMENTS

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1. INTRODUCTION

The increasing use of artificial replacement parts in humans has emphasised the problem of implant biocompatibility. The biocompatibility of an implant has said to be defined by an implant exhibiting physical integration with the tissue, discouraging excess cell proliferation and maintaining cell differentiations. The material properties that govern biocompatibility are still not clear. The literature indicates that the human body is capable of reaction both to implant geometry and to the chemical reaction products from the implant material. This work thus seeks to find a suitable test with which to decide whether it is the physical or the chemical properties of an implant that produce the major effects at the body interface. The test was used to examine whether lymphocytes recognise the presence of implant materials and to attempt an understanding of the interaction between the lymphocyte and a foreign surface.
2.1 Review of the Literature to find the Present State in Biocompatibility Testing

2.1.1 Introduction to the Problem

One of the greatest controversies existing in the bioengineering literature concerns the human body's response to implanted synthetic materials. Whilst many materials have the suitable physical properties, they may prove to be incompatible with the host tissue. The increased use of such implant materials has necessitated the investigation of their effect on the body.

There are three main types of reaction cited in the literature, i.e. immunological, cytotoxic and inert. There are also three main types of materials commonly implanted in the body, i.e. metals, plastics and ceramics. It has not yet, however, been found that a specific tissue reaction is found with a specific group of materials.

2.1.2 The Biological Responses Observed to Implanted Materials

(i) Introduction

Foreign body reactions are due to the physical, chemical (i.e. leaching to give toxic or immunological stimulatory agents) and the immunological properties of the implant surface. The primary problems in current implantation procedure are infection and pain, both of which may require removal of prostheses. The following review assesses the current understanding of how the three different groups of implant materials (plastics, ceramics and metals) differ in the reactions that they may produce when implanted.
(ii) Reported Biological Responses to Polymers

A general attitude to the implantation of polymeric materials has been that they have complete biocompatibility with the human body. Several workers have, however, reported biological responses to polymer implants.

(a) Immunological and Secretory - Oppenheimer used sheets of high density polyethylene to examine the effect of implant surface area on the body responses to it. He found that plastics induced increased tumour formation in rats compared with implants of a less continuous surface. Hueper reported that highly polarized, water soluble or residual monomers can cause a body response. Giant cell neoplasms, according to Hoffman were numerous in the presence of irregular margins or by an implant with a large uninterrupted surface area.

Salthouse et al have conducted interesting implantation work, applying enzyme histochemical techniques to investigate cellular changes due to the effect of implanted polymers. This work shows that polymer properties can induce varying secretory responses in surrounding tissue. He used this approach because cellular changes during the inflammatory response of the body are accompanied by characteristic enzyme patterns. He implanted material in rabbits and detected reactivity by a rise in acid phosphatase at the implant site. Another interesting observation by Salthouse has emphasised the importance of implant geometry and surface area, triangular specimens exhibiting a far higher hydrolase activity than a round specimen. This may be caused by the increased tissue damage, due to movement, or it may be related to the contact angle of the tissue at triangular corners. Also, the presence of certain additives can elicit a higher lysosomal enzyme response and Salthouse suggests the
possibility of enzymes causing material degradation and also interfering with the function of drug release polymers. It is interesting that this author, in contradiction to Oopenheimer and Hoffman, reports an increased response with increasingly rough surfaces. Rough surfaces produced locally dividing cells whereas a known toxic specimen caused an inward migration of macrophages (which was not seen with the rough surface). Occumpaugh and Lee\(^9\) have also tried to classify body responses in terms of implant materials. Occumpaugh and Lee stated that the foreign body reactions due to plastics can be those due to the implants' physical properties; those due directly to the chemical properties of the implant; and those due to immune reactions, i.e.:

<table>
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<th>Cause</th>
<th>Symptoms</th>
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<td>Physical Properties</td>
<td>Epithelial encapsulation, thickening of capsule, giant cells.</td>
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<tr>
<td>Chemical Properties</td>
<td>Epithelial hypertrophy, inhibition of epithelial growth, connective tissue inflammation and vacuolisation of test tissue.</td>
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They reported that responses in work on dogs could be classified but, however, gave no explanations. PTFE, polyurethane and graphite caused a reaction that could be related to the physical properties of the implant. Polyelectrolyte coated materials, epoxy resin and experimental adhesives caused a response due to the chemical properties of the material. So it can be seen that different implant properties can vary in importance with different materials. There was an absence of plasma cells, i.e. not an immune response. It was noted that Occumpaugh said that because plastics do not contain
protein or carbohydrate moieties they cannot stimulate an immune response. However, the evidence that plastics do not stimulate an immune response is uncertain\textsuperscript{10} and cases of delayed hypersensitivity have been reported. Charnley\textsuperscript{11} reports phagocytosis of plastic particles by giant cells (i.e. possibly an antigenic response). Rigdon\textsuperscript{12} who embedded polyethylene and polyurethane specimens in mice and rabbits, found a much more severe response from the polyethylene, which is considered to be non-toxic\textsuperscript{13,14}. In comparison to Occumpaugh, Rigdon stated that many of the local, basic inflammatory reactions associated with plastics are similar to those associated with bacteria. Rigdon also found a result variation between mice and rabbits. He related this to different plasma proteins in different animal species. He explains the polyethylene demonstrated a greater reaction, the polyurethane being coated by proteins faster than the polyethylene and hence being isolated from the tissue quicker. However, according to Fromageot et al\textsuperscript{15} an initially formed monolayer of plasma protein may not offer complete cover against the activity of minor plasma proteins because displacement and adsorption can occur and layers can be penetrated. It is possible that polyethylene is not so inert as generally supposed and Oppenheimer\textsuperscript{15} reported signs of degradation of ultra high molecular weight polyethylene after 26 weeks by C\textsuperscript{14} labelling and implantation.

(b) Toxicity - The toxicity of polymers has been widely reported and such toxicity is generally related to the presence of additives or residual monomeric materials\textsuperscript{14}. Apart from the work to be reviewed, using cell culture methods to test bio-plastics, polymer toxicity has also been studied by animal implantation work. Ratner et al\textsuperscript{16} related cell adhesion to biocompatibility, particularly after previous protein lay down on the surface. To further study the toxic effect of polymers
Kinzl\textsuperscript{17} implanted polyacetal, polyester, PTFE and polyethylene rings for four and twelve week periods and the cylinders of tissue from the rings (free from mechanical irritation) were examined. Polyethylene gave the least cell proliferation and reaction, and polyester gave the greatest. These workers then reimplanted the same rings and found that the tissue stimulation was not significantly lower on this second implantation. On this basis, Kinzl et al concluded that residual monomer on the polymer surface was not responsible for the tissue reaction since all the monomer should have been 'removed' during the initial implantation. He made no attempt, however, to investigate the presence of monomer on the 'once implanted' surface nor did they consider the monomer in the material bulk that may have continued to have a stimulatory effect. Gifford\textsuperscript{18} observed reduced tissue reaction from radiation cured pure silicone rubber compared with radiation cured silicone with a silica filler, but did not relate that to altered material properties.

(c) Passive Responses Observed - Polymers are said to never show an inert nature on implantation although they may be biocompatible. The adsorption characteristics of proteins on polymeric materials has been reported by several workers\textsuperscript{15,19,20}.

So it can be seen, in conclusion, that polymers can initiate a variety of responses on implantation.

(iii) Reported Biological Responses to Metals

Metal reactions may lead to in vivo responses e.g. corrosion leading to localised deposits of the corrosion product, dissociation of the corrosion products and the specific action of the ions with different toxicity levels. Corrosion and wear products may also
produce mechanical irritation. Steinman\textsuperscript{21} reported three types of tissue reaction due to metals, i.e. (i) toxic resulting in a sterile abscess, e.g. cobalt, nickel, copper and vanadium, (ii) capsule formation, e.g. aluminium, iron, and molybdenum and, (iii) loose connective vascular tissue, e.g. platinum, zirconium, niobium. He also stated that a very small amount of corrosion could be significant to large changes in the local pH. This was related to crevice and fretting corrosion.

(a) **Immunological** - Metal sensitivity is a common problem in implant patients and can be a cause of much pain, distress and subsequent removal of an implant. It is common practice to screen prospective implant receivers for metal sensitivity by patch testing and by the MIF/lymphocyte test. It has, however, been reported that an increased incidence of metal sensitivity has been found in patients with metal joint replacements\textsuperscript{22,23}. Also the toxic effects of cobalt, leached from hip arthroplasty has been reported\textsuperscript{24,25}.

(b) **Toxic** - Corrosion causes osteolysis and osteoclasis resulting in implant loosening\textsuperscript{14}. Tissue reaction, studied by Laing\textsuperscript{14}, to long term implantation of Co/Cr/Mo, is said to be minimal. Some reaction does, however, occur and instances of rather severe reactions have been reported\textsuperscript{26,27}. Corrosion may be initiated by infection which may raise or lower the pH. Low pH can remove the protection of the surface chromic oxide and wound pH can be as low as 4\textsuperscript{28}. Titanium and its alloys is also said to show good corrosion resistance\textsuperscript{27,29} and a minimal tissue reaction. Titanium, however, is always found in the adjacent tissue\textsuperscript{30} to the titanium implant. Also the inhibitory effect of titanium on alkaline phosphatase production has been reported\textsuperscript{31} and fibroblasts are said\textsuperscript{32} not to grow on a titanium
substrate. Several workers have attempted to define the most important alloy property to endow biocompatibility.

Thull\textsuperscript{33} has stated that the ideal metal for implantation has a short repassivation period with a mechanically indestructible surface oxide. He has tested 316L, Ti and MP35N interface impedance and related the mixed potential to the mechanical shear force. Black et al\textsuperscript{34} related the specific surface area of an implant, with respect to the body weight, to the implants reactivity in the body. This is particularly important with porous implants, so again both chemical and physical material properties appear to be important.

Gerber\textsuperscript{35} cultured rat femurs in the presence of soluble metal salts and solid metal implants at increasing concentrations for 10 days. He found, not surprisingly, that with increasing copper chloride concentration the deformation of the cultured femurs increased. Copper is a very biologically toxic metal. Implanted nickel, molybdenum and gold caused no effect and iron, cobalt and nickel, and copper caused colour changes and deformation. The cartilage cell density increased with increased reaction.

Plenk et al\textsuperscript{36}, at the same meeting, reported the use of fibroblast proliferation to test metal powders. He tested alumina, titanium tantalum, steel and dental gold. None of the materials showed an obvious cytotoxic effect and none totally inhibited cell growth. The ceramic debris decreased the growth rate slightly and steel and gold caused a significant inhibition of cell growth. In this case it would be suggested that the metal powders were behaving in an inert manner. It is interesting to speculate why Pappas and Cohen\textsuperscript{65} demonstrate metal powder cytotoxicity and Plenk et al do not. Again this must underline the importance of experimental specifications.
(iv) Reported Responses of the Body to Ceramics

Ceramic implants have been shown increasing attention during the past ten years, having good physical and chemical biocompatibility. The literature has dealt fully with the physical properties (wear, fatigue strength, toughness, etc) of ceramic implant materials but it is only recently that their chemical properties have been investigated to any extent. Although the general view is that such ceramics (usually high density alumina) lie inert in the body, causing no undesired responses in the surrounding tissue, there have been some observations made to the contrary which are worthy of reporting here.

(a) Immunological Responses - Welsh and McNab\textsuperscript{38}, while studying ceramics, observed a large number of inflammatory polymorphonuclear leukocytes in the surrounding tissue which is indicative of an immune response to the implant. They, however, explained that this was not a direct response to the presence of the ceramic but to the infection inadvertently introduced by it. The only other positive response observed in tissue surrounding a ceramic implant was seen by Plenk et al\textsuperscript{36}. They found that the degree of calcium resorption in the bone (which results in weakening of the bone) was proportional to the ceramic debris found in the tissue. The failure of the ceramic implant, resulting in ceramic debris, appears to be due to the metal screws used in fixation. Plenk suggested that such debris may induce adverse tissue reaction and interfere with the normal bone ingrowth found with satisfactory ceramic prostheses. It is obvious that the differing response initiated by material debris to that caused by a solid implant is a very important area of biocompatibility. It must be said, though, that this observed inflammatory response was
again due to misuse of the implant rather than a direct response to a properly prepared material.

(b) **Toxic** - No reports have been found to date of alumina or other bioceramics producing cytotoxic effects in surrounding tissue. However a slight decrease in lymphocyte thymidine uptake was seen (16,000 cpm control; 15,200 cpm test) when freeze/thawed macrophage debris and their ingested alumina particles were added to the culture. This was related to a possible material specific cytotoxicity corresponding to silicose formation which occurs only after the alumina passed through macrophages.

(c) **Passive or Beneficial Responses** - The passive properties of bioceramics have been emphasised by several workers. In some cases, however, a degree of reactivity of the ceramic with the surrounding tissue in which it is implanted have produced beneficial effects.

The adsorption behaviour of high density alumina has been studied and it has been found that alumina can adsorb synovia (the fluid found in joint capsules) and that salt adsorption depends on the orientation and polarity of the surface. This adsorption of synovia was postulated to explain alumina's excellent wear properties, the adsorbed layer acting as a lubricating film. Several workers have considered the dynamic surface chemistry of various alumina ceramics. Such properties are thought to induce the histological changes at the implant/bone interface that would normally occur if the implant were not present in stable interfacial osteogenesis. The formation of bone can be stimulated by calcium and phosphorous leached from certain bioglass ceramics. Clark and Hench mention,
however, that the ion release must be carefully controlled. Too few or too many ions cause bone resorption. They also found that calcium and phosphorus release into soft tissue causes inflammatory response and rejection. Pantana\textsuperscript{44} considered bioglass surface structures using Auger spectroscopy. He found a silica rich layer under a calcium phosphate rich layer of up to 10 μm. Calcium fluoride and calcium phosphate additions to the ceramic increased the silica concentration on the surface while boron oxide reduced it. Calcium fluoride and boron oxide also controlled the growth of the calcium phosphate layer. Calcium fluoride increased its formation. The sodium released from the ceramic surface permitted an alkaline pH to be maintained (for healing), overiding the acidic enzymes. Collagen was said to infiltrate the silica and calcium phosphate and subsequently mineralise. Decreasing phosphorus content decreased the bonding layer, decreasing interfacial strength. Excess phosphorus initiated macrophage attack. This work emphasises the importance of an implant surface and the way in which varying the surface properties can vary the responses induced in the body.

2.1.3 The Test Methods Used in the Past on New Prosthetic Materials

(i) Introduction

It is unlikely that a thorough understanding of immune responses caused by implant materials, with such a variety of material, animal and body responses reported above (section 2.1.2) can be gained. A first requirement for a programme of work is a test method which is sufficiently specific to indicate the nature of any particular interaction in the chain of events determining the relationship between a material and the host. Moreover, animal vivesection is commonly used to investigate biocompatibility whereas prostheses are inevitably
implanted in humans. These points are to be born in mind when reviewing the available test methods.

(ii) General Review

Several approaches have been used to consider material compatibility with the internal environment.

Blood compatibility studies have been extensively reported\textsuperscript{45,46,47} and surface roughness, surface charge and other material properties have been related to the clotting of blood. Adsorption and configuration changes of extra-cellular proteins and liquids in contact with artificial materials have also been studied\textsuperscript{48,49}. Currently the most widely used method for testing implant materials involves animal vivisection\textsuperscript{50,51,52,53,54} which is expensive, time consuming, introduces the possibility of the results varying with the species used\textsuperscript{55,56} and reduces the number of analytical techniques available.

The need was felt for a test which would allow a more direct investigation of the interaction with a material and the surrounding cells, preferably using a system closely related to the human internal environment. A cell culture seemed to be a good way of achieving this end and would also be easily replicated, easily quantified in both responses and numbers and give a fairly rapid response to any stimuli, being a closed system. A cell culture would also allow the novel use of in vitro techniques to study the cellular response to materials. Other workers have recently used cell and tissue cultures in an attempt to produce a quick, cheap and simple biocompatibility (toxicity) test. Their work was reviewed with the aim of taking cell culture testing further to attempt an investigation of the interfacial responses between the implant material and the human body,
(iii) The Use of Cell and Tissue Culture Testing

The majority of the work using cell and tissue culture to measure biocompatibility considers cell death or cell proliferation as a measure of the toxicity of a material. Little attempt has been made to study other cellular effects.

(a) Using Cell Culture to Test Polymers - Taylor et al.\(^{57}\) cast polymers into thin films and grew L (lung) or HeP (larynx carcinoma) cells in direct contact or in agar overlay on them. There were few conclusions. Cells showed no growth or attachment to natural rubber, which was thought to be toxic. Because the plastics had been recast into thin films they had not been tested in the form normally implanted in the body. HeLa (human carcinoma of the cervix) cells were used\(^{58}\) to test a polyamide, cast as a coverslip. This became coated with cells after three days, and the mitotic index of the test culture compared well with that of the control culture. Another secondary cell line, WI38, has been used for testing polymers\(^{59,60}\) which were either put in direct contact with the cells or a medium (or cotton seed oil) elution from the materials was added to the culture. The results were compared with systematic injection tests in mice and intracutaneous tests in rabbits. The material toxicity was demonstrated by using vital staining (i.e. Neutral Red) and positive toxicity was indicated by rubber, copolymers, polyvinyl chloride and paper.

Polymers may be manufactured by different routes and the various modes of manufacture can be a factor governing the compatibility. This aspect of material compatibility was considered by Dillingham et al.\(^{61}\) using mouse fibroblast cells in agar overlay. Solid and
liquid samples of PMMA* (of varying formulations) were tested using polyethylene and polyvinyl chloride as controls. Toxicity detection was by the loss of a previously applied vital stain. In vivo animal tests were run in parallel, where haemolysis, intradermal irritation and systemic toxicity were used as the indications of poor material compatibility. In vivo the PMMA formulation had little effect, but in vitro gave a wide range of toxic responses. This could indicate that either the cell culture provides a more sensitive method for detecting the effect of implant materials or the cell cultures may give misleading results. Such a misleading result could be due to toxic substances building up (if allowed) in the cell culture whereas the body systems are often capable of removing them. Wennberg et al. devised a system to test material specimens, culturing HeLa (human epithelial) and L929 (mouse fibroblast) on Millipore filters. The specimens of bone cement were thus not in direct contact with the cell monolayers and the leachable cytotoxic elements from the materials could be investigated. Cytotoxicity was assessed using enzyme histochemistry. Acrylic resin showed a cytotoxic effect when freshly prepared and silicate cements were cytotoxic after setting periods of 24 hours.

(b) Using Cell Culture to Test Metallics - Metals have also been put in contact with cell and tissue culture to observe the responses that they induce. The growth of cells in plasma clot around a piece of metal was considered by using cell area photoplanimetry, mitosis counts and observation of changes in the cell morphology. Periodic cell counts were taken, but the use of plasma clot precluded the accurate resuspension and, hence, enumeration of the cells. The commonly used implantable alloys were not tested and no attempt was made to demonstrate any leaching of ions into the medium, or corrosion products within the cells. Pappas and Cohen continued the study of

*PMMA (polymethyl methacrylate)
metal toxicity using metal powders in KB (nasal carcinoma) and P1534 (mouse leukemia) secondary cell lines. The comparison of cell counts was used to monitor the material reactions. Vitallium, which has been reported to be less toxic than stainless steel $^{53,66,67}$ showed a greater degree of cell death than the stainless steel. Hulbert and Klawitter $^{67}$ tested several metallics using chick embryo and saw no response from titanium. Stainless steel 316 showed little response and stainless steel 304 gave an increased response. Wrought vitallium gave a greater response than that of stainless steel. Cell growth inhibition has been shown when cells are cultured on a titanium base $^{68}$. Lymphocyte cell cultures have also been used to detect metal sensitivity in patients prior to receiving implants, using the "macrophage inhibition factor" test $^{69,70}$.

(c) Conclusions - There is much to be learnt from the previous work using cell and tissue culture for the testing of implant materials. The method has promise for studying the fundamental aspects of material compatibility and material reactivity can be monitored fairly routinely by the use of in vitro techniques on the cells. Past work in this field can, however, be criticised. Animal secondary culture lines were used (presumably for convenience). Such animal cells could introduce species variations into the results and also secondary lines will be of mutated cells, which must make the in vitro to in vivo relationship even more obscure. From this it was concluded that this work should use a primary culture of human cells, if possible from a single source, to eliminate differing individual responses.

(iv) Conclusions from Review of Past Test Methods

The point has been made that in designing a new system for the examination of the interaction between materials and the body it
would be desirable to try and use a system taken from the body, in order to attempt to model the human internal environment as closely as possible.

In test methods that have been used previously there have been too many steps between the initiation or stimulus and the observed response. This is always the case in in vivo experiments and in this thesis it is intended to study the initial interaction, which may then lead to further responses. For example, on recognition of an antigen or mitogen (which is an initial interaction), a lymphocyte may either transform to blast and memory cells or (if a B cell) produce plasma cells which secrete antibodies\(^7\). Once this occurs other events are triggered in the immune and inflammatory system (section 2.2.3) by cells involved in the protection and subsequent healing of the body tissue. Mergenhagen,\(^7\) and also Bourne (in the Biochemistry and Physiology of Bone, I, Acad. Press 1972) have shown that sensitised, mononuclear leucocytes are capable of causing damage to the surrounding tissue and bone. (Osteoclast production may be stimulated resulting in bone resorption.) Lymphocytes act as mediators to such cells and also act in their own right, producing antibodies and some hydrolytic enzymes. It is these subsequent events which may lead to inflammation and pain and so are an important factor in replacement surgery. The key interaction, however, is that of the lymphocytes, and it is probable that the major vehicle for the immunological response is the leucocyte population. It is this response which will be investigated in the following work.

Inorganic materials are said to be non-antigenic unless hapten combinations occur. However, the specific or non-specific interaction of lymphocytes with inorganic and organic implant materials has not been studied to date. In this thesis, a suitable way of examining the possibility that all three classes of materials may be recognised
by the lymphocytes is considered.

2.1.4 Conclusions from Literature Review

The literature indicated that a response of some sort to implant materials is widespread. There was, however, disagreement on which type of response may be caused by specific materials and the factors influencing specific responses. Some of this could possibly be eliminated by accurate experimental specifications and the testing of materials in the form in which they are normally implanted.

There was evidence that the shape and size of an implant can influence the body's responses to it and also that the material's chemical properties are important to biocompatibility, i.e. the surface properties and the chemical reaction products (the body being an aggressive environment).

Present test methods have not fully allowed the study of the key interactions that govern biocompatibility. Also the use of animals and their cells have introduced the possibility of species variation into results. In vitro work using human lymphocyte cultures appears suitable for studying the initial key interaction. Apparently no work has yet made use of primary cultures of human lymphocytes to study biocompatibility.

Objective of the Work

The objective of the work is to find whether a human lymphocyte primary culture can recognise implant materials (specifically or non-specifically) and to investigate if it is the physical or chemical material properties that control any such recognition.
2.2 Techniques

2.2.1 Introduction

The literature has shown that animal implantation and animal cell cultures can be used to examine implant material biocompatibility. Although cell culture was used only to study cytotoxicity it is apparent that such an approach allows further analysis of biological responses. This can be aided by eliminating variation in results by removing any species variation by using a human cell culture (preferably primary so to closely model the human body condition) and by using replicate culture - both of these would be difficult to achieve in vivo. A single source of cells would eliminate the slight variation between individual responses. Primary cultures of human cells have not been seen to be used by other workers to test implant materials, presumably because of the availability of easily cultured secondary animal cell lines from commercial sources, so this would be a completely new approach to biocompatibility studies.

As previously stated, because of its key involvement in the immunological responses of the human, the lymphocyte was chosen to model the body as far as possible in cell culture.

Having chosen a suitable experimental system a series of techniques must be selected for the examination of the cells and the materials. It was important to be able to quantify any cellular responses observed. Repetitive experiments to study any factors involved must also be possible. Such a technique could involve radiolabelling combined with liquid scintillation counting. The lymphocyte blast transformation is a good indication that the immune response has occurred. It is particularly useful that such transformation can be caused by specific and non-specific means, which thus allows the study of the spectrum of mitogenic responses. It is possible that the
implant material may induce a mitogenic or antigenic response in the cell population. The degree of cell growth or transformation can be measured by radiolabelling the newly synthesised cellular material (a more detailed account of the blastic response is given in Section 2.2.3). The uptake of radiolabel can then be quantified by liquid scintillation counting. There are other methods for studying lymphocyte behaviour, for example surface antigens and their release of antibodies can be studied by marker techniques (e.g. fluorescent labelled antisera, Ig-labelled latex particles, sheep red blood cells etc.,) but these do not provide such a straightforward means of general surveillance of the lymphocytic responses. Microscopy, (both light and electron) however, is useful to study cell and material morphologies.

2.2.2 Lymphocyte Structure

We have large (9-15 μm) and small lymphocytes. Small lymphocytes have a high nuclear/cytoplasmic ratio (=9/10) with a few free ribosomes, small number of mitochondria and an inactive Golgi apparatus. They are usually quiescent and are important for immune surveillance. The large lymphocyte usually shows a nucleus indented with the cell centre network and a few plates of track like endoplasmic reticulum.

Lymphocytes are spherical and move by uropod formation. The surface of lymphocytes in their normal condition is rarely smooth and generally exhibits ruffling and microvilli. These cells can undergo micropinocytosis and some of the microvilli reflect this. Lymphocytes cannot phagocytose large particles but rapidly engulf all sorts of molecules and small numbers of particles by micropinocytosis. It is still not sure that the ingested particles can serve as antigens but
it is possible that the molecules entrapped in vacuoles are degraded there. Acid phosphatase has been found in these vacuoles.

2.2.3 The Lymphocyte Function

The lymphocytes are a sub-population of leucocytes, with a two-lymphocyte model of immunity being established in the last ten years, with two central lymphoid organs—the bursa-equivalent and the thymus. These produce lymphocytes independently of antigen and seed them out to the peripheral lymphoid organs, (i.e. lymph nodes, spleen and gut-associated lymphoid tissue) where they await the stimulus to differentiate into 'effector' cells (Fig. 1). Those lymphocytes derived from the thymus are designated T-cells and those from the bursa-equivalent are B-cells. They are morphologically undistinguishable. Lymphocytes migrate from the blood to lymphoid tissue via post capillary venules and then back to the blood via the lymphatics and thoracic duct. They are important for immune surveillance.

Immune Response to an Antigen (Fig. 1)

These responses are classified as either cell mediated (i.e. the reactions are directly transferable by cells e.g. graft rejection and delayed hypersensitivity (T)) and humoral (i.e. the reactions that are transferable via the serum or antibody producing cells (B)). B cells have antibody molecules (Ig) as membrane receptors, for antigen, which are identical to the antibody which the cell or its progeny will eventually secrete. The Ig class of receptors, and that of the ultimately secreted antibody, may not, however, always be the same. T cells also have receptors of some type on their surface membrane for antigen recognition but whether it is Ig is disputed.

When an antigen combines with a lymphocyte by membrane interaction
THE LYMPHOCYTE RESPONSE TO ANTIGENS

FIGURE 1.

1. Stem cell
2. Primary lymphoblast
3. Thymus (Primary Colony) Stimulation / Antigen
   - Virgin T cells
   - B cells

4. Memory cell
5. Blast cell
6. Plasma cell
   - Helper cells to B cells
   - Secretes Antibodies

7. Phagocytic
   - T cell
   - Killer cell
   - MIF
   - Other Lymphokines
one of three things can happen to the lymphocyte. It may be paralysed, it may be unaffected or it may undergo the blast transformation with subsequent mitosis. It is this latter response that is of interest in this work. (The cell division and differentiation is undertaken as a response to an antigen so that both memory and effector cells are produced. Memory cells allow a quicker response on subsequent re-exposure to the same antigen and effector cells increase the available population able to deal with the specific triggering antigen.) Mitogens will also produce a blast response non-specifically. The enlarged transformed cell is called a \textit{lymphoblast} and is over twice as big as a normal, small lymphocyte, with a higher cytoplasmic to nuclear ratio\textsuperscript{76} (Fig. 2) (Plate 1). As the stimulated cell grows it must synthesise DNA and to do this it must take up the base, thymidine, from the surrounding medium. It is the monitoring of this uptake which will form the basis of the experimental work of this thesis. The base is tritiated and the level of thymidine uptake can be measured and related to the degree of DNA synthesis and hence the population growth. The $\beta$ emissions from the tritiated thymidine are measured using the liquid scintillation technique (Section 2.2.6).

In transformation, soluble substances termed lymphokines are produced, e.g. MIF, chemotactic factors, cytotoxic factors, so when in vivo the sensitised lymphocyte arrives at an antigen site mediators are produced which will lead directly or indirectly to tissue damage. Stimulated T-cells may also produce killer cells, which are attracted to target cells, or helper cells, which help B cells respond to different determinants on the same immunogen\textsuperscript{71}. It is possible that macrophages are involved in this co-operation, actually noting the
Antigen binds to cell wall (T) and lectins act nonspecifically on 80% pb1s.

DNA synthesis

5-7 μm

Cell takes thymidine from surroundings

(L3) H

14-20 μm

Lymphoblast
shed T-receptor and presenting the antigen to the B-cells. T-B cell co-operation is not always necessary.

Finally the presence of some enzymes (particularly lysosomal enzymes) in lymphocytes has been documented\textsuperscript{76} but is controversial.

2.2.4 Lymphocyte Culture

(i) Introduction

Most of the leucocyte culture work reported in the literature is concerned with the study of genetics. It was useful to this work, therefore, only in the development of the techniques necessary to grow leucocyte cultures. The following review of the literature is concerned with the information collected on the growth of cultures, and in particular, on the growth of lymphocyte cultures.

(ii) White Cell Separation

The use of peripheral venous blood was suggested\textsuperscript{82} as a source of lymphocytes, being easily accessible without excessive trauma to the donor. For good cell viability the blood must be used within 4 hours of extraction. Several methods can be used to separate white blood cells from whole blood (Fig. 3). Busch\textsuperscript{83} suggested low speed centrifugation (800 rpm for 20 minutes) or column separation to remove the red blood cells. Also gravity sedimentation (A)\textsuperscript{84}, plasmagel sedimentation (B)\textsuperscript{84}, density gradients\textsuperscript{83,84,85}(C) and (D) and chromatographic filtration methods were reported. Plasmagel, polyvinyl pyrrolidone and Eicol/Triosil mixtures have been used to form density gradients (D). Commercial systems are now also available.

For successful culture the elimination of erythrocytes has been emphasised and the optimum white cell population was stated to be
Separation techniques

(A) Serum

(B) White cell layer

(C) Ficoll & Triosil

(D) Lymphocytes

Erythrocytes and polymorphs
5-15 \times 10^6/ml. The medium RPMI1640 supplemented with non-
human serum, and with a pH of 6.9-7.2, is said to be most suitable
for human leucocytes.

(iii) Culture Environment

Cultures have been grown in glass or plastic vessels, e.g. tubes,
petri dishes. The glassware must be well cleaned and sterilised
to eliminate bacterial contamination. Plastic tubes are disposable.
Hollow, semi-permeable fibre systems have also been used. These
are manufactured from polysulfone or from poly vinyl chloride/acrylic
copolymers. Fibres, 200-350 µm diameters, bundled in a glass
cylinder, are perfused with medium so that waste products do not
build up and the nutrients are constantly replenished. When large
replication cultures are required, e.g. for statistical analysis,
microtitre plates are used.

2.2.5 Dye Exclusion Technique

This is a method that can be used to assess the viability of a
cell population. In experiments looking for the cellular responses
to implant materials it is important to know both the initial culture
viability and the cytotoxicity of the materials being tested. Loss
of cells because of cytotoxicity of the materials would consequently
undermine other results.

If a cell is viable it is able to maintain the osmotic pressure
difference between its cytoplasm and the surrounding environment.
Any dye in the environment would thus be excluded from the cell and
the cell would not be stained. If, however, the cell is dead or
dying, its membrane is unable to exclude such a dye and the cell
becomes stained. This is the basis of the dye exclusion technique.
Method

Cell suspensions are incubated at 37°C 1:1 with dye for 2 minutes. Either 2% Trypan Blue in buffered salt solution or 2% Nigerosine in buffered salt solution were used as the dyes. At the end of the incubation time the cells are counted on an Improved Neubau Haemocytometer and the percentage of dead or dying cells is assessed. At least 100 cells are counted for each result and this is repeated several times.

2.2.6 Scintillation Counting

(i) Introduction

The use of isotopes for labelling chemicals is widely used in biochemistry, and chemistry, and is an extremely versatile technique. By the incorporation of a radio-labelled element into a synthesised molecule the movement of the molecule can be monitored.

(ii) Lymphocyte Incorporation of Thymidine

In this work tritiated methyl thymidine was used to label the DNA of the lymphocyte when the lymphocyte is immunologically sensitised, (transformed).

(iii) Liquid Scintillation Counting Technique

The technique of liquid scintillation counting relies upon the emitted radiation being absorbed by a fluorescent material in the surrounding cocktail and re-emitted as light photons which are detected by a photomultiplier. A scintillation cocktail holds the specimen (in various forms) and consists of an aromatic hydrocarbon solvent, e.g. toluene, with primary and secondary scintillants to
match the photomultiplier sensitivity, e.g. PPO and dimethyl POPOP. Often a detergent, e.g. Triton X-100 is added to aid the miscibility of the specimen with the cocktail.

(iv) Specimen Preparation and Count Efficiency

There are two main directions in the preparation of a tissue/cellular specimen for liquid scintillation counting. Either the compound can be converted into a more soluble form by means of a digesting agent (solubiliser) or the tissue is counted on a solid support, e.g. filter paper.

Counting may be low and irreproducible when counting tritium on a solid support. The specimen preparation is, of necessity, kept as constant as possible and the aim in the preparation is to give a high counting efficiency, where

\[
\text{Counting efficiency} = \frac{\text{counts per minute}}{\text{disintegration per minute}} \times 100
\]

and a low quench, i.e. loss in counting efficiency which is due to either a decrease in the energy transfer from the isotope to the fluor or a decrease in the light output between the fluor and the photomultiplier tube (Fig. 4). These decreases can be caused by chemical quenching, self quenching, surface absorption, etc. Other important aspects for good counting efficiency are the count geometry and the specimen volume. All of these factors are important when using a low energy isotope such as tritium (a \( \beta \)-emitter). It is felt that efficiency can be as low as 5% when counting tritium on a solid support due to quench effects. This is said to be even worse if the molecule is small enough to penetrate into the support matrix to give a large pulse height shift related to the thickness and absorptivity of the matrix. McKenzie & Gholson, however, showed...
FIGURE 4

Disintegration Events

\[ \text{increased quenching} \]

\[ \text{KeV} \]
that the use of a solid support will give good results providing that either everything is solubilised, i.e. with Biosolv BBS-3 or if all the labelled molecules are kept on the support. They felt that problems arose with a half and half situation with some molecules left on the support and the remainder taken into the cocktail. This is the approach used, in that specimens were not agitated but handled with care to ensure that labelled material remained on paper. Takahasi used a commercial solubiliser (Hyamin 10-x) to elute the protein hydrolysate from the paper to the counting vial. By elution from the support the controversy over the importance of the orientation of the support in the vial was overcome. Malt and Miller stated that trichloroacetic acid (TCA) precipitated protein may be counted on glass fibre discs in a toluene based cocktail with validity equal to counting ammonium hydroxide digests on Millipore filters in Bray's eluting cocktail. The former approach was also considered in this work. It is generally felt that glass fibre is the best material for solid support scintillation counting, giving a higher efficiency than cellulose paper. Thus it was used throughout the experimentation.

If a solid support specimen is not used for biological liquid scintillation specimen preparation then the protein is generally TCA precipitated and then digested. The literature states a variety of solubilisers. Other workers suggest the use of biological specimen support that is either combustible or soluble.

(v) Time

The time course of isotope dosing has been considered by Fujiwara and also Thurman et al. Fujiwara felt that the lymphocytes should be dosed in the log phase of culture and the
Thurman work\(^98\), in agreement with this, states a maximum cell stimulation between 3 and 6 days with the cultures reaching a maximum incorporation of thymidine around 1 \(\mu\text{Ci}/\text{culture}\). A pulse duration test showed that an 8 hour pulse gave the optimum count.

(vi) Quench Corrections

It is generally felt that channels ratio is the most suitable method for the determination of count efficiency for solid support liquid scintillation counting. However, since this method is complicated and also because of recommendations\(^{95,104}\) that the external standard ratio method is valid for use with glass fibre support counting this correction method was concentrated on. Also although the methods and recommendations in the literature were appreciated and borne in mind the method used was largely developed after personal discussions.

External Standard Corrections

The external standard is used as a source of radioactivity. Its count rate is first determined in each of a series of quenched standards whose counting efficiency is known and the count rate of the external standard is then related to this efficiency. The external standard count rate obtained in a sample of unknown activity can then be used to give the efficiency of counting for that sample,

\[
  r = \frac{\text{CPM}_{\text{STD2}} - \text{BG}_{\text{STD2}}}{\text{CPM}_{\text{STD1}} - \text{BG}_{\text{STD1}}}
\]

BG = sources is in its shield.
CPM = counts/minute
BG = background
STD = standard
2.2.7 Material Analysis Techniques

(i) Introduction

The material specimens for testing were prepared as discs, powders and solutions. Elutions were tested to isolate any reactive effect of the chemical reaction products from the materials. Powders were used to investigate the effect of wear debris on cells. As far as possible the specimen sizes, shapes and finishes should be kept constant, and their compositions and preparations clearly specified.

(ii) The Relevance of the State of a Material Surface

The surface composition of a material does not necessarily reflect the composition and nature of the bulk material. Various factors can influence the state of a surface and it is very important to bear these in mind in this work. It has been noticed in the literature that although it is the implant surface that is in intimate contact with the host tissue the importance of characterising this surface has never been recognised.

The factors which can effect a surface include alloying additions, cleaning materials and sterilisation methods. To increase the relevance of this work to in vivo implantation the manufacturers recommended cleaning procedures were followed, after which the surfaces were analysed. Cleaning materials, such as detergents and polishing compounds could obscure the true surface. Even a 'clean' surface always has a several ångström thick organic carbon contamination layer to the atmosphere.

(iii) Methods of Analysis

Electron microprobe analysis has a depth of analysis up to 2μm
whereas photoelectron spectroscopy is a purely surface analytical technique, looking at the first few atomic layers. The latter technique was thus used for characterising the nature of the discs of material. X-ray bombardment of the materials will produce low energy photoelectrons. Only those from the upper surface reach the detector, because of their low energy, and these electron kinetic energies are characteristic of an element and its valence state.\textsuperscript{107}

The material elutions could be characterised by atomic absorption spectrometry or mass spectrometry. Mass spectrometry detects both organic and inorganic materials and would be suitable for the analysis of both the metal and the plastic elutions. The eluting medium was, however, loaded with proteins and amino acids making any information from a mass spectrometer difficult to interpret so the atomic absorption spectrometer was used. This technique is quantitative rather than qualitative so selected elements were searched for rather than the whole range. A liquid sample is sprayed into a standardised acetylene or nitrous oxide flame. The vaporised ions emit a characteristic wavelength of radiation which is observed through a standardised light source.

The energy absorption by the ions then allows quantification of the amount of ions present in the solution. Sensitivity varies with different elements and by the use of standards quantification is possible.

Not only the chemical nature of the surface is important. Recent work\textsuperscript{108} has suggested that implant geometry is important in the inflammatory process and so the physical nature of the surface should also be characterised. Surface roughness and specimen area are obvious candidates for measurement and other interesting parameters are surface
charge, contact angle, degree of cleanliness, electropotential, and coatings. Several of these were measured. Scanning electron microscopy, which allows the visualisation of surface topography via secondary electron emission, and the Tallysurf, a direct surface roughness measurement, can be used to determine specimen topography and roughness. Gas adsorption can be used for determining the surface area of a specimen but was unsuited to this work because there was not enough material available for analysis.

2.2.8 Conclusion from the Review of Techniques

The lymphocyte blastic transformation was chosen as the response most suitable for monitoring the lymphocyte reaction to the materials in vitro. The lymphocyte blast formation can indicate both specific and non-specific responses of the lymphocyte.

The literature was then reviewed to assess the state of the understanding of the lymphocyte structure and function with particular reference to the immune response of lymphocytes to antigens and mitogens. The literature also indicated the most suitable ways of culturing lymphocytes and using the liquid scintillation technique.

It appears that other workers testing biocompatibility have not emphasised the importance of the state of the material surface. This work intends to fully characterise the cleaned and prepared material surface and photoelectron spectroscopy will be employed. Other methods will be used to define physical characteristics and the constitution of the other specimen forms.
3. SPECIMEN PREPARATION FOR A TYPICAL EXPERIMENT

3.1 Setting up the Cell Culture

Following the literature review (2.2.4) suitable methods were investigated with the aim of establishing lymphocyte cultures.

3.1.1 Blood Source

Peripheral venous blood from a single donor was removed from the cephalic vein using 19 gauge sterile needles. The blood was gently mixed with \( 10^{-3} \) heparin per 20 ml of blood in a sterile container. When acid phosphatase activity was being studied EDTA was used as an anticoagulant, heparin being said\(^{109}\) to interfere with histochemistry of the enzyme stain.

3.1.2 Lymphocyte Separation

Whole blood consists of both red and white cells, platelets and plasma. To grow a lymphocyte culture it is generally accepted that the other blood components must be removed. Several methods of white cell separation were tried in order to find a method which gave a population in the optimum range with good cell viability. These included gravity sedimentation to remove the red cells (the serum being pipetted off) or dextran-aided red cell sedimentation followed by the use of a Ficol/Triocil density gradient.

The method that was finally adopted involved mixing the whole blood with an equal volume of Hanks buffered salt solution. This was then layered onto Ficol-Pague (a commercial density gradient) and centrifuged at 100 g for 45 minutes. The lymphocytes then appeared as a white band covered with serum and overlaying the band of the erythrocytes and polymorphonuclear cells and the opalescent density
gradient. The lymphocytes were removed with a pipette and washed three times, spinning at 100 g for 10 minutes, in Hanks buffered salt solution. The cells were then resuspended in the supplemented culture medium and the population adjusted to the required cell concentration. Cell counting was conducted on a Neubau haemocytometer.

3.1.3 The Culture Environment

The function of the growth medium for cell and tissue culture is to provide the conditions, pH, osmotic pressure, required for cell survival, and to provide the nutrients which the cell cannot synthesise e.g. amino acids, carbohydrates and vitamins. Commercial media such as Eagle MEM (Burrough Wellcome), RPMI 1640 (Flow) and TC199 (Burrough Wellcome) were all tried to find the most suitable medium for growing lymphocytes. Finally supplemented RPMI 1640 was used. The media supplements added are shown in Table 1.

<table>
<thead>
<tr>
<th>Supplement</th>
<th>i</th>
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<tbody>
<tr>
<td>Foetal Calf Serum</td>
<td>10</td>
</tr>
<tr>
<td>Penicillin</td>
<td>100 iu</td>
</tr>
<tr>
<td>Steptomycin</td>
<td>200 iu</td>
</tr>
<tr>
<td>L- Glutamine</td>
<td>1%</td>
</tr>
<tr>
<td>RPMI 1640</td>
<td>Balance</td>
</tr>
</tbody>
</table>

Sterile, distilled or deionised water or Hanks buffered salt solution were used for the preparation of all the solutions. The antibiotics were added to RPMI 1640 to inhibit the growth of bacteria and L-glutamine is believed to enhance cell viability.

It was found that the pH of a cell suspension could be kept slightly acidic by the excreted carbon dioxide in the culture growing vessel. If the ullage is too large the partial pressure of the carbon
dioxide never reaches the level necessary to sustain the acidic pH required for good cell viability. The optimum ullage was investigated in a series of experiments, utilising lymphocyte cultures and reported in the author's MSc thesis. The best cell viability was obtained using either 2 ml polypropylene tubes or tissue culture coated microtitre plates (Plate 2), maintained in a 5% carbon dioxide atmosphere. (It was found that although the excreted carbon dioxide in the culture tube ullage improved the lymphocyte viability, extra gassing was needed to achieve a normal level of lymphocyte transformation response (Section 3.3).) After separation from the whole blood 200 µl of lymphocytes at 2-3 x 10^6/ml were added to each microtitre well or 1000 µl of lymphocytes at 1 x 10^6 cells/ml were added to the polypropylene tubes. These cultures were then incubated, both with and without material specimens, at 37°C.

3.1.4 Aseptic Technique

In order to reduce the contamination and subsequent proliferation of bacteria in the cell cultures all the cell manipulations were carried out in a laminar flow cabinet. All the apparatus involved in cell contact was sterilised.

3.2 Implant Materials

A range of commonly implanted materials were obtained from their manufacturers. The range chosen covered metals (3), plastics (3) and ceramics (1), (see Table 2).
TABLE 2

<table>
<thead>
<tr>
<th>Material</th>
<th>Finish</th>
<th>Specification %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Titanium (Ti)</td>
<td>Lap</td>
<td>79.2Ti; N&lt;0.07; C&lt;0.1; Fe&lt;0.3; H&lt;0.0125</td>
</tr>
<tr>
<td>Titanium 318 (318)</td>
<td>Lap</td>
<td>Al6; V4; Ti balance; Ni&lt;0.05; C&lt;0.08; Fe&lt;0.25</td>
</tr>
<tr>
<td>Vitallium (Vi) High Density</td>
<td>Lap</td>
<td>Cr27-30; Mo5-7; Ni&lt;2.5; Fe&lt;0.75; C&lt;0.35</td>
</tr>
<tr>
<td>Polyethylene (PE)</td>
<td>As received</td>
<td>Possible traces of Ti and Al catalyst [CH₂=CH₂]</td>
</tr>
<tr>
<td>Polypropylene (PP)</td>
<td>Milled</td>
<td>Medical grade [CH₃]</td>
</tr>
<tr>
<td>Polyvinylchloride (PVC)</td>
<td>As received</td>
<td>Medical grade [CH₂=CH]</td>
</tr>
<tr>
<td>High density Alumina (HOAL)</td>
<td>Lap</td>
<td>Medical grade</td>
</tr>
</tbody>
</table>

Titanium and titanium 318 were kindly supplied by Imperial Metals Industry. The remainder of the materials were given by Thackerays Ltd.

3.2.1 Discs

6 mm x 1 mm discs of the materials were prepared by workshop techniques polished to 6 µm and then cleaned and steam sterilised. Cleaning (which was recommended by the material manufacturers) involved degreasing with Decon 90 detergent, followed by copious hot and cold water rinsing. Sterilisation was at 120 psi for 30 minutes.

3.2.2 Powder

It was considered to be useful to test some of the materials as powder to simulate any response to wear debris. Chromium, cobalt, vitallium and titanium powders were tested. The vitallium powder was the product of a previously conducted pin on disc wear test. All the powders were cleaned and sterilised as previously described in Section 3.2.1.
3.2.3 Elutions

Elutions of the materials were also prepared by immersing \(1 \text{ gm} \times \frac{\text{days}}{37^\circ \text{C}}\) of the solid per \(1 \text{ ml}\) of RPMI 1640 for 3 and 5 days at \(37^\circ \text{C}\). Elutions were also prepared in sterile, deionized water.

3.3 Characterisation of the Specimens

3.3.1 Cell Culture

(i) Method

In order to realise fully the benefits of using a cell culture to model the human body it is necessary to characterise its normal in vitro responses in the absence of material interactions. Lymphocytes were cultured at \(1 \times 10^5/\text{ml}\) in \(1 \text{ ml}\) aliquots in polypropylene tubes (Nunc) for 8 days and cell deaths, numbers, appearances and transformations were monitored at 24 hour intervals. Dye exclusion was used to assess cell viability (Section 2.2.5). The counts taken from different tubes were compared in order to examine the reproducibility and standard deviation of such visual measurements.

Light microscopy was used to consider the distribution of the cell population and scanning electron microscopy was used to look for any adhesion of the cells to the culture tube walls.

(ii) Results

The cell concentration with respect to time and the percentage of dead or dying cells are shown in Figure 5. Each point represents 10 counts. It can be seen that the cell numbers remain relatively constant over the first 60 hours and then the cell concentration falls away quite rapidly. 72 hours has been stated to be the optimum...
FIGURE 5
Variation of Cell Concentration and the Percentage Dead with Increase in Culture Time.
response time for human lymphocytes. As this covers the constant part of the cell concentration with respect to time 72 hours was chosen as the test period.

It can be seen that the cell numbers fell at a greater rate than that indicated by the cell death curve. It was initially thought that this discrepancy in the results could have been caused by cells adhering to the tube walls and so not being considered in the count. When the tube surfaces were examined by Nomarski differential interference contrast and by SEM this was found not to be the case. The only other possible explanation is that the toxicity was greater than observed and a large percentage of the dead cells completely disintegrated. If cells disintegrated they would be neither counted into the population or rated as dead or dying. This is only of academic interest because the drop in cell population occurs after the period of culture chosen as the test period.

As it can be seen the degree of cell death was very small (=6% of the total population) over the first 72 hours in culture. This again conveniently allows 72 hours to be used as a test period without a high degree of inherent cytotoxicity in culture interfering with any other measured results. By 8 days the cell death and the cell number curves approach one another indicating that very few lymphocytes will survive more than 5 days in primary culture. On this basis no experiment run for such a period would be valid with this culture system.

As it can be seen in Table 3 less than 10% of the total population were cells other than lymphocytes. To purify the population further would have involved extra manipulations to the cells including column separation and antigen recognition. It is obvious that such
extra manipulations would further introduce cell damage and also would bring the cells into contact with more uncontrolled materials (the test materials having been carefully characterised and thus controlled).

**TABLE 3**

**Showing the type of cells making up the total cell population**

<table>
<thead>
<tr>
<th>Cell Culture Population</th>
<th>% of Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lymphocyte</td>
<td>&gt; 90</td>
</tr>
<tr>
<td>Granulocyte</td>
<td>&lt; 5</td>
</tr>
<tr>
<td>Erythrocyte</td>
<td>&lt; 5</td>
</tr>
<tr>
<td>Monocyte</td>
<td>= 2</td>
</tr>
</tbody>
</table>

Because of this the level of lymphocytes in the population was felt to be high enough and it was accepted that for any cellular effect to be significant it should be greater than 10% (the level of contaminating cells).

Plates 3, 4 and 5 show typical, isolated human lymphocytes examined by light microscopy, scanning electron microscopy and transmission electron microscopy. Small lymphocytes have a high nuclear to cytoplasmic ratio, with a few free ribosomes, a small number of mitochondria and an inactive Golgi apparatus. There is a continuous cell size variation between 6 and 10 μm.

3.3.2 Materials

(i) Method

The prepared disc surfaces were examined by SEM, to check their
surface topography, and by XPS (electron spectroscopy), to find the composition of the surfaces with which the cells will be in contact. The spectra were also checked for any residues of the detergent, Decon 90, that may remain after rinsing. Such residues could obscure the true surface from the cells and are indicated by the presence of sodium citrate, potassium hydroxide, sodium lauryl sulphate and detergents of the sulphonic acid group.

The peak to peak surface roughness and the centre line average of the discs were measured using a Taylor-Hobson MkIII Tallysurf.

The elutions were analysed by atomic absorption spectrometry to determine the ions leached from the material surfaces. Both acetylene and nitrous oxide flames were used and the solutions were analysed for titanium, aluminium, vanadium, cobalt, chromium and molybdenum.

Finally the active atomic sites on a range of the material surfaces were studied by ionic labelling followed by XPS analysis. Cleaned discs of high density polyethylene and titanium 316 and pieces of microtitre well material were immersed in solutions of free ions. Separate specimens were soaked in lime water, bromine water and ammonium chloride solution for several hours. The specimens were washed in deionised water and allowed to dry overnight in a dessicator. After quantitative analysis by XPS for the major elements present on each surface the atomic percentage of calcium, nitrogen and bromine bound to each surface was calculated and related to the active atomic sites.
(ii) Results

Figures 6 to 19 show representative wide and narrow scan spectra obtained from the material surfaces. Table 4 shows the atomic percentage of those elements found on the surface.

No Decon 90 residues were detected (those being indicated by the presence of sodium citrate, potassium hydroxide, sodium lauryl sulphate and detergents of the sulphonic acid group). Calcium, sodium, chlorine and nitrogen were often found, however, and it was suspected that these elements were contaminations from the cleaning procedures (hard and deionised water - Section 3.2.1). Consequently all future washes, solutions and sterilisings were made in distilled water.

The fact that surface composition is seldom the same as a material's bulk composition is confirmed by Table 4. Carbon and oxygen were invariably the most predominant elements found on the surfaces. These are due to atmospheric and hydrocarbon contamination although, with the metal alloys and the alumina ceramic, some of the oxygen peak will be contributed by the metal oxide found on the material surface. For example (Figs. 6, 7, 8 and 9), the shape of the spectra indicate the presence of chromium sesquioxide and cobalt oxide on the vitallium surface. Selective oxidation of alloying elements will thus occur, altering alloy surface compositions from that of the bulk composition. The charge balance on the disc surfaces could be calculated by taking the normalised peak height units (Table 4) and converting these to charge units, knowing the elemental valencies. It can be seen from the final column of Table 5 that the net charge indicates a OH⁻ rich film on the surface of vitallium and titanium. The contamination layer on the titanium
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th>ST</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.80</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8.90</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6.50</td>
<td>2.90</td>
<td>6.00</td>
<td>2.90</td>
<td>6.00</td>
<td></td>
</tr>
<tr>
<td>4.00</td>
<td>6.90</td>
<td>2.10</td>
<td>6.90</td>
<td>2.10</td>
<td></td>
</tr>
<tr>
<td>0.30</td>
<td>6.90</td>
<td>2.10</td>
<td>6.90</td>
<td>2.10</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>HIGH DENSITY ALUMINA</strong></td>
<td><strong>Polyvinyl Chloride</strong></td>
<td><strong>Polyethylene</strong></td>
<td><strong>Polypropylene</strong></td>
<td><strong>Titanium 318</strong></td>
<td></td>
</tr>
</tbody>
</table>

Table 4
318 surface was thinner and it appears likely that the film was $O_2^-$-rich.

<table>
<thead>
<tr>
<th>Anion</th>
<th>Cation</th>
<th>Net Charge</th>
</tr>
</thead>
<tbody>
<tr>
<td>$OH^-$</td>
<td>38.6</td>
<td>21.8</td>
</tr>
<tr>
<td>$O_2^-$</td>
<td>76.5</td>
<td></td>
</tr>
<tr>
<td>$OH^-$</td>
<td>80.8</td>
<td>10.5</td>
</tr>
<tr>
<td>$O_2^-$</td>
<td>160.8</td>
<td></td>
</tr>
<tr>
<td>$OH^-$</td>
<td>34.8</td>
<td>46.4</td>
</tr>
<tr>
<td>$O_2^-$</td>
<td>69.6</td>
<td></td>
</tr>
</tbody>
</table>

It must be noted that the implication of the calculations made from Tables 4 and 5 is that a thick contamination layer remained on all the materials, even after washing. This has the effect of making the outer atomic layers of all these material discs chemically similar. Surprisingly little titanium was detected on the titanium surface (Fig. 10), probably because of the surface layer of contamination, and although there is 6% aluminium in titanium 318 (Fig. 11 and 12) none at all was detected on this alloy's surface. Titanium and vanadium were, however, both detected. The Vn surface composition is the same as its bulk so it is all involved in surface passivation.

Although the polyethylene (Fig. 13 and 14) and polypropylene (Fig. 15) specimens were medical grade several impurities were found on their surfaces. The fluorine found on these surfaces could have originated from the mould release agent during the material manufacture. The aluminium on the polypropylene was probably a residue
from the Zeigler-Natta catalyst. The high density alumina appeared to have a small amount (2.8%) of silicon on its surface, although the signal may have derived from the underlying sellotape (Fig. 16).

When these results are compared with the material elution results (Table 6) it can be seen that it is those elements composing a surface that are selectively dissolved from the surface by an eluting medium. Although, however, there was a higher percentage of titanium on the titanium alloy surface than on the titanium surface, the titanium alloy surface was more protective. 43 parts per million of titanium was leached from the pure titanium whereas only a trace of titanium was leached from the alloy. Vitallium forms an exception in that no molybdenum was found on this alloy’s surface (Fig. 6), but a trace of molybdenum was found in its elution. Obviously the vitallium wear debris exposed a larger surface area to the eluting medium than the vitallium discs. Thus high levels (2.0, 0.4 and 5 ppm of Co, Cr, and Mo) of the elements were found in the elution.

The deionised water elution results (Table 7) show the importance of correct experimental design. The RPMI-1640 eluting medium was more aggressive than the eluting water and doubtlessly modelled the physiological environment better.

Table 8 shows the physical characteristics of the materials. A nitrogen adsorption technique was used to measure the surface area of the powders but insufficient material was available for an accurate result to be obtained.

The surface roughness results obtained using a Tallysurf are shown in Table 9. The metals, being harder, had smoother surfaces than the polymers. The high density alumina had a porous surface
<table>
<thead>
<tr>
<th>Metal</th>
<th>5 ppm</th>
<th>0.4 ppm</th>
<th>0.25 ppm</th>
<th>43 ppm</th>
<th>47 ppm</th>
<th>Material</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mo</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>ND</td>
<td>ND</td>
<td>Mo</td>
</tr>
<tr>
<td>Cr</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>ND</td>
<td>ND</td>
<td>Cr</td>
</tr>
<tr>
<td>Co</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>ND</td>
<td>ND</td>
<td>Co</td>
</tr>
<tr>
<td>Ti</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>ND</td>
<td>ND</td>
<td>Ti</td>
</tr>
<tr>
<td>Al</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>ND</td>
<td>ND</td>
<td>Al</td>
</tr>
</tbody>
</table>

**Detection Limits**

<table>
<thead>
<tr>
<th>Metal</th>
<th>Mo</th>
<th>Cr</th>
<th>Co</th>
<th>Ti</th>
<th>Al</th>
</tr>
</thead>
<tbody>
<tr>
<td>ppm</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

**Key**

- ND = not detected (not analyzed)
<table>
<thead>
<tr>
<th></th>
<th>Mo</th>
<th>Cr</th>
<th>Co</th>
<th>Ti</th>
<th>Al</th>
<th>2.5 ppm</th>
<th>ND</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mo</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Cr</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Co</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Ti</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Al</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

Detection Limits:
- not analyzed
- not detected

Key:
- Mo - Molybdenum
- Cr - Chromium
- Co - Cobalt
- Ti - Titanium
- Al - Aluminum

Table 7

Atomic Absorption Spectrometry Results for 3-day Fluctuations at 37°C in Deionized Water
Table 8

<table>
<thead>
<tr>
<th>Specimen</th>
<th>( \text{Nm}^2 \text{ area} )</th>
<th>Wt gms</th>
</tr>
</thead>
<tbody>
<tr>
<td>Disc</td>
<td>( 5.66 \times 10^8 )</td>
<td>0.1</td>
</tr>
<tr>
<td>Powder</td>
<td>No data</td>
<td>0.1</td>
</tr>
</tbody>
</table>

Table 9

<table>
<thead>
<tr>
<th>Tallysurf</th>
<th>Peak to Peak Nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Titanium</td>
<td>0.75</td>
</tr>
<tr>
<td>Titanium 318</td>
<td>0.50</td>
</tr>
<tr>
<td>Polyethylene</td>
<td>10 ( \mu )m</td>
</tr>
<tr>
<td>Polypropylene</td>
<td>10 ( \mu )m - 5 ( \mu )m</td>
</tr>
<tr>
<td>Vitallium</td>
<td>Undetectable</td>
</tr>
<tr>
<td>Well</td>
<td>Undetectable</td>
</tr>
<tr>
<td>( \text{Al}_2\text{O}_3 )</td>
<td>2.50</td>
</tr>
<tr>
<td>PVC</td>
<td>1.75</td>
</tr>
</tbody>
</table>
with a peak to peak roughness of at least 2.5 Nm. Although it would have been ideal to keep all the surface roughnesses identical this was impossible because of material variations. The differences in surface roughness can be taken into account, if need be, when the results are analysed.

The size of the material specimens was chosen to make any effect of the culture tube and well walls insignificant. Any effect caused by the tube or well material was taken into account by controls and also the effective area of the discs was greater than that of the culture vessels, when roughness factors were considered (the culture vessels were tissue culture coated and had very smooth walls when viewed on the microscope). The roughness of the discs surfaces caused their effective area to be 3-4 times greater than their geometric area. The disc specimen faces were $10^3$ times larger than a cell diameter and had approximately $3 \times 10^7$ times greater surface area. The disc was about 3 times the weight of $2 \times 10^5$ cells. By this design it was hoped to amplify any experimental effects and hence increase experimental accuracy. The atomic percentage of calcium, bromine and nitrogen found on the three materials immersed in solutions containing these ions is shown in Table 10.

**Table 10**

<table>
<thead>
<tr>
<th>Material</th>
<th>Atomic Percent (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ca$^{++}$</td>
</tr>
<tr>
<td>High Density Polyethylene</td>
<td>1.9</td>
</tr>
<tr>
<td>Titanium 318</td>
<td>0.7</td>
</tr>
<tr>
<td>Well Material</td>
<td>2.2</td>
</tr>
</tbody>
</table>
The binding sites available for different ions are obviously different on the three materials. Bromine ions label the hydroxyl groups and calcium and ammonia label the acid groups. It can be seen from Table 10 that titanium 318 behaved, as expected, as a basic surface (e.g. Ti(OH)), picking up anions, whilst the polyethylene surface, as expected, showed itself having acid sites (e.g. COOH\(^{-}\)). The well material behaved in a similar way to the polyethylene in its pick up of calcium. (The acid sites on the well surface were probably due to the silicone coating) but was neutral to bromine and ammonia.

3.3.3 Conclusions

The characterisation of the cellular and material specimens has served to define the system used in the experiments. The cell culture cytotoxicity was low and a 72 hours test period was chosen. The material specimens have been examined and the constituents and physical characteristics of their surfaces is known. The importance of the surface analysis was shown by the difference between the bulk and the surface compositions.
Figure 16

Binding Energy

High Density Alumina

$3 \times 10^4$

C[1s]

Al[2p]

Si[2p]

Si[2s]
4.1 Response of the Lymphocyte Cultures to the Preliminary Tests

4.1.1 Experimental Methods

(i) Toxicity Testing

The implant materials were tested for cytotoxicity by measuring the death or damage of the cells cultures, as described in Chapter 3, in contact with them. The dye exclusion method for the detection of dead or damaged cells was applied using Trypan Blue or Nigerosine. Plate 6 shows several cells stained in this way lying on a haemocytometer grid. Nigerosine is said to be the less toxic of the two stains so both have been used at 2%, pH 7.2, isotonc with the cells. Cells were cultured with a range of material discs in polypropylene tubes at 37°C, 5% carbon dioxide atmosphere. Positive and negative controls were set up using polyvinyl chloride as the positive control, and cells cultured without materials as the negative control. The cultures were then sampled at 30 hrs, 80 hrs and 168 hrs. The cell samples were incubated 1:1 with 2% stain for 3 minutes at 37°C and then examined on a haemocytometer where the percentage of dead or dying cells for each material was determined. All materials were tested in triplicate on 3 occasions, nigerosine being used on the last. A minimum of 100 cells were observed for each count and each culture was sampled several times.

(ii) Experimental Method for Measuring Immunological Responses.

A typical run involved setting up the lymphocyte cultures as already described (3.3.1) with 4 x 10⁶ cell/ml in RPMI-1640 medium in microtitre plates (Plate 7) with 2 x 10⁵ cells per well. One disc of material was placed in each well of the microtitre plate, as shown in Figure 20, with a minimum of six replicate wells for each material.
A SECTION THROUGH MICROTItte
WELLS SHOWING CELLS & MATERIALS

$2 \times 10^5$ Lymphocytes
in suspension

Material Specimens

FIGURE 20
The cell suspensions lay above the material. Wells were also set up containing no material specimens. After incubation for 72 hours at 37°C in a 5% carbon dioxide atmosphere the cell suspensions were with 2.5 μCi/ml tritiated methyl thymidine and pulsed for 16 hours. After this time the cells and washings were harvested, using the water pump and Buchner funnel arrangement shown in Figure 21. The culture was pipetted from the wells and deposited carefully onto 1 cm diameter Whatman GF/A glass fibre discs, lying in the sintered glass funnel. The wells were then thoroughly washed by aspiration of water and all the washings also deposited onto the filter disc. The wells and materials were then checked to ensure that no cells remained on them. Finally the filter discs were washed 5 or 6 times with clean water to remove the background radiation and then allowed to dry.

The filter-discs holding the radiolabelled cells were then put into poly(propylene), disposable scintillation vials with 5 ml of scintillation cocktail (1 litre toluene containing 4 gm PPO, 0.1 gm POPOP and 10% Triton-X-100). The vials were then counted within a few days on a Packard 81000 liquid scintillation counter. Each vial was counted for 600 seconds and the external standard quench correction was used with a 60 second standard count. The print-out of counts per minute could thus be directly related to disintegrations per minute using Figure 22. By this method the β-emissions from the cultures were monitored and these were related to the methyl thymidine uptake of the cells.

The harvesting system that has been described above was essentially a single channel version of the multiple analysis sample harvester (MASH), which is widely used. Runs were carried out both on a MASH (courtesy of the Chester Beatty Institute for Cancer Research, Sutton) and on the single channel version at the University of Surrey. It can
DOSED CELLS

WHATMAN GF/A FILTER

SINTERED GLASS (MASKED)

TO THE TAP

WASHINGS

FIGURE 21
be seen by comparing the left and right hand sides of Figure 23 that the results from control cultures are directly comparable using either harvesting system. Not only did the two sets of results compare well when the lymphocytes were cultured as normal, and with a toxic control material (polyvinyl chloride, PVC) but also when a known mitogen was added (PHA, phytohaemagglutinin). In both cases, the stimulant gave the same order of dpm (disintegration per minute) increase above the control value. The polyvinyl chloride dpm values were low compared with the control because of the cytotoxic effect of the material. The comparibility between the harvesting technique developed for this study with the commercial system used for wide scale assay gave confidence in the functioning of the equipment to be used.

The experimental system described above was used initially to examine three main responses. These are listed below:

(a) The Effect of Material Form

The cell cultures were challenged with the range of implant materials in order to determine whether the lymphocytes recognised any material as antigenic. The material samples were tested as discs, powders and elutions (Chapter 3.3) to look for any differing response with changed material characteristics. Also 1 ppm and 2 ppm of both aluminium sulphate and cobalt sulphate were added to the RPMI-1640 prior to culture in order to ascertain any possible concentration effects of metal ions in solution.

(b) The Effect of Materials on the Mitogenic Response

A series of experiments was also conducted to examine the effect of materials on the known mitogenic effect of the lectin, phytohaemagglutinin (PHA). Cells, with an addition of 10% PHA, were cultured with the range of materials in disc, powder and elution forms and, after labelling as before, with tritiated thymidine, prepared for liquid scintillation counting as previously described.

(c) The Influence of Culture Environment

Finally the influence of the culture environment on the results
COMPARING THE RESULTS FROM TWO HARVESTING METHODS.
obtained was examined using both culture tubes (Nunc) and microtitre plates as vessels and examining the effect of gassing the culture with 5% carbon dioxide.

(iii) Secretory Response

The cell cultures were challenged with the materials to see if they would cause the inflammatory response characterised by enzyme production which may then be related to any immune response observed. The method of Ackerman was used for the alkaline phosphatase determination. Cells were again cultured in the presence of discs of material for 72 hours. Films of cells, fixed in cold 10% methyl formalin for 1 minute, were then prepared and incubated in Naphthol AS-MX Phosphate at room temperature for 30 minutes As a result of phosphatase activity Naphthol AS-MX was liberated and immediately coupled with the diazonium salt (Fast Blue RR) present in the solution forming an insoluble, visible pigment at the sites of phosphatase activity. The slides were then gently washed and counterstained in Mayer’s Haematoxyline. By observing the cells at about 900 x magnification in bright field illumination the cells displaying alkaline phosphatase could be rated from 0 to 4 for blue intensity and counted with the aid of an automatic stage movement point counter. The leucocyte alkaline phosphatase activity (LAPA) was obtained by multiplying the number of cells counted by their rating. The sum of this gave the LAPA score.

A series of controls were used in parallel to the test cultures set in contact with material. Blood from a pregnant woman in the 3rd trimester was used as a positive control (Plate 8) showing an elevated LAPA and a negative control was prepared by immersing a normal smear in boiling water for one minute to destroy enzyme activity
(Plate 9). Slides were also prepared from normal separated white cells cultured for 72 hours. By using these slides for comparison it was possible to remove some of the doubt from the very subjective rating. After each slide had been allocated an LAPA for 600 cells (each test was duplicated), colour prints were taken on Kodak Professional film. These were mounted to allow easy comparison of the observed enzyme sites.

4.1.2 Culture Response Results (Preliminary)

(i) Toxicity Testing

Figure 24 shows the measured relationship between cell death and time after contact with a range of test materials in disc form. The observations were made using a Trypan Blue stain. When the experiment was repeated using a Nigerosine stain, although a good comparative agreement was obtained, there was 10% decrease in detected cell death when compared to the Trypan Blue results (Figure 25). Since Trypan Blue has been reported to increase cell death by 20% after 10 minutes 70 (Nigerosine is said not to have this effect) it seems reasonable that those results obtained using Nigerosine were the more reliable of the two. It is important to note that, apart from the positive control using polyvinyl chloride, no material caused a significant high level of cytotoxicity above the control. Any differences between the materials were small and at 72 hours no material caused more than 10% greater cell death than observed in the control in the 3 day period.

(ii) Immunological Responses

(a) The Effect of Material Form

The uptake of tritiated thymidine gave a direct indication of the cellular immune response to the materials. The absolute values of the
The Variation of Percentage Cell Death with Different Times and Materials
dpm (disintegrations per minute) from the cells are shown in Figures 26, 27, 28 and 29, as uncircled dots. (The circled dots shown on these Figures indicate the effect of the materials on the cellular mitotic response. These are discussed in 4.1.2 (ii)b). Figure 26 shows the results from run A, where lymphocytes were cultured with discs of the materials and harvested using MASH. Runs B, C and D were all harvested using the single channel version of MASH and correspond to the response of the lymphocytes to the material as disc, elution and powder forms respectively (B, C and D). When Figures 26 and 27 are studied it can be seen that the disc specimens caused a lowering of cellular activity in all cases and on both runs. That is, there was less thymidine uptake than normal and the material type seemed unimportant to this result. Figure 26 shows how the presence of polyvinyl chloride caused a drastic ten-fold reduction in the dpm, reflecting its cytotoxic nature. In both runs A and B it can be seen that the polypropylene discs caused the smallest alteration of the lymphocytes control response and high density alumina caused one of the largest.

The effect of culturing the lymphocytes in material elutions is shown in Figure 28. In this case the cellular activities were raised above the control, i.e. the elutions were stimulatory to the lymphocytes. Again the polypropylene caused no alteration of dpm compared to the control, and the polyvinyl chloride elution caused a reduced dpm compared with the control (reflecting its cytotoxicity when eluted). The effect of different elution times is shown in Figure 29. It can be seen that the longer the titanium 318 was eluted in medium, the larger was the stimulatory effect of its elution on the culture dpm.

The results obtained when the materials were tested as powders are shown in Figure 30. Again a depressed cellular activity was seen
RESULTS FROM EXPERIMENT 'A'
FIGURE 27
DPM (logarithmic scale)

KEY
As Figure 26

RESULTS FROM EXPERIMENT 'B'
FIGURE 28

DPM (logarithmic scale)

100,000

10,000

1,000

100

Control PP  AL  318  PE  TI  VI  PVC

RESULTS FROM EXPERIMENT 'C'
THE EFFECT OF TIME OF ELLUTION ON THE DPM
KEY
VI Vitallium
TI Titanium
AL Alumina
CO Cobalt
MO Molybdenum

RESULTS FROM EXPERIMENT 'D'
in the presence of the powders but it must be noted that it was extremely difficult to harvest the cells from the powders. It is felt, therefore, that the dpm values shown could be artificially low (due to loss of cells in harvesting) and hence difficult to interpret. They have been presented here for the sake of completeness. Finally, Figure 31 shows the effect of the aluminium and cobalt sulphate on the cells uptake of thymidine. There appears to be little affect.

To ease the comparison of results from different runs a ratio R was calculated for each material, in all its forms, by normalising the mean dpm value with respect to the relevant mean control dpm value. It can be seen from the bar charts (Figures 26, 27, 28 and 29) that all the control values fell into the same range so such a ratio R would reflect the absolute dpm values well, whilst allowing comparison of results from different runs using slightly different cell size populations. Table 11 lists the RI values which indicate the modification of cellular activity in the presence of materials. Columns A and B record the results obtained using disc specimens (Run A was harvested on MASH and run B was harvested on the single channel version). Columns C and D record the results measured using elution and powder specimens respectively. The level of depressed cellular thymidine uptake in the presence of discs can be clearly seen in column A and B whereas the stimulated uptake can be seen for material elutions in column C.

(b) The Effect of Materials on the Response to a Mitogen

The absolute dpm values measured when phytohaemagglutinin stimulated lymphocytes were cultured in the presence of the materials are shown as the circled dots on Figures 26, 27, 28 and 29. Again these four figures correspond to the materials being present as discs, elutions or powders (A and B, C or D). It can be seen from these charts that the
FIGURE 31
DPM [logarithmic scale]

100,000

10,000

1,000

100

ppm Aluminium Sulphate

1  2  Control

1  2 ppm Cobalt Sulphate
<table>
<thead>
<tr>
<th></th>
<th>SINGLE CHANNEL</th>
<th>MASH</th>
<th>CONTROL</th>
</tr>
</thead>
<tbody>
<tr>
<td>POWDER</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>CO. ELUTION</td>
<td>1.54</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>BR. DISC</td>
<td>1.02</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>POLYVINYLCHLORIDE</td>
<td>0.4</td>
<td>0.33</td>
<td>0.02</td>
</tr>
<tr>
<td>POLYPROPYLENE</td>
<td>1.38</td>
<td>1.29</td>
<td>0.68</td>
</tr>
<tr>
<td>ALUMINA</td>
<td>2.79</td>
<td>0.14</td>
<td>0.31</td>
</tr>
<tr>
<td>HIGH DENTISTRY</td>
<td>0.72</td>
<td>0.2</td>
<td>0.38</td>
</tr>
<tr>
<td>TITANIUM 316</td>
<td>0.29</td>
<td>0.17</td>
<td>0.48</td>
</tr>
<tr>
<td>TITANIUM MATERIAL</td>
<td>0.026</td>
<td>0.48</td>
<td>0.48</td>
</tr>
</tbody>
</table>

**KEY:**

- POWDER
- CO. ELUTION
- BR. DISC
- A. DISC

**Value of**

- TABLE II

- $R_t$ Values
phytohaemagglutinin (PHA) caused a large increase in thymidine uptake when the results are compared with the uncircled (non-PHA) controls, even when the materials were present. It can be seen, however, from Figures 26 and 27, that the material discs again caused a decrease in the stimulated uptake of thymidine. This decrease in cellular activity below the control was not as marked as in the non-PHA containing cultures. It was interesting to note that the presence of polyvinyl chloride, which is cytotoxic, upset the action of PHA completely.

The material elutions (Figure 28) did not seem to significantly enhance the thymidine uptake of the cells when cultured with the mitogen PHA. The results obtained using powder specimens again are difficult to interpret because of the harvesting problems.

A table of $R_2$ values (Table 12) was constructed (as the table of $R_1$ values (Table 11) in 4.1.2 (ii)) to allow the comparison of results from different runs. The ratio $R_2$, was obtained by normalising the mean dpm values for each material with respect to the mean dpm value for the PHA control. It indicates the modification in the stimulated cellular activity in the presence of materials. The nomenclature is the same as described for Table 11. The stimulation indices, $R_3$, produced by PHA in the absence of materials are shown in Table 13. It was considered important to also calculate $R_4$ values which are shown in Table 14. The ratio, $R_4$, was given by normalising the mean dpm of PHA-stimulated cells cultured with materials with respect to the non-stimulated control value. This then indicates the level of effectiveness of the PHA on the cells when they were cultured with material specimens and is an important check on whether the PHA was stimulating the cells to thymidine uptake. It can be seen from Table 14 that on all the runs (A, B and C) the PHA produced the desired transformation in the cultures but this was modified in its degree by the presence of the materials.
<table>
<thead>
<tr>
<th>Material</th>
<th>SINGLE CHANNEL</th>
<th>MASH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.05</td>
<td>0.10</td>
</tr>
<tr>
<td>Polyvinylchloride</td>
<td>0.05</td>
<td>0.10</td>
</tr>
<tr>
<td>Polypropylene</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alumina</td>
<td></td>
<td></td>
</tr>
<tr>
<td>High Density</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Titanium 316L</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Titanium</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Material</td>
<td></td>
<td></td>
</tr>
<tr>
<td>R² Values</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.73</td>
<td>4.7</td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>B</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>D</td>
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TABLE 13
<table>
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<th>MATERIAL</th>
<th>A</th>
<th>B</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitallium</td>
<td>3.12</td>
<td>2.49</td>
<td>2.95</td>
</tr>
<tr>
<td>Titanium</td>
<td>1.11</td>
<td>2.36</td>
<td>8.09</td>
</tr>
<tr>
<td>Titanium 318</td>
<td>6.25</td>
<td>3.72</td>
<td>0.02</td>
</tr>
<tr>
<td>High Density Alumina</td>
<td>6.6</td>
<td>2.6</td>
<td>5.29</td>
</tr>
<tr>
<td>P (Propylene)</td>
<td>4.09</td>
<td>2.58</td>
<td>2.82</td>
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<tr>
<td>P (Ethylene)</td>
<td>2.3</td>
<td>3.32</td>
<td>-</td>
</tr>
<tr>
<td>P (Vinyl Chloride)</td>
<td>0.01</td>
<td>0.4</td>
<td>3.34</td>
</tr>
<tr>
<td>Control</td>
<td>4.75</td>
<td>4.3</td>
<td>7.04</td>
</tr>
</tbody>
</table>

TABLE 14
(c) The Influence of Environment on Lymphocyte Transformation

The effect of growing the lymphocytes under a 5% carbon dioxide atmosphere is shown in Figure 32 using an early set of data which should be disregarded for other purposes. It can be seen that the non-gassed culture did not respond to the phytohaemagglutinin stimulus and the majority of the results fell below the 10,000 dpm level. When the run was repeated, however, using gassing those cultures containing phytohaemagglutinin gave the expected increase in thymidine uptake whereas the non-stimulated cultures again produced results below 10,000 dpm. The efficacy of the single channel harvesting system had been proven (Section 4.1.1) so it was concluded that gassing of the lymphocyte culture was necessary to obtain their normal behaviour. Consequently the cultures were placed in gassed Maxwell jars during incubation. It was also interesting to note that the lymphocytes did not respond to the presence of the materials in the ungassed culture but normal cell growth and response is obviously necessary for subsequent response to the materials.

(iii) Secretory Response Results

The leucocyte alkaline phosphatase activity (LAPA) score for the cells in contact with materials and for the controls are shown in Figure 33. Plates 8 to 12 show typical cells observed during examination for sites of enzyme activity and emphasise the problems of the technique. The positive control shows clear evidence of alkaline phosphatase sites in the cytoplasm (Plate 8) and produced an elevated LAPA. Plate 9 shows the negative control. The cells in contact with the materials, however, showed lower activity, i.e. very pale blue granules, which was difficult to rate (Plates 10 and 11). (The control culture cells are shown in Plate 12). It can be seen from Figure 33 that titanium and
FIGURE 32
DPM [logarithmic scale]
100,000

THE EFFECT OF GASSING ON MITOGENIC TRANSFORMATION
Although titanium 318 induced slightly lower LAPA in the cell cultures than that found in the control, and polyvinyl chloride and high density alumina induced a slightly higher value than that found in the control. Although this has proved to be a weak test for reduced cell activity when cultured with materials it can be seen that there is no evidence of increased activity in the cells in contact with materials.

4.1.3 Summary of Culture Response Results

The results reported in 4.1.2 show that an unexpected effect of the materials on the lymphocytes was found. The expectation that the lymphocytes would recognise the materials as mitogenic or antigenic and thus become stimulated, as occurred with the elutions, was not realised. Instead the opposite effect was exerted by the material discs and a decrease in cellular activity (compared with the control) was observed in all cases. It was interesting to observe a slightly smaller degree of alkaline phosphatase activity in cells cultured with titanium and titanium 318. It is possible that this was another indication of depressed cellular activity but seen by a different method. The following part of this thesis sets out to investigate this effect and to postulate its possible cause.

Reduction in dpm could be a result of cell death, cell damage, loss of cells in harvesting or the bulk removal of essential nutrient from the medium limiting cell growth.

The toxicity testing showed that none of the materials produced more than 20% cytotoxicity within 3 days. (The normal level of cell death in the cultures was about 10% of the cell population after 3 days.) Thus toxicity alone does not account for the 50% decrease in cellular activity.
Bulk adsorption of essential nutrients from the medium could cause the lymphocytes not to grow and behave normally. If this were the case the cells would probably be unable to respond to the mitogenic stimulation of PHA. The result (Table 14) showed that the cells cultured with the materials did respond to PHA so depletion of the nutrient by the materials appeared to be unlikely. The level of stimulation was altered by the presence of materials but it is hard to draw any conclusions on the effect of any specific material.

The material discs and the wells were examined by SEM (Scanning Electron Microscopy) after the cells had been harvested. No cells appeared to have been overlooked (Chapter 5) so the loss of cells in harvesting causing dpm decrease, seems unlikely.

This leaves the possibility of material induced cell damage causing the reduced cellular activity.

Although the solid material caused a depressed uptake of thymidine by the lymphocytes the material elutions caused an enhancement. This suggested that the material surface was instrumental to causing cell damage and thus decreased activity. A second series of experiments were thus undertaken to investigate this hypothesis and to add more to the understanding of the role of a material surface in biological responses. All these experiments, apart from the last two, were conducted on disc specimens of titanium 318. This material caused a large and similar response to all the other materials and is also of interest since it is a relatively new material for use in prosthetics. The second series of experiments and their results are reported in Chapter 4.2.
4.2 The Role of the Material Surface when Considering Lymphocyte Responses

4.2.1 Experimental Method

(i) Impact Damage

In order to study the cell damage effects, reported in Chapter 4.1, two experiments were conducted to examine the possibility of impact damage to cells which could reduce their turnover of thymidine. The first of these experiments involved increasing the material surface available for cell contact. The second involved culturing the cells in media of varying viscosity to look at the effect of cell immobilisation.

(a) The Effect of Material Surface Area on the Cellular Thymidine Uptake

Lymphocytes were separated in the normal way and then cultured with an increased area of titanium 318. This was achieved by changing the specimen from a 6 x 1 mm disc to a 7 x 5 (ID.) mm bucket giving a surface area increase of 2.6 times. The cells were cultured at 4 x 10^5/ml in these buckets and held in microtitre wells for 72 hours. After dosing and pulsing the lymphocytes were harvested and counted on the liquid scintillation counter.

(b) Cell Immobilisation

The amount of cell immobilisation was varied by culturing the cells in a non-toxic medium with a range of viscosities. Sterile polyvinyl pyrrolidones (PVP), of molecular weights 10,000, 40,000, 160,000 and 360,000 were made into 15% W/W solutions in RPMI-1640. 20 μl of cell suspension at 21 x 10^6/ml were put into each microtitre well and 200 μl of the prepared PVP was added. (Because of their high viscosity volumes of the 360 K solution was difficult to measure.) Each molecular weight PVP was tested six times, and controls and PHA stimulated
cultures in PVP were set up in parallel. After 72 hours the cells were dosed and pulsed and harvested. Harvesting was also difficult because of the media viscosity. Counting of the dpm from each culture was then conducted in the normal way on a liquid scintillation counter.

The dye exclusion technique was applied to those cells cultured in PVP after 80 hours in order to assess any obvious cytotoxicity of the PVP.

(ii) Contact Angle Measurements

The contact angles of the material surfaces were measured in order to further the understanding of any interaction between the lymphocytes and the material. Contact angles are a major factor when considering adhesion.

Clean, sterile material surfaces were prepared and their contact angles measured using a liquid drop reflection goniometer. A light beam was made to hit the water drop on the surface along the same axis as the line of sight. A reflection from the drop was observed which moves down the drop as the eye and light source are lowered. As the incident light beam reaches the edge of the drop, there is no more reflective surface and so the light beam disappears. The angle the beam makes with the vertical is equal to the contact angle.

The range of materials were examined in this way and 3 readings were taken for each material disc.

(iii) Surface Coating

The possibility of passivating the material surface with proteins from the medium was considered. This would help determine whether
direct contact with the material surface was necessary for cell activity changes to occur. Prepared discs of titanium 318 were held in serum-supplemented RPMI-1640 for 7 days at 37°C prior to their addition to the usual lymphocyte culture. After 72 hours the cell culture was dosed and pulsed as before and counted on the liquid scintillation counter.

(iv) Cell Secretions

(a) The medium in which cells had been cultured was tested for an effective cellular secretion which could be causing the response inhibition effect. Cells were thus cultured in contact with discs of titanium 318 for 72 hours in the usual way. The suspension was drawn off, centrifuged (600 g, 15 min), and the supernatant medium collected. This was resupplemented with 10% fresh medium and then used for the culture of freshly separated lymphocytes. The uptake of thymidine was studied in the usual way, controls were set up in parallel, i.e. cell cultured alone for re-use of medium, and cells cultured with titanium 318 to check the occurrence of the depressive effect.

(b) Time

Another test culture was set up and the suspensions harvested at 1, 3, 11½ and 72 hours. The medium from each sample was again retrieved and resupplemented with 10% fresh medium. Newly separated cells were cultured in each sample and examined for uptake of tritiated thymidine.

(c) Dilution

The 72 hour contact sample of medium was progressively diluted with fresh medium to give 1.1, 2.2 and 8.8 times the initial concentration. Newly separated cells were cultured in these media and examined for uptake of thymidine.
(v) Testing the Microtitre Well Material

A disc of the microtitre well material was prepared and tested in the previously described manner. The cells cultured in contact with it were examined for uptake of thymidine.

4.2.2 Results from the Experiments to Determine the Role of the Material Surface

(i) Investigating the Possibility of Impact Damage to the Cells

(a) Increased Material Surface Area

The increase of material surface available to the cell culture resulted in further reduction of thymidine uptake by the cells. This is shown in Figure 34. The presentation of titanium 318 to the cells as buckets rather than discs increased the material surface by 2.6. This caused a 1.4 further reduction in cellular activity measured by the uptake of thymidine. The result for titanium powder has been included on this figure also as an indication of the effect of a powder (although it has already been stated that the powder results are suspect, 4.1.2 (ii).) This powder caused a 10 fold reduction in cellular activity. It is thus apparent that any relationship between surface area and uptake of thymidine may be non-linear.

(b) The Effect of Immobilisation of the Cells using PVP (polyvinylpyrillidone)

The bars, reading left to right, on Figure 35 indicate the results obtained when increasing molecular weight PVP was added to the cell culture medium. That is, the cells were grown in increasing viscosity medium. It can be seen that a possible rise of the thymidine uptake by the cells was measured with respect to increased medium viscosity. It can also be seen from Table 15 that the higher the medium viscosity the smaller the percentage of cell death. This could indicate that the
FIGURE 34

DPM [logarithmic scale]

1,000,000

10,000

1,000

100

Control Disc Bucket Powder Soaked disc

THE EFFECT OF MATERIAL SURFACE AREA ON THYMIDINE UPTAKE
THE EFFECT OF INCREASED CULTURE MEDIUM VISCOSITY ON DPM
cells underwent less 'impact damage' against the well walls when movement was restricted. However the experimental problems were such that the high viscosity cultures were extremely difficult to handle and harvest. It is difficult, therefore, to rely too much on any results obtained.

The circled dots indicate the effect of PHA on the cells in the different viscosity cultures. These results are also difficult to interpret, presumably because of the experimental problems.

TABLE 15 At 96 Hours of Culture

<table>
<thead>
<tr>
<th>PVP Molecular Weight</th>
<th>% Cell Dead</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 K</td>
<td>23</td>
</tr>
<tr>
<td>40 K</td>
<td>10</td>
</tr>
<tr>
<td>160 K</td>
<td>4</td>
</tr>
<tr>
<td>360 K</td>
<td>Healthy</td>
</tr>
</tbody>
</table>

(ii) Contact Angle Measurements

The contact angle values (θ) for the range of material discs is shown in Table 16.

(iii) The Effect of Pre-Soaking the Discs in a Serum-supplemented Medium before Testing

It can be seen by comparing the second and final bars of Figure 34 that any lay down of protein during prior exposure on the titanium 318 discs did not increase the subsequent uptake of thymidine by the cells. That is, the disc surface was not passivated with respect to the cells since the same order of dpm was measured as with the unsoaked discs.
### TABLE 16

<table>
<thead>
<tr>
<th>Material</th>
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<tbody>
<tr>
<td>Titanium</td>
<td>25.0 ± 7</td>
</tr>
<tr>
<td>Titanium 318</td>
<td>22.6 ± 2</td>
</tr>
<tr>
<td>Vitallium</td>
<td>45.3 ± 10</td>
</tr>
<tr>
<td>High density polyethylene</td>
<td>0</td>
</tr>
<tr>
<td>Polypropylene</td>
<td>76.0 ± 3</td>
</tr>
<tr>
<td>High density alumina</td>
<td>0</td>
</tr>
<tr>
<td>Polyvinylchloride</td>
<td>80.0 ± 6</td>
</tr>
</tbody>
</table>

(iv) Cell Secretions

The effects obtained when the lymphocyte culture supernatants were cultured with fresh cells for 72 hours are shown in Figures 36 and 37. Figure 36 shows the large drop in dpm obtained between the normal control cells and those exposed to a titanium 318 culture supernatant, with a 10% dilution. The difference is of the order of 10 fold. (The control dpm can be seen to be of the same order as those previously obtained for a labelled 3-day culture.)

Figure 36 also shows how dilution of these supernatants reduced their efficacy in suppressing the cellular uptake of thymidine, such that with 80% RPMI-1640 the dpm value of the test cultures approaches that of the culture with the added supernatant from an original control culture.

All these results have also been converted to $R_1$ values showing how the culture supernatants (produced both in the absence and the
Figure 36: DPM (logarithmic scale)

100,000

10,000

1,000

100

---% of fresh medium added---

Normal control 10% 20% 40% 80% 10%
to titanium 318 culture S.N. to control S.N.

72 hours test period
THE EFFECT OF SUPERNATANT SAMPLE TIME ON DPM.
presence of titanium 318) modified the behaviour of normal cultures. To obtain $R_1$'s the mean control dpm was divided into the mean test dpm and the values are shown in Table 17. The effect of the supernatant dilution on these $R_1$ values is shown in Figure 38, which indicates a linear relationship.

**TABLE 17**

<table>
<thead>
<tr>
<th>Dilution</th>
<th>Initial Contact Time (hrs)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>1.1</td>
<td>0.7</td>
</tr>
<tr>
<td>2.2</td>
<td>-</td>
</tr>
<tr>
<td>4.4</td>
<td>-</td>
</tr>
<tr>
<td>8.8</td>
<td>-</td>
</tr>
</tbody>
</table>

It is also interesting to note that whereas the cells cultured with material disc itself exhibited an $R_1$ of about .48, those cells cultured in the supernatant from the "disc cultures" exhibited an $R_1$ of about 0.085 (which was reproducible).

The time relationship of the supernatant efficacy was also studied and these results are shown in Figures 37 and 39. Figure 37 again presents the dpm values on a bar chart with the bottom axis showing the time the supernatant was in contact with the initial test culture for titanium 318 discs. Of each pair of bars the left hand bar represents the control dpm for each supernatant contact time and the right hand bar represents the test values. The far left bar is the result for the normal 72 hour control culture. The 5, 11.5 and 72 hour contact control supernatants all lie within this normal control value. The 1 hr contact control lies somewhat below the normal control value.
The Effect of Variation of Secretion Dilution on the Modification of Lymphocyte Activity (RI)

FIGURE 38

DATA POINT NOT SIGNIFICANT

SEE FIG. 35
FIGURE 39

With addition of sample of culture supernatant taken at intervals.

Showing the variation of the modulation of lymphocyte activity (RI).
In each case the test supernatant caused some reduction in thymidine uptake compared to its own control and this reduction increased with contact time. Figure 39 shows the $R_1$ values (i.e. the modification of the mean 'time control' dpm value by the presence of a "disc contact supernatant") for these supernatant tests with respect to time. It appears that after an initial rapid increase in the supernatant activity after 4 hours it levels off by 72 hours. The increase in activity was about sixty fold.

(v) Well Material

When a piece of the well material was tested against the normal cell culture it was found to have no effect on the cellular activity, the control and the test dpm being both about 1300 dpm.

4.2.3 Discussion of Results from Chapter 4.2

The hypothesis was made in Chapter 4.1.3 that the material surface was instrumental in reducing normal lymphocyte activity. The importance of having any material surface present on this effect was demonstrated by the dpm results when the surface area of the material was increased (4.2.2). By increasing the material available for cell contact the cellular thymidine uptake was further reduced from the control. Although this is a good indication that the material surface initiates the alteration in cell behaviour the possibility of impact damage of the cell on the material is still a consideration. This consideration similarly cannot be dismissed when the results of cell immobilisation are examined. Although there were experimental problems in the run using cell media of increasing viscosity it was interesting to note a rise in cell dpm with increasing PVP molecular weight (media viscosity). This rise could just be indicative of the problems of washing away the background radiation, a problem which would increase with increased
However it must be noted that there is a possibility of PVP adsorption to the well walls causing protection. Such a factor could not be excluded when using a macromolecular solutions.
matrix viscosity. The rise in dpm, however, could also indicate that
the more a cell is immobilised the less its chance of impingement damage
on the well walls. Such impact damage to a cell could result in its
decreased thymidine turn-over and is more of a possibility when it is
seen that the toxicity measurements indicated that cell viability was
better with increased immobilisation of the culture.

The role of the material surface was further reinforced when the
contact angle results were studied. The ability of cells to adhere
to materials will be effected by the contact angle of the materials,
the higher the material contact angle, the lower the cell adhesion\textsuperscript{110}
When the contact angles were compared to the $R_\text{1}$ values presented in
Chapter 4.1.2 (ii) a relationship could be seen, with the higher
contact angle materials producing the least depressive effect on the
cellular metabolism. This observation is another indication that
cell/material contact is necessary for any depressive cellular effect
to occur.

It was at first surprising that coating the material discs with
adsorbed proteins from the medium prior to testing did not inactivate
the material surface. However communications\textsuperscript{111} have shown that such
a layer of adsorbed protein is dynamic and not necessarily adequate to
screen material surface effects.

Because impact damage to the cells by the materials was still a
possible cause of the reduced thymidine uptake, the supernatant tests
in the absence of the material surface were most interesting. It was
exciting to find that the supernatants from the titanium 318 test
cultures produced a depressed activity in a new culture whereas the
supernatants from control cultures produced a lesser effect (remembering
that all the supernatants were refreshed with nutrients prior to testing). This
means that the test supernatants contained an active ingredient not found in the control supernatants. It also means that the presence of the material surface is not directly required for cell damage to occur, although the material surface must be indirectly responsible. It was shown by diluting the supernatants that the activity of the factor was reduced by dilution until by 8.8 x dilution the test supernatant dpm was similar to that obtained with the control supernatant. This means that the cells secrete an active factor in response to the presence of foreign surfaces. By sampling test supernatant at various times it was seen that the cells produced this active factor between 4 and 12 hours indicating that the cells were secreting this factor at a certain time of their cycle.

So this discovery now left the possibility that impact damage to the cells caused their initial secretion of an active factor, which on addition to subsequent cultures continued to reduce the cellular uptake of thymidine. This possibility can be fairly conclusively dismissed when it is remembered that specimens of the microtitre well material, when added to the culture, did not appear to affect the cells at all. If impact damage to the cells was occurring it could equally well happen against a piece of microtitre well, as it could against a metal, polymer or ceramic disc.
5. MICROSCOPY

5.1 Introduction

Once it had been established that the material discs were exerting an effect on the lymphocyte behaviour a possible explanation must be sought. Microscopy, in its various forms, was thus used to look for cellular morphology changes which might be related to the altered cellular activities. Microscopy was also used to find whether any sub-lethal forms of cell damage were caused by their contact with the materials. For example, the cellular ultrastructure and the cell coat were studied for signs of damage. Also the possibility of chemical DNA damage having occurred was examined by looking for intranuclear repair processes by autoradiography. Finally, energy dispersive analysis was used on intracellular granules in order to estimate the degree of pick up of metal ions from solution or from surfaces by the cells.

5.2 Experimental Method

5.2.1 Light Microscopy

(i) Viability

Light microscopy was commonly used in this work to assess the viability of the lymphocytes. This was done after the separation of lymphocytes from whole blood, and during culture (with or without implant materials).

The method used was that of dye exclusion, which has been fully described in Chapter 3, and the cells were viewed in bright field.
(ii) **Morphology**

The lymphocyte cultures were also prepared by staining on glass slides in order to classify the cell population. The cell suspension was applied to a glass slide as a smear and one of a series of stains was applied. The stains used included Leishmuns, May-Grunwald/Giems, Mayers Haematoxylin and Haematoxylin/Eosin. These are described in reference 76 and the results reported in Chapter 2.

The cell population morphology was also studied after 3 days of culture. Stained smears were again prepared for both the control cultures and for cells cultured in the presence of the range of disc material specimens. These were viewed in bright field in order to see any possible cell morphology alterations due to the presence of the materials. Phase contrast microscopy, which allows the observation of living cells, was also applied to samples of the cell cultures. This was however found to be of less use, giving poor resolution and contrast. Nomarski differential interference contrast microscopy was also applied to both the cells and to the material discs in the early stages of this work. It was found that this method did not add any further information to that obtained on the scanning electron microscope (SEM) and so was abandoned.

(iii) **Autoradiography**

Lymphocytes were separated and cultured as already described in contact with discs of titanium 318. Control cells were cultured under the same conditions in the absence of titanium 318. After 72 hours at 37°C the cells were dosed with 2.5 μCi/ml tritiated methyl thymidine and pulsed for 16 hours. At the end of this time the cells were collected from the medium by centrifugation (100 g, 10 minutes), washed in fresh medium, resuspended in buffered bovine serum albumin
(to facilitate a good smear) and smeared onto hydrofluoric acid washed slides. These were then air dried and fixed for 10 minutes in 4°C methanol. After transportation in a dessicator to Ciba Geigy, the slides were carefully dipped in K2 (Kodak) autoradiography photographic emulsion after being separated into 2 groups. The first group was left to expose in a light tight box for 4 days and then, after developing the emulsion, stained using Haemotoxylin and Eosin. The second group were exposed for 10 days, and after development, were stained using Mayers Haemotoxylin. The slides were then observed by bright field transmission light microscopy and assessed for uptake of tritiated thymidine which appeared as dense granules at the site (Plates 13-16). The degree of high level (>20 grains/nuclei) and low level (>3 grains/nuclei) uptake of thymidine of each group of cells was then measured and any differences between the appearance of the test and the control slides was noted.

5.2.2 Electron Microscopy

(i) Introduction

Electron microscopy allows a higher resolution of observation of cell morphology than light microscopy. It is thus an important tool when studying the responses caused in the cell culture by the material specimens, since pathobiology may be applied to any observations of changes in cell morphology. Electron microscopy allows the study of a range of cell organelles including the cell coat, membrane, mitochondria, etc. The specimen preparation of the cells for electron microscopy is, however, more demanding and great care must be taken in the interpretation of observations made, especially at higher magnifications. Preparation artifacts and cellular changes are a constraint problem to the electron microscopist.
(ii) Scanning Electron Microscope (SEM)

(a) Introduction

A beam of electrons is directed at the specimen which in response produces X-rays, secondary electrons, backscattered electrons etc. The secondary electrons are used in the image formation for the SEM. The action of the SEM is fully described in the literature\textsuperscript{112} and it allows the study of surface relief and morphology to a maximum resolution of 80 Å. The SEM was used in this work to check the roughness of the material discs after preparation and to ensure the entire removal of cells from the discs and wells after harvesting for scintillation counting. Cell surface morphology is said to alter in response to stimuli\textsuperscript{113,114} so it was considered useful to study the number and morphology of those cells sitting on the range of material discs by SEM. The SEM was also used in sequential time studies of the surface of titanium 316 after contact with the lymphocyte culture in order to study the adhesion behaviour of the cells on the material.

A limitation to electron microscopy when dealing with soft biological material is the possibility of fixation and drying artifacts. In preparation for SEM the drying technique is said\textsuperscript{112} to be a most important influence on the observed morphology of the tissue. After fixation and washing the tissue must be dehydrated by either air, critical point or freeze drying. The literature suggests that the most acceptable technique is that of critical point drying. This work has used both critical point and air drying (the latter when critical point drying equipment was initially not available).

(b) Specimen Preparation

Cells, either as suspension or lying on the material discs, were fixed in 2% glutaraldehyde in a 0.1 M sodium cacodylate buffer at room
temperature and pH 7.2 for 1 hour. At the end of this time the cells were carefully washed in sodium cocodylate buffer and then post fixed in 1% osmium tetroxide for 30 minutes. After washing several times the specimens were dehydrated. The initial dehydration was effected by moving the specimens through a 50%, 70%, 95% and 100% series of ethanol with a stay of 10 minutes in each. If the specimens were air dried they were then stored in a dessicator until examination. If critical point drying was used the ethanol content of the cells was substituted by acetone by moving the specimens through a 2:1, 1:1 and 1:2 ethanol/acetone series with 10 minutes in each. They were then placed in 100% acetone over molecular sieve until critical point drying was applied (critical point drying is described in Reference 112). The control cell suspensions were put on glass slides prior to critical point drying. The specimens were then put on studs, coated with sputtered gold/palladium and viewed on a Cambridge MII at 20 kV with a 45° specimen tilt. Photographs were taken on FP4 film with a 100 second time scan.

This basic specimen preparation technique was used in a series of experiments.

(c) To Examine the Morphology of Cells sitting on the Range of Material Discs

Separated lymphocytes were cultured as previously described with the range of material discs for 72 hours at 37°C. At the end of this time the discs were carefully removed from culture and prepared for SEM examination as described above in Chapter 5.2.2(ii)(b). During SEM examination the morphology of the cells on the materials was studied and photographed.
(d) The Degree of Cell Contact to the Material Surfaces

Cultures were again set up containing the range of implant material discs. After 3 days the discs were removed and prepared for SEM examination. Photographs were taken of each material and the percentage of each disc surface covered by cells was calculated. This experiment was repeated with cells cultured in the presence of PHA.

(e) The Time Course of Cell Adhesion to Titanium 318 Surfaces

Titanium 318 discs were used to measure the percentage of the cell culture population lying on the surface with respect to time. Lymphocyte cultures were prepared and titanium 318 discs added. The discs were then carefully removed from culture after 4, 30, 50.5, 74 and 94.5 hours contact, and prepared for SEM examination. Under SEM, six successive photographs were taken of each specimen in order to produce a montage of each surface at 500 x magnification. The shape, size and distribution of the cells was noted and the cells were counted. The number of cells adhering in the 0.25 mm² photographed area was related to the number of the cell population originally in culture.

(iii) Transmission Electron Microscopy

(a) Introduction

Image production in this type of microscopy is by differential electron scattering of thin (=500 Å) sections of prepared material. There is a possible maximum resolution of 5 Å and so it is possible to study any internal alteration of the lymphocyte structure. The limitations of the technique again lie in the preparation artifacts and the interpretation of results. It is especially difficult to prepare a suspension cell culture for TEM because of the problem of holding the cells together before they are embedded in resin. A method was devised for this.
(b) Specimen Preparation

The cell suspension was spun for 15 minutes at 100 g and the resulting cell button resuspended in one drop of 10% bovine serum albumin \(^{138}\) in phosphate buffered saline. This drop was placed on a clean piece of PTFE (polytetrafluoroethylene) and \(\frac{1}{3}\) drop of 12% glutaraldehyde in 0.1 M sodium cacodylate was added. This was left to gel. The blob of gel was then sliced with a scalpel and fixed for 1 hour in 4% glutaraldehyde. The pieces were washed 3 times in buffer and post fixed in 1% osmium tetroxide for about an hour. (The osmium tetroxide also acts as a heavy metal stain). After washing twice in distilled water the cells were stained in uranyl acetate and dehydrated. Dehydration was effected by putting the gel pieces through an alcohol series followed by epoxypropylene. Epoxy resin was used for embedding and the blocks were sectioned on a microtome. The sections were lead citrate stained and carbon coated (for stability under the beam) and then viewed on a Jeol 100B TEM at 80 kV. The use of the transmission electron microscopy is explained below.

(c) To Consider any Cell Morphology Alteration due to Culture in the Presence of the Range of Material Discs

Lymphocyte cultures were set up both as controls and in the presence of the material discs. After 3 days the cells were aspirated off and the cell suspensions were prepared (as previously described) for TEM examination. Any signs of cell damage and necrosis were noted and selected organelles (such as the mitochondria and the cell membranes) were examined for signs of injury. All the sections were studied for any visible differences between the test and control cells. Dead and dying cells were counted so that the results obtained by dye exclusion and light microscopy could be verified.
(d) The Effect on Lymphocytes of Direct Surface Contact with
Titanium 318 Morphology

Specimens were also prepared for cells washed directly from the
titanium 318 discs after 3 days in culture. These cells were examined
for any visible morphology changes compared to the control cells and
a series of micrographs were taken.

(iv) Scanning Transmission Electron Microscopy (STEM)

(a) Introduction

This is a relatively new form of microscopy which combines aspects
of both TEM and SEM. Thicker specimens can be observed than with TEM
which allows more accurate energy dispersive analysis while a
maximum resolution of \( \approx 12 \text{ Å} \) is still maintained. STEM was particularly
useful in this work because it allows the observation of unstained
sections, the contrast manipulation being by electronics. This means
that any inclusions in the cells may be analysed without any inter-
ference from heavy metal stains. Heavy metal stains raise the
Brehmsstrahlung and obscure any small coincident peaks. The instrument
used was a JEOL 100CX with a Link X-ray spectrometer.

(b) Specimen Preparation

The cells were prepared, as for TEM, omitting all the heavy metal
stains. After embedding the blocks were cut to 1 \( \mu \text{m} \) thick sections,
carbon coated and viewed.

(c) Experimental Method

The cells were removed directly from the titanium 318 disc surfaces
after 3 days in culture. They were prepared as described and viewed
by STEM. Any obvious inclusions in the cells were analysed for the
presence of titanium 318 trace elements (i.e. titanium, aluminium and
vanadium). Control cells were analysed in parallel.
5.3 Results from Microscopy

5.3.1 Light Microscopy

(i) Viability

The dye exclusion technique was found to be useful in assessing the viability of cells after separation and during culture. These results have been reported and discussed in Chapter 3.

(ii) Morphology

There were no obvious differences in the appearance of cells cultured with different materials after staining and viewing with bright field microscopy, with the exception of those cells cultured with vitallium. There was more appearance of cell debris in these cultures. The cells cultured with cytotoxic polyvinyl chloride appeared damaged with signs of cell lysis and damage. There was much cell debris apparent.

(iii) Autoradiography

By observation in bright field with a x40 objective and a x2 optiviar the level and the position of the tritium uptake of the labelled cells could be assessed. The percentage of cells taking up any thymidine in culture was also measured by point counting the appearance of radiation on 1000 cells per slide. The radiation, if present in a cell, appeared as dark grains as shown in Plate 13. The percentage of cells exhibiting thymidine uptake (at any level) when cultured with and without the presence of titanium 518 are shown in Table 18. It can be seen from Table 18 that those cells cultured in the presence of material showed a general level of labelling at 58% of that of the control cells. This is in agreement with the scintillation counting results.
shown in Table 11.

**TABLE 18**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>9.1 ± 0.9</td>
</tr>
<tr>
<td>Cultured in the presence of titanium 318</td>
<td>5.36 ± 0.2</td>
</tr>
</tbody>
</table>

It was also interesting to study the level and type of uptake in the two cultures. Elastin transformation in culture is indicated by S-phase cells, which exhibit a very high level of thymidine labelling. The schematic diagram (Figure 40) indicates the different appearances of thymidine uptake observed. Drawing 'A' shows such a S-phase cell, as does Plate 14. It was noted that the control cultures exhibited less S-phase cells than the cultures with added titanium 318. It was also seen that those cells in the control culture exhibiting any thymidine uptake had only about 10 grains per nucleus (Figure 40, C,D), although the overall labelling was greater than that in the titanium 318 cultures. This is probably due to normal thymidine cycling (Plate 16). Those lymphocytes in the test culture exhibiting thymidine uptake were mainly S-phase cells with only about 3% of the labelled cells showing low level labelling (Plate 15). (This compares with about 80% in the control culture). Cytoplasmic uptake of radiation was apparent in those cells cultured with titanium 318 but was not seen in the control cells (Figure 40, E).

The expectation that a high percentage of overall low level labelling in the test culture was not realised. Such low level labelling would be indicative of unscheduled DNA synthesis due to DNA repair in the cells115.
THE DIFFERENT APPEARANCES OF LABELLING

FIGURE 40
5.3.2 Electron Microscopy

(i) Scanning Electron Microscopy (SEM)

(a) The Appearance of the Materials

When the material discs were examined by SEM it was apparent that, although they had all been lapped by the same degree, differences were obvious. The high density alumina surface was very porous (presumably closed pores). The metal surfaces were smoother with approximately 0.5 μm peak to peak scratches. The SEM results were borne out by the Tallysurf results. Undulations could be seen on the polyvinyl chloride disc surfaces formed in the belt manufacture and the filler particles could be seen. The polypropylene discs having been milled in preparation had rather rough, filamentsed surfaces. The high density polyethylene discs also looked rough compared to the metals.

It was thought important to observe the specimen surface finish because of its effect on specific surface area and also because of the noted effects of specimen finish and shape on other peoples biocompatibility results. Plates 17-24 show scanning electron micrographs of all the materials surfaces.

Harvesting - After all the cells had apparently been harvested for liquid scintillation counting by the method previously described, the wells, tubes and discs were checked for remaining cells. No cells were found by SEM indicating that none of the cell culture was being lost at this stage of experimentation and hence artificially lowering the measured cpm of each culture.

(b) The Morphology of Cells sitting on the Range of Material Discs

Control - The normal, circulating mature lymphocytes were seen to be
spherical with a diameter range of 3.5-7.5 μm. The lymphocyte surface is covered with numerous microvilli, 0.2-1.2 μm long and 0.2-0.5 Nm thick. Plate 25 shows a typical normal peripheral blood lymphocyte. Plate 26 shows a typical normal monocyte showing the characteristic deeply ruffled cell surface. The macrophages were generally larger than the lymphocytes, having extended pseudopodia and exhibiting spreading.

Polypropylene - The adhesion of cells to polypropylene was virtually non-existent. The cells sat isolated rather than in groups and appeared to have extremely smooth membranes (Plate 27). Numerous small blobs and microfilaments of protein were seen on the polypropylene surfaces (Plates 27 and 28).

Vitallium - More cells stuck to the metal/alloy surfaces than they did to the polymers and ceramic. The cell behaviour on vitallium was no exception. As it can be seen (Plate 29) relatively large numbers of cells (lymphocytes and some monocytes) were seen on the vitallium disc after 3 days. The cells were slightly ruffled but lacked microvilli. They were surrounded with bloppy, proteinaceous debris.

Titanium 318 - A fairly high level of cell adhesion was also seen to the vanadium-aluminium-titanium alloy, titanium 318. Adhesion to the titanium 318 was however less than that to the vitallium. After 3 days contact with the culture the cells on titanium 318 were spreading and many cells had extended pseudopodia. These cells were probably macrophages and appeared to be more abundant on this surface than on the other surfaces. All the cells looked healthy but the lymphocytes had lost most of their microvilli (Plate 30).

High Density Alumina - Adhesion of the cells to this material was variable, in one case being about 1% of the surface covered and in another about 20% of the surface was covered. About 20% of the cells lying
on the high density alumina showed the ruffled membrane characteristic of monocytes. Some signs of damage were apparent with about 10% of the lymphocytes having smooth outer membranes. Blebs were seen on the material surface. Most of the lymphocytes had microvilli and looked healthy (Plate 31).

Titanium - Again a healthy appearing cell population was seen on the titanium surfaces. About the same percentage (10%) of smooth (damaged) lymphocytes were seen on the titanium discs as on the alumina discs but less monocytes were identified. 60% of the cells had some form of a villous surface although on 20% of these the villi looked "stubbly" (Plate 32).

The level of cell adhesion to the titanium surface was of the same order as to the vitallium surface but the adhering cells looked much healthier on the titanium with less blebbing and more microvilli (Plate 33).

(c) The Degree of Cell Contact to the Range of Material Surfaces

The percentage of the total disc surface covered by cells for each material is shown in Table 19.

TABLE 19

<table>
<thead>
<tr>
<th>Material</th>
<th>Observations</th>
<th>% of Disc Area covered by Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitallium</td>
<td>Cells grouped so reading may be high</td>
<td>13.8 ± 6.0</td>
</tr>
<tr>
<td>Titanium</td>
<td></td>
<td>7.0 ± 2.5</td>
</tr>
<tr>
<td>Titanium 318</td>
<td></td>
<td>3.0 ± 1.5</td>
</tr>
<tr>
<td>Polypropylene</td>
<td>Isolated cells</td>
<td>0.6 ± 0.2</td>
</tr>
<tr>
<td>High density Alumina</td>
<td>Cells merge into material so reading may be low</td>
<td>1.6 ± 0.3</td>
</tr>
</tbody>
</table>
Phytohaemagglutinin added to the lymphocytes cultures seemed to reduce the cell adhesion to all the materials. This was not quantified.

(d) The Time Course of Cell Adhesion to Titanium 318 Surfaces

Figure 41, curve III shows the number of cells lying on the titanium 318 with time. It can be seen that there was a sharp rise in cell numbers sticking to the surface in the first few hours and this was followed by a slow decay. Cell counts had previously been conducted on this culture to determine the number of cells in suspension and these are shown in curve II. This curve describes an almost exponential decay. Curve I was produced by summing the cells on the surface and the cells in suspension and so should describe the concentration of the total population.

Plate 34 shows the cell population on the titanium 318 after 3 hours. The population is healthy and several red blood cells can be seen amongst the villous, ruffled lymphocytes. A proteinaceous deposit had already been laid down from the medium. As it can be seen on Plate 35 there is little change in the appearance of the cell surface population after 30 hours, although more damaged red cells were seen in the small red blood cell population. By 50.5 hours the number of cells lying on the titanium 318 surface had levelled out. Few red blood cells were seen and the white cells looked healthy with ruffled surfaces (Plate 36). Macrophages, 20-30 μm diameter, exhibiting pseudopodia, were seen. After 74 hours (Plate 37) there was obviously a mixed population on the material surface. The cells are looking less healthy and more mis-shapen. Plate 38 shows the surface after 94 hours. Less cells with pseudopodia were seen but the lymphocytes looked distorted. It can be seen that the material was thickly coated with a proteinaceous-like deposit in the area of
Figure 41

Both in suspension and on the surface with increase in time, showing the variation of cell numbers in contact with titanium 318,
adhering cells. The deposit seems to be cracking away. After 171 hours the cells were very indistinct due to the increased thickness of this deposit.

(ii) Transmission Electron Microscopy

(a) Control

Plates 39-46 show cells from the control cultures. A normal small lymphocyte is shown in Plate 40. It appears quiescent with few mitochondria. Plate 39 also shows a small lymphocyte on the right side with an eosinophil on the left side (the only one ever detected in the course of this work). Plate 41 shows a typical monocyte. 10% of the separated cell population had this appearance. A cell in stage 7 of necrosis is shown in Plate 42 being highly vacuolated, myelinated and swollen. No neutrophils were seen by TEM. Plates 43 to 46 show low magnification views of control cultures after 72 hours. The cells look mainly healthy with a small (15-20%) number of dead or dying cells. The dying cells were recognised by a combination of nuclear granulation, membrane detachment, vacuolation and blebbing. The cell membranes had small villi and ruffles. About 14% of the lymphocytes appeared highly villous. The lymphocytes were about 80% nucleus and the cytoplasm contained only a few (one or two) small mitochondria, a small amount of endoplasmic reticulum and generally a few small holes. The nuclear chromatin did not appear badly marginated.

Cell Morphology Alteration due to Culture in the Presence of Material Discs - There were no great differences between the appearance of cells cultured in the presence of different materials and it was difficult to draw any conclusion. The cytotoxicity measurements were in agreement with those made by dye exclusion (Chapter 4).
(b) The Effect of Direct Surface Contact with Titanium 318 on Lymphocyte Ultrastructure

Plates 47 to 50 show cells which had been removed from the surface of titanium 318 and viewed by TEM. A similar population distribution to the control cultures was seen. This was in some ways surprising because one might expect a higher concentration of macrophages on a surface than in suspension. Also SEM results showed many spreading cells on the surface of titanium 318 and these were initially thought to be macrophages. A possible explanation to this small percentage of macrophages seen by TEM could be that they remained on the titanium 318 surface after cells had been removed for the TEM preparation. But when surfaces of titanium 318 after cell harvesting had been previously examined by SEM no cells were seen to remain!

The lymphocytes which had lain on the titanium 318 surface looked different to the control cells from 72 hour cultures, in several ways. The test cells exhibited a more villous surface than the control cells and had a larger number of vacuoles, some of these vacuoles held dense particles. About 35% of the test cells held dense particles, in the cytoplasm, which were not seen in the control. These were later analysed using STEM observation. Signs of nuclear membrane detachment and cytoplasmic vacuoles were commonly seen in the test culture and these are indicative of the early, sub-lethal stages of cell necrosis.

(iii) Scanning Transmission Electron Microscopy (STEM)

(a) The Experimental Technique

The technique of not heavy metal staining the specimens was successful to a limited extent. The background radiation on spectra was reduced and dense particles in the cells appeared clearly against
the background. Unfortunately it became increasingly difficult to find the cells on the grids because the contrast was low. (Once the cells had been located the contrast could be adjusted to see the cells more clearly.) Because of this problem of cell location later work used a 1% osmium tetroxide fixation and stain, omitting the lead citrate and uranyl acetate stains. Another early problem was keeping these thick (1 μm) unstained sections on the grids during evacuation of the specimen chamber. This was overcome by using carbon support films on the grids followed by a final carbon coating of the grids and its sections. This final coating was also helpful in maintaining the specimen stability under the electron beam, being conductive.

(b) The Observation of Cells removed directly from the Titanium 318 Surface

Many sections from control and test cultures were viewed and any electron dense regions were analysed. Although some interesting observations were made none of these could be statistically verified. No real evidence of metal ion accumulation on the cells was found although in just two cases some titanium was detected in a cytoplasmic dense particle (Figure 42). The spectrum shown in Figure 42 shows a peak for titanium and should be compared with Figure 43 which is the trace for the intracellular background showing no titanium. On these figures the elements which were detected are circled on the bottom axis. The copper peak is due to the copper grid and the chlorine and silicon peaks are contributed by both the cellular material and by the embedding medium (the trace for the embedding medium is shown in Figure 44). Further analysis made of the intracellular backgrounds showed similar spectra, although when mitochondria were analysed a slightly higher chlorine content was found.

It was interesting to find that in many cases dead cells and cell
Figure 4.3: Analysis of cellular background and Titanium 31B- Eu-Ture

Key:
- Take off angle
- Seconds
- Sample
- Researcher
- Date
- Laboratory
debris showed a higher silicon content than the healthy cells. Also it was initially found that the dark particles seen in the cytoplasm of cells which were removed directly from the titanium 318 surface proved to be calcium phosphate. Such a trace is shown in Figure 45. Calcium phosphate particles were not detected in the comparable control cells.

Finally, it was interesting to see, in many cases, how the membrane around the microvilli on the lymphocytes removed directly from titanium 318 appeared to be damaged on their tips (Plate 55). Although this may be a preparation artifact it should be borne in mind when discussing the results. Plates 51 and 52 show STEM images of control cells and Plates 53 and 54 show STEM images of cells removed from titanium 318 after 3 days.

5.4 Summary of Microscopy Results

Although there was little difference in the appearance of the cell populations which had been cultured in the presence of the different materials by light microscopy, electron microscopy gave a little more information. Those cells sitting on the surfaces of titanium, high density alumina and polypropylene gave all the appearances of health. They were not distorted and possessed microvilli. The cells observed on vitallium lacked microvilli but were spherical and, although the cells on titanium 318 looked healthy, they gave the appearance of being predominately macrophages. (TEM results did not however confirm this).

When the ultrastructure of lymphocytes removed directly from the surface of titanium 318 were studied cell membrane alterations were seen compared to the control cells. The cells from the material surface appeared more villous, with cytoplasmic vacuoles and evidence
of pinocytosis. It was disappointing that no statistically worthy results were obtained from the analysis of intracytoplasmic granules seen in these cells. It was noted, however, that the initial indications were that these granules were calcium phosphate. The observations of calcification and cell surface alterations taken together, however, did indicate that the lymphocytes were at stage 2 or 3 necrosis. At this stage protein synthesis is inhibited and injury involving primary attack on the cell membranes causes mitochondrial calcification.

When the autoradiography results are examined it can be seen that much of the low level thymidine labelling of the cells cultured with titanium 318 was present in the cytoplasm. This gives an indication that membrane alteration happened to those cells cultured in the proximity of titanium 318 allowing direct uptake of the media and the radioactive label. This is confirmed by the TEM observations and is possibly a precursor to blastic transformation.
6. DISCUSSION

The most important and unexpected finding has been made that when lymphocytes are cultured in the presence of discs of implantable materials their activity is reduced, and their normal level uptake of thymidine is depressed. The implication of this should it occur in vivo, would be that lymphocytes in the proximity of prostheses are less able to fight any infection introduced during surgery. Infection is one of the major reasons for implant removal because of pain. If this is the case it may be possible, by reviewing the results presented in Chapters 4 and 5, to interpret the effect the materials have on the lymphocytes and thus suggest possible ways in which these materials may be improved.

At the conclusion of the literature review certain well defined objectives were stated and tests have been carried out with these objectives in mind. It was anticipated that the lymphocytes would recognise the implant materials as either stimulatory or inflammatory and react accordingly, or even not at all. The materials were recognised but with the reverse effect. Because of the importance of this finding it is considered wise at this point to retrospectively review the techniques used in order to ensure that no extraneous factors were responsible.

The cell culture technique was successful, producing viable lymphocytes for the test period chosen. The cellular morphology agreed with that seen in standard textbooks showing that no undue cell damage occurred during preparation. The results of the observations of the normal lymphocyte cultures were reported and discussed in Chapter 3. It was seen that the lymphocytes behaved normally in all respects with good viability in culture. The normal cellular responses both to mitogens (i.e. phytohaemagglutinin and
material elutions) and to cytotoxic factors (i.e. polyvinyl chloride) were as expected. The experimental system was, therefore, accepted as having sufficient sensitivity to factors arising from material subsequently added. The question which now arises from the culturing technique is whether the contaminating cell species in the cultures could have produced artifacts in the results. For example, granulocytes lyse easily after a short time in culture, releasing hydrolysing enzymes which could damage the rest of the cell population. Only a very small number of granulocytes were ever seen and they were all intact. Also, if the granulocytes had been releasing enzymes in the cultures any subsequent effects would have been accounted for by the depression of activity in the control cultures. Macrophages give another example of how cellular contamination could cause interpretation problems. These cells are morphologically similar to lymphocytes, although distinguishable by TEM, and will automatically adhere to surfaces. It is obviously important to the understanding of the interaction between the material and the cell culture to be able to identify the cell population colonising the material surface. The presence of macrophages in culture made this difficult but an attempt has been made to state the percentage of macrophages seen both on the material surfaces and in TEM sections so this factor could be taken into account. Reviewers\textsuperscript{113,114} have described the topographical differences between lymphocytes and monocytes seen by SEM but these appear to be controversial. It is said\textsuperscript{113} that a monocyte membrane is ruffled, unlike the villous lymphocyte membrane. However, the villous appearance of the lymphocyte depends on the preparation method, fixation temperature, cell damage or cell activity. Any of these can cause the lymphocyte membrane to look slightly ruffled and apparently\textsuperscript{76} the possession of microvilli is a common feature of
many blood leucocytes. Spreading on surfaces is a feature exhibited by macrophages but even this is not a positive means of identifying the macrophage population because lymphocytes have also been seen to exhibit differential spreading, depending on the surface of contact. It must be said that less than 10% of the culture population was identified to be macrophages by TEM observations but it is possible that macrophages were lost in the TEM preparation. Macrophages can be removed from a leucocyte population by allowing them to stick to a glass surface for 3 days. Unfortunately, by the end of this time the lymphocyte viability is dropping and also the procedure involved in the purification of the lymphocyte culture would introduce unknown material variables into contact with the lymphocyte. Such unspecified materials could interfere with the analysis of the results.

Turning now to the materials, it was found that hydrocarbons in a thin (<2 nm) layer were on the surface of all of them. It might be thought possible that, allowing for the leaching of toxic elements, the cells would find all the surfaces chemically similar. In fact, it was found in the immunological response results that all the material surfaces caused reduced thymidine uptake. It was also seen, however, that the degree of reduction was related to contact angles of the surfaces and the more hydrophobic they were, the greater was their effect. Therefore, the cells did not recognise the materials as chemically identical, presumably because of incomplete hydrocarbon overlays.

Thus, even in hindsight, it does seem that the depression of metabolic activity, seen in responses assessed by both tracer (Chapter 4) and microscopic means (Chapter 5) is quite valid. In the following sections the mechanism for the depression is discussed
therefore in terms of lymphocyte material interaction.

The Effect of Material Specimens in Different Forms on the Behaviour of Lymphocytes in Culture

The uptake of tritiated thymidine by the lymphocytes was found to be changed about the control level when the lymphocytes were cultured with specimens of material. A surprising effect was that the material elutions caused a stimulated cellular uptake of thymidine, whereas the disc and powder materials caused a depressed cellular uptake of thymidine. The materials were initially tested in this way in the expectation that the cells would recognise certain materials (e.g. the metals) as antigenic or mitogenic and also be able to distinguish between different types of materials. In fact, when the cells were cultured with disc specimens, the uptake of thymidine was depressed below the control value and the metals appeared to produce more effect than the polymers, so there was an indication that the lymphocytes recognised the various groups of materials as chemically different. The level of depression was the same for each individual material on both runs so the effect was specific.

In some ways the material elutions, however, effected the cell behaviour as initially expected. In part, the results were, however, surprising. Although antigens are mainly complex macromolecules (antigens are the substances capable of inducing antibody production by B-cells and of reacting specifically with the antibody) a compound need not be extremely complex to be antigenic. Low molecular weight compounds (such as metal salts) may be antigenic if they are conjugated to a carrier (usually a protein). These compounds are called haptens. It was thus thought at the start of this work that there was a
possibility that by eluting the materials any leached ions from the materials may then undergo hapten behaviour. Even the polymers were eluted for testing because it was thought possible that any residual catalyst ions etc., may also be leached out and cause an immune response. In the light of all this it may not be thought to be surprising that the material elutions were stimulatory. The atomic absorption spectrometry of the elutions, however, indicated that the only materials that demonstrated any elemental leaching (within the detection limits of the instrument) were titanium and the alloy, vitallium. If this is the case then no elements were available for the hapten reaction and no stimulation would occur. It must, therefore, be supposed that elements were leached from all the materials but were present below the detection limits of the instrument. This is especially likely when one considers that the polymer elution did not significantly cause any cell activation and a polymer is unlikely to have large enough amounts of metal ions present for any leaching to occur. The stimulatory effect of the elutions was completely masked when the material discs were present in culture showing the powerful effect on the cells of the presence of the solid material.

It was found that the longer the time a material (Titanium 318) was eluted the greater was the elutions' stimulatory effect. Presumably this is because the longer the elution time, the more free ions there are in solution for hapten conjugation and thus more cells are stimulated. It would be expected that this effect would show a time dependent relationship, being limited by the number of cells available for activation and by competition between the diffusion of the ions into solution and their removal by hapten formation.
The Role of the Material in Causing Damage to Lymphocytes in Culture

It appears that it is the physical contact between the lymphocytes and the material surfaces which is the most important factor in producing the observed effects. By increasing the surface area of the materials available for cell contact the cellular thymidine uptake was further reduced from the control. The contact angle measurements showed that the more hydrophilic the surface, the less the cells adhered (which agrees with the literature\(^{110}\)), and the less the materials affected the thymidine uptake of the cells. It was also found that the depression of thymidine uptake of PHA-stimulated cells in culture, with material discs, was less marked than with non-stimulated cells. Phytohaemagglutinin increases the contact angle of cell membranes and, thus, decreases the cells ability to adhere to surfaces and to phagocytose. This was supported by microscopy, and so again it is seen that physical contact with the material surface is necessary for a marked depression in cellular thymidine uptake to occur.

It was, therefore, of interest to study the cell adhesion measurements in the light of their importance. It was exciting to find that cell colonisation increased on the surface of the material for the first few hours in culture. After this time, and coincident with the time that an active factor appeared to be present in the solution, the cells started to fall off the surface. This is most important because the implication is that the active factor, produced in response to the materials, could have been altering the cell membranes such that their adhesive ability is reduced. Alteration in the structure of the cell membranes has been observed by both SEM and TEM. The TEM results, in particular, showed that contact with the materials caused the cell membrane to look more villous with evidence of pinocytosis. STEM observations showed that,
in some cases, the microvilli of cells removed from titanium 318 appeared to have damaged membranes on their tips. It, therefore, appears that membrane alterations, induced by material contact, either directly or indirectly (by the subsequent secretion of an active factor by the cells) is likely. Such membrane changes then cause the cells to move back into suspension. The active factor must also have an effect on the cells in suspension. It has been shown that just 14% of the cell population lay on the titanium 318 surface at 72 hours. But the inhibition of thymidine uptake has been measured to be about 50% (or 329% if the stimulation due to the material elutions is considered). This means that a possible further 36% of the cells in suspension are also inactivated. It is most revealing that it was found by TEM that about 35% of the cells cultured with titanium 318 appeared to be highly villous. These figures tie together surprisingly well. Taken together with the finding that materials did not need to be physically present to induce an effect, they confirm the suspicions that a factor capable of damaging cells is produced by the material/cell interaction.

The Nature of the Chemical Damage Caused to the Lymphocytes by the Material Surfaces

The description of chemical damage could cover all manner of things. For example, cytotoxic elements leaching from the materials could cause chemical damage and even death to the cells in the proximity. Other mediators of chemical damage could be produced by the culture itself in response to the presence of a material, which subsequently causes damage to the cells. These possibilities will now be discussed.
(a) Material Cytotoxicity

Examination of the results in the preceding chapters has eliminated the possibility that the materials were directly cytotoxic to the cells. When the cytotoxicity results shown in Chapter 4.1 were studied it could be seen that none of the materials were particularly cytotoxic compared to the control. The maximum material induced cell death was measured to be about 10% above the control, so this alone would not account for the 50% cell damage. It is possible that the materials directly induced sub-lethal damage to the cells disabling their ability to turn over thymidine. It was, however, seen that elutions of the materials were stimulatory to the cell population and anything but cytotoxic. Material induced cytotoxic damage can thus be eliminated.

(b) Culture Induced Damage

The experimental evidence is such that cell self-induced damage to the culture as a result of material contact is the only possible explanation for the lymphocyte responses recorded in this thesis.

The lymphocytes are responding in some way to the presence of the materials such that an active secretion is produced in the medium. This active factor is then effective both on the culture originally producing it and, subsequently, on cultures that it is added to. The following part of the discussion must now consider in which way the cells are interacting with the materials and then investigate the constitution of the active factor.

The Constitution of the Active Factor Produced by Cells in Response to Materials

Other workers have observed a depression of thymidine uptake
and blastogenesis in lymphocytes. Such observations have either been related to the presence of metal salts\textsuperscript{117,118,119} in culture or to the addition of other factors. The effect of the metal salts (e.g. cobalt, mercury, cadmium, zinc, etc.), was said to be one of cytotoxicity so is not relevant to this discussion. It is interesting, however, that Williams\textsuperscript{120} states that the mode of cobalt cytotoxicity is one of damaging the cell membranes and the reduction of phagocytic ability by reducing the glucose 6 phosphate dehydrogenase activity. Pegrum and Thompson\textsuperscript{101} found that bald lymphocyte nuclei inhibited DNA synthesis in lymphocytes by 12 and 96\% but again this is not relevant to this discussion since no bald nuclei were seen.

The main question under discussion is the nature of the active factor. It has been established that the active factor is produced by the cells in culture and so it is more relevant to find whether any of the possible cellular secretions could cause a similar effect to that observed.

Under various stimuli and antigen recognition, T-lymphocytes produce various factors active in the hypersensitivity response. These include lymphokines and lymphotoxins. Also, when involved in phagocytosis, granulocytes and monocytes can release lysosomal hydrolases. The following discussion will consider the possibility that either lymphotoxins could be the active factor or, because of the contaminating species in culture, whether the damage could be due to hydrolytic enzymes.

**Lymphotoxins**

The cytotoxic effects of lymphotoxins in culture were first described by Gouaerts\textsuperscript{121} and Rosenau and Moon\textsuperscript{122} in the early 1960's. They are produced against target cells and their nature and action
are still under investigation. Early experiments conducted by Ruddle and Waksman used tuberculin-sensitised rat lymphocytes which produced lymphotoxins on coculture with PPD antigen. The supernatant from the rat lymphocyte culture was diluted 50% with fresh medium and applied to a rat fibroblast culture. Cell killing was demonstrated by cells dropping from the dish. An important point is that Schuler et al. found that the presence of a silicone prosthesis, in rats, caused an increase in the cytotoxicity of lymphotoxins. This is the only reference that could be found on the effect of implant materials on lymphotoxin production.

Lymphotoxins cause growth inhibition or direct cytolysis of target cells in vitro. Both specific and non-specific responses cause lymphocytes to release them as part of the host defence mechanism. Because lymphotoxin secretion can be triggered by both mitogens and immunogens its production is thought to be controlled by a surface receptor reaction. The effect of lymphotoxins on target cells has been monitored by the target cell 14C incorporation or by the loss of adhesion of fibroblasts. As in this work, cell damage was detected by depressed nutrient uptake or by loss of cellular adhesion, and so the action of lymphotoxins is worthy of further investigation because of their similarity in action to the active factor described in this thesis.

Although lymphotoxins have a high activity they are present in prepared supernatants in very small amounts, which has made them difficult to study. Various fractionation techniques have previously been applied to lymphotoxin-containing serum and four molecular weight classes have been found. The techniques used involved a combination of cellulose column chromatography, sephadex chromatography, polyacrylamide disc gel electrophoresis, density gradients
and Peviken block electrophoresis. It would have been useful in
the present work to analyse for the presence of lymphotoxins in
the culture supernatants by such techniques. Sadly these sophisticated
facilities were not available for the present investigation.

Ruddle and Waksman found a similar time course of lymphotoxin
secretion as that found for the active factor in this work. Lympho-
toxins were detected after incubation of lymphocytes with the antigen
after eight hours by these workers and Williams and Granger found
that lymphotoxin production reached a steady state by twenty-
four hours, which again is consistent with the observation made on
the active factor in this thesis. Aksamit and Leonard also found
that lymphotoxins started to accumulate at a low rate at six hours
and then the rate increased sixfold. In my work the unknown active
factor started to be effective after about four hours and then
increased rapidly until twelve hours when its efficacy levelled off.
It is thought that certain lymphocytes are precommitted for
lymphotoxin release, secreting it very rapidly on activation. The
mechanism of cytolysis has been studied by Granger and co-workers. It
appears to result from the degradation of the target cell plasma
membrane and is non-specific. The active factor produced in the
response to materials in culture also appeared to be effective on
cell membranes. Although lymphocytes are said to be the cells
most resistant to lymphotoxins, if high enough levels of lymphotoxins
are obtained, lymphocytes are also destroyed. The speed, specificity
and sensitivity of target cell cytolysis is based on the concentration
of lymphotoxin in the medium, the physiological state and the tissue
source of the cells. Table 18 shows the change in counts per
minutes reported by two workers, Granger and Smith, when
lymphotoxin-containing supernatants were added to fresh cultures
containing $3 \times 10^6$ cells.
Table 18

<table>
<thead>
<tr>
<th>Culture</th>
<th>Counts per Minute</th>
<th>Worker</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>9,000</td>
<td>Granger^134 using 14C uptake</td>
</tr>
<tr>
<td>Control + supernatant</td>
<td>400-4,000</td>
<td></td>
</tr>
<tr>
<td>PHA stimulated culture</td>
<td>150,000</td>
<td>Smith^126 et al using 3H</td>
</tr>
<tr>
<td>PHA stimulated culture + supernatant</td>
<td>24,000</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>14,000</td>
<td></td>
</tr>
<tr>
<td>Control + supernatant</td>
<td>2,000</td>
<td></td>
</tr>
</tbody>
</table>

Table 19 shows the change in disintegrations per minutes (i.e. counts per minute x count efficiency) reported in this thesis when the supernatant from a culture containing titanium 318 was added to a fresh culture containing 1 x 10^6 cells.

In Granger's work the R1 value, which indicates the modification of the cellular carbon 14 uptake by the presence of lymphotoxin can be seen from Table 18 to range from 0.04 to 0.4. Smith showed the modification ratio R1 to be 0.14. When these are compared to the value obtained in this work (0.08), using the supernatant from the titanium 318 culture, they can be seen to be comparable. The thymidine uptake of the stimulated lymphocytes was also seen to be of the same order but the depression in activity of the stimulated
cells caused by the titanium 318 was found to be less than that caused by lymphotoxins in Smith's work. It has, however, been seen in this thesis that the active factor is even more effective in a fresh culture than it is in the material containing culture - perhaps because it is present at different stages in the cell cycle.

Table 19

<table>
<thead>
<tr>
<th>Culture</th>
<th>Disintegration per min.</th>
<th>Worker</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6,262</td>
<td></td>
</tr>
<tr>
<td>Control + supernatant</td>
<td>530</td>
<td></td>
</tr>
<tr>
<td>PHA stimulated culture</td>
<td>45,000</td>
<td>Doyle</td>
</tr>
<tr>
<td></td>
<td></td>
<td>using</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3H</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1 x 10^6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>cells/ml</td>
</tr>
<tr>
<td>PHA stimulated culture +</td>
<td>24,000</td>
<td></td>
</tr>
<tr>
<td>titanium 318</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Finally, the other piece of evidence to be noted is the high activity of both lymphotoxins and the cell secretion found in this work. Granger found that cell inhibition was detectable at a lymphotoxin dilution up to 1:50,000 although it was commonly detectable at 1:50 dilution. The cell secretion produced in a response to material in the culture was still detected at 1:40 dilution.

**Hydrolytic Enzymes**

Whenever a foreign object is implanted in living tissue an
inflammatory response occurs. If uncomplicated the acute inflammatory response changes after three days and the population of neutrophils is predominantly replaced by monocytes, plasma cells and lymphocytes. At implant sites the enzymes are generally the hydrolytic enzymes\(^7\) e.g. phosphatases and the oxidoreductase enzymes (e.g. dehydrogenases). The former are usually lysosomal enzymes and associated with mononuclear macrophages and giant cells. Thus macrophages can cause tissue damage and degradation at the sites of chronic inflammation. It is said\(^7\) that lymphocytes contribute no demonstrable hydrolytic enzyme activity at implant sites so if the active factor is an enzyme it must be produced by the contaminating macrophages in their attempt to phagocytose the material surfaces.

All the work considering the induction of enzyme production in response to foreign materials has been conducted in vivo. Consequently there are no in vitro results showing the effect of enzymes on thymidine uptake to compare with the results obtained in this work. It is, however, suspected that lysosomal enzymes may also inhibit the nutrient uptake of cells because initially the lymphotoxin investigators\(^132\) thought that lymphotoxins could be non-specifically acting lysosomal enzymes. This idea was eventually dismissed when it was found both that lymphotoxins could be neutralised by antibodies directed against them and that they lacked proteolytic activity.

Acid phosphatase activity is said by Salthouse\(^135\) to be noticeable at five days and reaches a maximum at 14 days. Alkaline phosphatase is associated with granulocytes and is usually observed from one to seven days. Neither of these fit in with the observations made on the cellular secretion in this work. Aminopeptidase is often predominant in areas of necrosis and tissue damage but its activity
is said, in the same review\textsuperscript{7}, to be minimal with most medical grade materials. Increased adenosine triphosphate activity is said to be associated with increased cellular metabolic activity so again it is unlikely that this enzyme is responsible for the depressed lymphocyte activity found in this work unless it is produced in response to the stimulus of the material elutions and then damages the rest of the cell population. It is also produced by fibroblasts which were not present in these cultures. Enzyme production at implant sites seems most likely if the implant material produces cytotoxic materials or if the prosthesis is mechanically irritating to the tissue. When this is the case older macrophage populations\textsuperscript{136} have been known to release lysosomal enzymes into the extra-cellular space. It would be necessary for such an event to be occurring therefore, as the macrophage response to the material discs in the culture used during this study. There is no real reason, however, why the material should be found either irritating (since they are stationary) or cytotoxic by the cells. Results have shown that the materials were not appreciably cytotoxic to the cultures. If anything the observation of the alkaline phosphatase in the few neutrophils in culture showed a slightly depressed or normal activity in contact with the range of materials, apart from those in contact with cytotoxic polyvinyl chloride, which had slightly elevated activity. Discussions at ICI\textsuperscript{137} (Toxicology Division) suggested that the possibility of enzymal DNA damage to the cells, which had been cultured in the presence of materials, could be investigated by the use of thymidine labelling and autoradiography. Such damage would be indicated by an unscheduled low-level, overall, radiolabelling in the cell nucleis indicating that the DNA was undergoing repair. This was not seen to be the case. There was a small amount of low-level labelling in both the control and the test cultures and there was more evidence of thymidine uptake
in the control cultures, although the test cultures produced more s-phase cells.

The Responsible Factor: Lymphotoxins or Lysosomal Activity?

In principle, either lysosomal enzymes or lymphotoxins could be responsible for the damage to the lymphocytes caused indirectly by the materials. However, the time scale involved in enzyme production does not seem to tie in with observations made in this work; nor do the culture conditions and the materials now seem to be the type which would encourage such enzyme production. On the other hand, all the experimental evidence so far seems to fit in well with the present knowledge of the action of lymphotoxins. The action of lymphotoxins has already been seen to be increased by the presence of an implant by another worker. Lymphotoxins are produced by stimulated T-cells and autoradiographic evidence is given in Chapter 8 that some such cells are found in the cultures prepared with added titanium 318. It is possible that the material contact and the material elution stimulated the T-cells to different levels. Those cells stimulated to the higher level by material contact then release lymphotoxins which then, being at a high enough concentration, effect the membranes of the lymphocytes and cause cytolysis. This is then visualised by the cells' apparent inability to turn over thymidine. It is possible that adhesion to the materials alters the cell membranes prior to lymphotoxin production such that the cells are then made more susceptible to cytolysis but this is not a necessary part of the thesis. Such prior damage would, however, explain why the PHA-stimulated cultures are not so strongly effected by the material discs.

The final question that must be asked is the pertinence of in vitro techniques to the in vivo situation. An 'in vitro' system is closed whereas in vivo the lymphocytes are
circulating and renewing. Also the body is often capable of protecting itself by encapsulating a foreign material whereas this would not happen in a lymphocyte culture. Even so it is still felt that the use of cell culture has been a very useful tool for the examination of the cell/material interaction, the experimental system being more easily characterised and the introduction of variables can be controlled. Also the responses of the body and its immune system are still not fully understood and it is possible that local reactions such as those found in this work, may have further consequences in the chain of the immune response. The importance of the contact angle of an implant material has now been shown and future work should investigate this further. The contact angle of the material could be systematically changed and measured. The materials, possessing a range of contact angles, could then be tested in direct contact with the lymphocyte cultures and the release of lymphotoxins monitored. By this method a series of materials could be designed for subsequent exposure and testing in animals.
7. CONCLUSIONS

(1) The validity of using a lymphocyte culture as a test medium for implant materials has been shown. Both the lymphocyte culture behaviour and the material specimens were characterised so that the precise nature of any cellular responses, and of the surface coming in contact with the cells, was known. The uptake of cellular thymidine proved to be a sensitive method of monitoring the lymphocyte response to materials in vitro.

(2) None of these implantable materials tested exhibited gross cytotoxicity to the lymphocyte cultures.

(3) Lymphocytes were stimulated, as expected, in a mitogenic way by both phytohaemagglutinin and by material elutions. Surprisingly, this response was elicited by the polymer and the ceramic elutions, as well as by the metallic elusions. It is possible that metal ions (suitable for hapten formation) were leached from the polymers and the ceramic but were below the detection limits of atomic absorption spectroscopy.

(4) Lymphocytes were shown to respond to the presence of material discs in culture by a lowering of their thymidine uptake, both from its normal level and from the level induced by mitogenic stimulation using phytohaemagglutinin. Any obvious explanations of this observation were investigated. It was found that cell contact with the disc surfaces was an important mediator in this response.

(5) A relationship was shown between cell/surface contact, the contact angle of the surface and the degree of reduction
of the cellular thymidine uptake from the norm. It was, however, found that when supernatants from cultures grown against titanium 318 were tested against fresh cultures the same, but larger, depression in the thymidine uptake of the culture was measured. The possibility of impact damage causing this effect directly could thus be dismissed. The indication was that the presence of materials in lymphocyte culture initiated an active secretion from the cells.

(6) The cell secretion was produced between 4 and 12 hours and its efficacy could be reduced by dilution.

(7) Lymphocytes cultured with materials showed membrane alterations and there was some evidence of stage 2 or 3 necrosis, i.e. that some sub-lethal damage was being caused by the materials. Possible types of cell secretions responsible for cytolysis were investigated and it was postulated that lymphotoxin production by stimulated lymphocytes subsequently caused damage to cells in suspension. It is also possible that adhesion to the materials, while stimulating the cells, also produced membrane alterations which caused an increased susceptibility of those cells to lymphotoxin damage.
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Plate 1  Transformed lymphocyte and small lymphocyte. Giemsa/
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Plate 2  Polypropylene culture tubes and material discs in universal
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Plate 21 Scanning Electron Micrograph of the titanium surface.

x 400.

Plate 22 Scanning Electron Micrograph of the titanium 318 surface.

x 400.
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x 400.

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x 400.
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x 3 K.

Plate 52  Control lymphocyte after 3 days culture viewed in STEM mode.  x 10 K.
Plate 53  Lymphocyte after 3 days culture, removed from a Titanium 318 surface.  x 10 K (STEM image).

Plate 54  Lymphocyte after 3 days in culture, removed from a Titanium 318 surface.  x 10 K (STEM image).
Plate 55 Lymphocyte membrane after 3 days in culture, removed from a Titanium 318 surface. (STEM image).