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Theoretical and practical aspects of testing potential biomaterials *in vitro*

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In vitro methods provide a necessary and useful adjunct to *in vivo* studies in testing potential biomaterials. One of the most important functions is the screening for toxic effects of the biomaterials. The spectrum of changes elicited ranges from cell death to alterations of cell adhesion, proliferation and biosynthetic activity. Such test systems may involve the direct contact of cells with the biomaterials or the use of soluble extracts of the latter. The rapid, cost-effective and highly sensitive *in vitro* methods have to be weighed against the problem of validity of extrapolation to the *in vivo* situation. The first step in testing potential biomaterials may be termed "general" biocompatibility testing and usually involves the use of various cell lines (i.e. transformed cells) which can be easily cultivated and passaged in the laboratory for long periods. Although the latter is convenient and highly sensitive for recognizing and excluding toxic materials at an early stage in the process of toxicity testing, this method cannot be regarded as exhaustive. It is proposed that such screening methods be followed by a second *in vitro* phase, in which primary and early passage cells of a type relevant to the proposed application of the medical device are used. This "specific" biocompatibility testing is an attempt to simulate the *in vivo* situation as closely as possible. A further component of such a testing scheme involves the use of relevant biological parameters, such as cell adhesion or the production of specific biosynthetic products by the relevant cell type. It is thus possible to construct a spectrum of *in vitro* changes, ranging from marked inhibition of growth with frank cell death ("not biocompatible") to marked stimulation of relevant growth and other cell biological parameters ("biocompatible and bioactive"). An expansion of *in vitro* testing methods can offer a method to "tailor" biomaterials for specific *in vivo* applications. In conclusion, it must be stressed that all *in vitro* experimentation, whether "general" of "specific", cannot replace the subsequent *in vivo* testing. Both components are necessary in an adequate scheme for testing potential biomaterials.

1. Introduction

The increasing use of biomaterials in medical and dental practice, as well as the increasing sensitivity of both legislator and community to environmental problems in the broadest sense, have made the need for effective and reproducible test systems for biomaterials greater than ever before. The striving to restrict animal experimentation to a minimum has heightened the interest in using *in vitro* systems to distinguish adequately between potentially useful biomaterials and those unsuitable for human application.

In this paper we discuss briefly some theoretical and practical aspects of such *in vitro* endeavour.

2. Scope and limitations of *in vitro* systems

The principal aim in adopting test systems for biomaterialas is to test biocompatibility, which may be defined as "the ability to perform with an appropriate host response in a specific application" [1]. This necessarily involves the exclusion of materials that exert a toxic effect on cells, and until recently this aspect has represented the single most important function of biocompatibility tests. As will be seen below, this is not the only aspect of such *in vitro* methods, neither indeed is it the only feature of biocompatibility itself. Toxicity *in vitro* is a negative or deleterious effect of an agent on normal cellular biochemical functions, i.e. a disturbance of cellular homeostasis. This may assume a spectrum of changes from frank loss of cellular vitality to very subtle alterations in cellular function, which can be detected only by biochemical means. Examples of toxicity *in vitro* are the following.

1. Cell death. This phenomenon is usually apparent by direct observation in the inverted microscope. The cells will round up and usually detach from the growth substratum. Before this, nuclear shrinkage (pyknosis) and cytoplasmic fragmentation may be seen, the latter being particularly detectable using phase-contrast optics.

2. Reduced cell adhesion. This must be clearly distinguished from cell death and may cause problems for the unwary, as both lead to detachment of cells in

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an established monolayer or the failure of the single-cell suspension to adhere to the growth substratum, either the biomaterial itself or a soluble extract of the biomaterial added to the cell suspension for adherence to a standard or reference surface, such as tissue culture plastic or glass. Thus, reduced cell adhesion to a biomaterial may be wrongly interpreted as evidence of cell death, and reduced cellular adhesion may or may not be a toxic effect. This is best stressed by presenting an example. If a medical device is constructed to be haemocompatible, i.e. to be as inert as possible on contact with blood (both cellular and non-cellular components), then the failure of platelets and other blood cells to adhere is most certainly not a toxic effect. On the contrary, failure to adhere is, in this case, evidence of biocompatibility.

3. Altered cellular morphology. This is often a prelude to the loss of cellular vitality. Of special significance is the increased vacuolation of the cytoplasm, which often involves the development of autophagosomes with accumulation of lamellar phospholipid membranes, that can be demonstrated in the electron microscope. A certain degree of vacuolation is, however, a normal *in vitro* phenomenon and represents intracytoplasmatic lipid accumulation which usually decreases once a post-confluent state has been reached in monolayer [2].

4. Reduced cell proliferation. This sensitive parameter of cell toxicity can be readily quantified by a variety of methods. The simplest of these is the growth curve obtained by counting the cell number in culture at various times after initiation of a passage. This usually involves enzymatic treatment of the culture to yield a single-cell suspension which is then counted, either in a counting chamber using direct microscopy, or in an automated particle counter.

An alternative is to pulse label cells in the *S*-phase of the cell cycle using, for example, tritiated thymidine or bromodeoxyuridine, followed by visualization of those cells undergoing DNA synthesis. In the case of radiolabels this can be achieved by autoradiography, quantifiable as a labelling index [3, 4] or, in the case of bromodeoxyuridine, using a monospecific antibody coupled to a visualization system such as the peroxidase–antiperoxidase method [5].

5. Reduced biosynthetic activity. Alterations in the biosynthetic activity of a cell may be subtle and may or may not be of biological significance with reference to biocompatibility. However, it is possible that a medical device may not exert a negative influence on cell proliferation *in vitro*, but may radically reduce the production of certain biosynthetic products. This may take the form of structural proteins within the cell or a specific component designed as a secretory product of the cell. This topic is discussed more specifically below.

As well as the recognition of deleterious effects of biomaterials on cells in culture, it is possible to detect stimulatory influences *in vitro*. This is part of specific biocompatibility testing. Fig. 1 summarizes the interpretative spectrum encompassing the palette of methods used, such as those listed above. Thus, a marked reduction in cell proliferation, coupled with

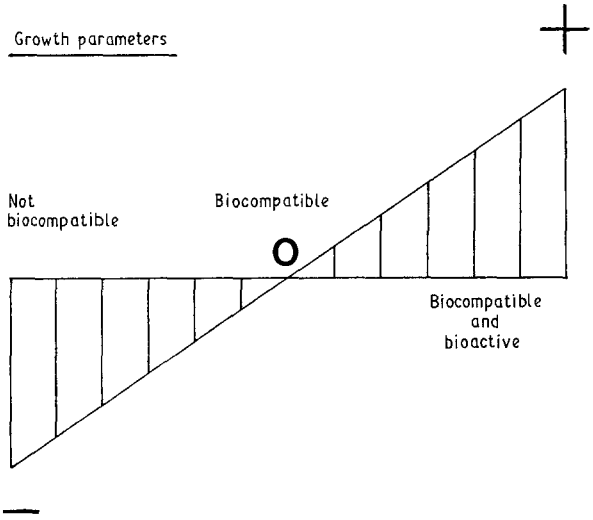

Figure 1 Spectrum of interpretation from studies of growth parameters *in vitro*.

inhibition of biosynthetic functions and especially cell death will place the tested medical device well towards the left, i.e. not biocompatible. Merely minimal alterations in cell proliferation and biosynthetic function with no evidence of cell death are regarded as acceptable and thus as biocompatible. If, however, certain parameters regarded as relevant and desirable for the cell types in contact with the medical device are stimulated, the biomaterial can be regarded not only as biocompatible, but as "bioactive". Useful parameters in this respect are cell proliferation, synthesis of specific cellular products and cell adhesion. An example of this is the increased adhesion of osteoblasts to a medical device designed for contact with bone. A specific form of bioactivity has been termed "biointegration" and involves incorporation of tissue into the medical device. Examples are the penetration of cells into or colonization of porous materials. This could take the form of osteoblast penetration of porous ceramics or myofibroblast colonization of the adventitial region of a porous vascular prosthesis. Thus, whereas biocompatibility involves a process of exclusion of negative effects of biomaterials in cells and tissues, and in the past has been solely concerned with this aspect, bioactivation and biointegration involve inclusion of positive or desirable stimulatory effects of the biomaterials and have to be considered in the new concept of biocompatibility.

In practice, one of the important problems is how to bring the biomaterials to be tested into contact with the chosen cell type. The latter is usually in the form of a single-cell suspension for monolayer culture or as an established monolayer. The available methods can be either direct or indirect. The direct (or contact) method is particularly useful for biomaterials, such as metals and polymers, which can be provided in foil form. This contact method is especially important in testing for potential biointegration, in which the cells must be allowed to interact directly with the (usually porous) material to be tested.

There are two important indirect methods for *in vitro* test systems. The first involves separating the

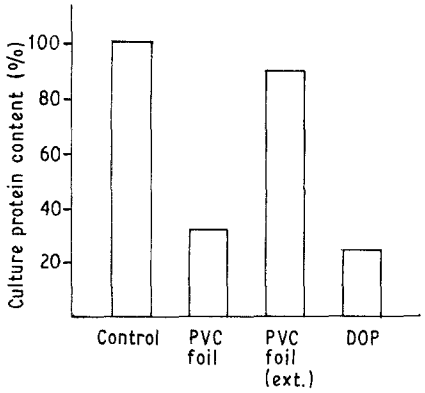

Figure 2 Culture protein content of HeLa cells grown on PVC foils with or without extraction, glass control surface or control surface in the presence of dioctylphthalate (DOP). Control surface set at 100%.

cells from the potential biomaterial, using a diffusion barrier. An example of this is given by Johnson *et al.* [6], who used cells in monolayer, covered by a thin layer of sterile agar, on which the biomaterial in foil form could be placed. Thus, soluble products can leach out of the biomaterial, diffuse across the agar barrier and influence the monolayer-cultured cells. The second form of indirect testing involves an extraction of the material. This may be performed in a Soxhlet apparatus, in which a solvent is distilled into a chamber containing the material to be extracted. We have found *n*-hexane to be an excellent solvent for use with polymers. The solvent can then be distilled off and the extract added to tissue culture medium. A further variation is to incubate the sterilized polymer with complete medium for various periods of time, e.g. 24 h, at 37° C. A fundamental principle of extraction methods in which various biomaterials are being compared is the use of identical surface areas of the material. It is evident that a thin foil of large surface area will permit a greater extraction of soluble components than a similar weight of the same material in spherical form.

Such extraction methods offer an excellent system to establish which components of a potential biomaterial may exert a toxic effect. Fig. 2 illustrates a typical experiment, in which a polyvinyl chloride (PVC) foil was tested by a direct contact testing method before and after extraction. The test parameter used was the total protein content of a cultured cell line (HeLa cells). Included in the experiment was the plasticizer, dioctylphthalate, used in the production process. The figure indicates that in this case the plasticizer was the component responsible for the inhibition of growth of the cells. Following a simple extraction step, the PVC foil gave merely 10% inhibition of protein synthesis, compared with the glass control surface.

3. Advantages and disadvantages of *in vitro* systems

Cell culture systems possess the great advantage of providing a rapid and cost-effective screening method for biomaterials. Coupled with this is the high sensitivity of the method, which enables potentially cytotoxic materials to be recognized and excluded at an early stage in the testing procedure. A further advantage is the easy evaluation of the growth parameters presented above, enabling multiple comparisons between various biomaterials with subsequent statistical evaluation.

At the top of the list of disadvantages of *in vitro* methods is the fundamental problem of extrapolation to the *in vivo* situation. This problem cannot be solved by any amount of philosophical discussion, and thus it should be stressed that *in vitro* testing represents only one phase in studying biocompatibility. These specimens classified as *in vitro* biocompatible must enter a further phase of testing, which requires *in vivo* observation. A second disadvantage is the problem of *in vitro*-specific sensitivity, which simply describes the phenomenon of deleterious effects *in vitro*, which do not occur in the intact organism. Expressed another way, *in vitro* test systems may lead to false negatives. We do not regard this disadvantage as serious, as it is unlikely that a material that causes rapid cell death in all cell types tested, both cell lines and primary isolated cells, will prove to be totally harmless *in vivo*. Thirdly, *in vitro* methods may be restricted by the choice of cell type. We regard this problem as more serious if only one cell line is used as the screening method. We favour the use of primary cultured epithelial and mesenchymal cells (see below), in addition to the use of a few established transformed cells in the form of cell lines.

A fourth disadvantage is the failure of *in vitro* methods to provide adequate information on biomaterial breakdown. These data can be collected only by studying host–biomaterial interactions *in vivo*. Nevertheless, it is possible to study limited, yet important, aspects of this matter by constructing simulation experiments *in vitro*. An example of such a study is given by Williams *et al.* [7], who devised experiments to test the ability of enzymes known to be produced *in vivo* to elicit polymer breakdown. ^{14}C-labelled polymers were treated with trypsin, chymotrypsin, papain or esterase. Albeit quantitatively small, radioactivity was released from the polymers, suggesting that the latter may be susceptible to enzymatic degradation. Undoubtedly, this aspect of biomaterial testing will gain in importance in the future.

4. Comparison of *in vivo* and *in vitro* systems

It is not the purpose of this paper to present a comprehensive review of studies *in vivo*, but rather to underline the reciprocal nature of both approaches and to highlight *in vivo*/*in vitro* correlations. Rice *et al.* [8] tested a variety of polymers in powdered form and used transformed mouse fibroblasts (L-929) as test system by estimating population doublings. These *in vitro* data were then compared with published data from various sources on *in vivo* toxicity. Although a few polymers which were classified as mildly toxic from *in vitro* studies showed moderate toxicity in rat intramuscular implantation studies, there was generally good agreement between *in vivo* and *in vitro* results.

In a detailed study Johnson *et al.* [6] investigated a

total of 20 test substances, both metals and polymers, on 12 permanent cell lines of fibroblastic or epithelial type. As well as a contact and agar diffusion method, they employed extracts of the materials at both low and high cell density. The value of this study lies not only in the spectrum of *in vitro* systems used, but also in the attempted correlation with *in vivo* data, obtained by implanting the materials for 5 days intramuscularly in the rabbit. This investigation indicated that of the *in vitro* methods used, the extraction method at low cell density represented the most sensitive method; that is, it gave the highest number of materials with growth inhibition. This may be explained by the fact that cells at low seeding density are particularly vulnerable, as the extent of cell–cell co-operation is at a minimum. In addition, Johnson *et al.* reported that the best correlation between *in vivo* and *in vitro* studies was provided by the agar diffusion method. This could be due to the more *in vivo*-like nature of this method, in which substances released by the potential biomaterial have to cross a diffusion barrier in order to exert their effect on the cells. This involves establishing a concentration gradient, such as could be expected in the *in vivo* situation. Later studies by Johnson *et al.* [9] compared cell lines with primary cell cultures, mostly of blood cells. They concluded that in material testing better reproducibility was provided by the established cell lines.

Burton *et al.* [10] described the use of bacterial bioluminescence, instead of cells, for acute toxicity testing of biomaterials. This method, based on the bioluminescence of certain bacteria, such as *Photobacterium phosphoreum*, in the presence of toxic compounds, was reported to be much more sensitive than a variety of *in vivo* tests, such as rabbit intramuscular implantation or systemic injection in the mouse.

The limitations of *in vitro* test systems are illustrated well by a more recent publication by Gross *et al.* [11], in which the performance of surface-reactive bone-bonding materials *in vitro* was compared with the *in vivo* situation. Using foetal rat osteoblast growth as *in vitro* parameter, they found that a bone-bonding ceramic was more inhibitory to growth *in vitro* than a non-bonding glass material, both materials being inhibitory when compared with an inert control substrate (Thermanox). However. morphological studies following implantation in the rat femur *in vivo* revealed excellent bone bonding for the ceramic, which according to the *in vitro* studies alone would have been classified as the less suitable material.

5. Guiding principles for specific biocompatibility testing *in vitro*

The following guidelines may be proposed for the use of *in vitro* methods in specific biocompatibility testing of medical devices.

5.1. The use of relevant cell type

Although it is clear from the literature that established cell lines provide an excellent screening method, which can be employed in even modestly equipped laboratories, the ideal situation is the use of human, non-transformed cells; that is, primary isolated cells used in early passage. We favour the use of both approaches and use established cell lines in a "phase 1" screening, in which new or modified polymers and other potential biomaterials are screened to exclude those giving marked inhibition of growth parameters. "Phase 2" screening follows, in which we employ human primary or early passage cells, chosen to be relevant to the purpose for which the potential biomaterial has been made (specific biocompatibility testing). Thus, an attempt is made to simulate the *in vivo* situation as closely as possible. This point is best illustrated by some examples. One of our endeavours is the development of new vascular prostheses, whose inner surface will promote cell attachment and growth [12]. In "phase 2" screening, use is made of endothelial cells, in this case the relevant cell type for the medical device. For the testing of dental materials, gingival fibroblasts and epithelial cells are suitable cell types. In studying biomaterials for implantation in bone, the cells of choice would be the osteoblast and fibroblast.

5.2. The use of relevant cell biological parameters

As is the case for choice of cell type, it is important to choose relevant parameters for the *in vitro* test method. Cell proliferation remains a mainstay in this respect. Cell adhesion is also of great importance and has been alluded to above. Reduced cell adhesion is desirable in haemocompatible surfaces. However, it is essential to distinguish between reduced cell adhesion in the course of a cytotoxic effect and reduced adhesion without cytotoxicity. The acid test here is the proof of cell vitality after exposure to the material and is best shown by the ability of the cell suspension to establish a monolayer culture following further passage (i.e. after removal from biomaterial contact).

In "phase 2" *in vitro* screening described above, specific gene products of the relevant cell type are particularly useful in monitoring the maintainance of cellular homeostasis in the presence of the medical device to be tested. Thus, in testing materials for orthopaedic application, in which contact with cartilage will occur, specific biosynthetic functions would include the production of type II collagen and proteoglycans. Returning to the testing of modified vascular prostheses, the endothelial production of antithrombotic agents such as prostacyclin [13], plasminogen activator [14] and thrombomodulin [15] could be used as relevant cell biological parameters.

6. Future perspectives

One of the very promising developments is the surface modification of biomaterials to promote desirable reactions, such as increased cell adhesion, or to prevent undesirable effects, such as the triggering of blood coagulation. An example of the latter is the attempt to make intravascular catheters more blood compatible by, for example, bonding heparin to the catheter surface [16]. *In vitro* methods provide an excellent tool to compare the efficacy of such modifications with the native (untreated) biomaterial. In this respect studies can be performed not only in static culture systems, but also under dynamic conditions.

Engbers *et al.* [17] coated catheters with a conjugate of heparin and albumin and were able to demonstrate that even incubation of the catheters with a solution of this conjugate enabled platelet adhesion in a subsequent assay to be reduced four- to five-fold. This assay took place under different wall shear rates in a perfusion system.

The modification of biomaterial surfaces adopted by Engbers *et al.* [17] represents a simple molecular adsorption on to the surface. This has also been used in attempts to improve endothelial cell adhesion to vascular grafts before implantation (so-called "preseeding"). Thus, conventional vascular graft materials such as polytetrafluoroethylene, treated with fibronectin and/or other components of the basement membrane, such as collagen, promoted a raised adhesion and/or growth of endothelial cells on the graft [18–22]. Other research groups have taken the surface modification procedure a step further. Thus, in the case of vascular prostheses, successful attempts have been made by our group to use spacer molecules to bind adhesion-promoting peptides covalently to a polyetherurethane surface [12]. Use has also been made of covalent bonding to immobilize heparin to the polyurethane of intravascular catheters [23, 24].

The increased sophistication of the methods being employed to modify the properties of biomaterials to "tailor" medical devices for specific purposes means that the use of a few easily obtained and easily handled cell lines is no longer acceptable in *in vitro* testing regimes. "Phase 2" screening methods are required to test specific cellular functions with the relevant cell types. In conclusion, it must be stressed that even the most elaborate and specific test systems *in vitro* do not obviate the need to perform subsequent tests in experimental models *in vivo*.

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