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Evaluation of the responses of human peritoneal macrophages to a range of biomaterials using reflectance light microscopy

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The local response to biomaterials consists of a balance between inflammatory and repairtive processes. Macrophages are one of the cell types involved in both these responses. Initially macrophage infiltration is necessary to phagocytose cellular and other debris which result from implantation of a device. Wound healing can then follow. If, however, phagocytosis cannot be completed the macrophages may become highly activated resulting in a chronic inflammatory response. In this paper we examine the activation of human peritoneal macrophages, cultured on a range of materials, by transmitted and reflected light microscopy. The cells were stained for non-specific esterase (NSE), a macrophage marker and indicator of activation. The results showed that cells adhered to all materials tested, except for polyvinyl chloride. Polypropylene caused the greatest degree of macrophage activation, while hydroxyapatite and polytetrafluoroethylene caused the least activation. Macrophages cultured with metal phagocytosed some of the material. We conclude that this technique gives a good indication of the activated state of these cells and can be used in the assessment of the biocompatibility of materials with respect to the immune system.

1. Introduction

Appropriate biocompatibility testing of biomaterials is an essential process in the development of implantable devices for use in medicine. A number of researchers have established *in vivo* test methods for assessing the biocompatibility of biomaterials. The difficulties encountered in *in vivo* testing are poor reliability, individual variability and ethical considerations. This has led researchers to develop *in vitro* techniques in an attempt to assess biocompatibility. *In vitro* testing, using cell culture techniques has a number of advantages, in that the response of a variety of cells can be compared and evaluated quickly in a reproducible and controlled manner. It also allows standardization of test methods and reduces the need for large numbers of experimental animals. One criteria for the biocompatibility of a material is that after implantation, it will not evoke an additional inflammatory response.

The macrophage is one of the most important cells of the immune system and its response may be elicited by implantation of a foreign material into the body [1, 2]. Activation of macrophages by bulk materials or particulate debris can result in phagocytosis or the release of a number of inflammatory mediators, cytokines, and lysosomal enzymes [3, 4]. To date, a number of researchers have studied the response to biomaterials, of macrophage cell lines and macrophages derived from animal sources [4]. The response of human peripheral blood macrophages have also been examined [5].

In this study we have used human macrophages from the peritoneal cavity of renal dialysis patients undergoing continuous ambulatory peritoneal dialysis (CAPD). Activated macrophages have distinct morphological features and can be recognized using microscopic techniques. One of the main problems with the evaluation of cellular response to biomaterials microscopically is that the majority are opaque and therefore adherent cells cannot be viewed by transmitted light microscopy. Scanning electron microscopy (SEM) can be used but this has a number of disadvantages: processing of the samples for SEM is not suitable for some materials, it is time consuming and detailed morphology and staining characteristics of the cells cannot be determined. In this study, we have used a mixture of both reflected and transmitted light microscopy with various stains, including non-specific esterase (NSE), to study macrophage responses to a range of materials.

2. Materials and methods

2.1. Biomaterials

The biomaterials tested were tricalcium phosphate (TCP), hydroxyapatite (HA), polyvinyl chloride (PVC), polytetrafluoroethylene (PTFE), polymethylmethacrylate (PMMA), polypropylene (PP), polyvinylidene flouride (PVDF), and ultra high molecular weight polyethylene (UHMW-PE) (Goodfellows), stainless steel, cobalt – chrome alloy, titanium alloy polished, titanium alloy – alumina blast, titanium

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alloy – HA coated, and titanium alloy – glass bead blast (kindly supplied by Orthodesign Ltd). The materials from Goodfellows were cut into squares measuring 10 mm × 10 mm, and the Orthodesign materials were supplied as 10 mm diameter discs. Thermanox discs were used as a control material.

2.2. Cell culture

Human peritoneal macrophages were obtained from the peritoneal washings of renal dialysis patients undergoing CAPD. The fluid was centrifuged at 1000 rpm for 5 min. The cells were washed in RPMI 1640 with 10% foetal calf serum, 100 U/ml penicillin, 100 mg/ml streptomycin and 2 mM L-glutamine (all GIBCO). The cells were plated on the materials at a density of 5×10^5/ml in 24-well tissue culture plates and incubated overnight at 37 °C in a humidified atmosphere of 5% CO_2. The medium was removed and the non-adherent cells washed off with phosphate-buffered saline (Sigma). 1 ml medium was added to each well and the plates incubated for a further 24 h.

2.3. Light microscopy

The cells were fixed in citrate:acetone:formaldehyde (25:56:8) before being stained for non-specific esterase (NSE) using an α-naphthyl acetate esterase staining kit (Sigma). The cells were viewed by a combination of transmitted and reflected light microscopy using

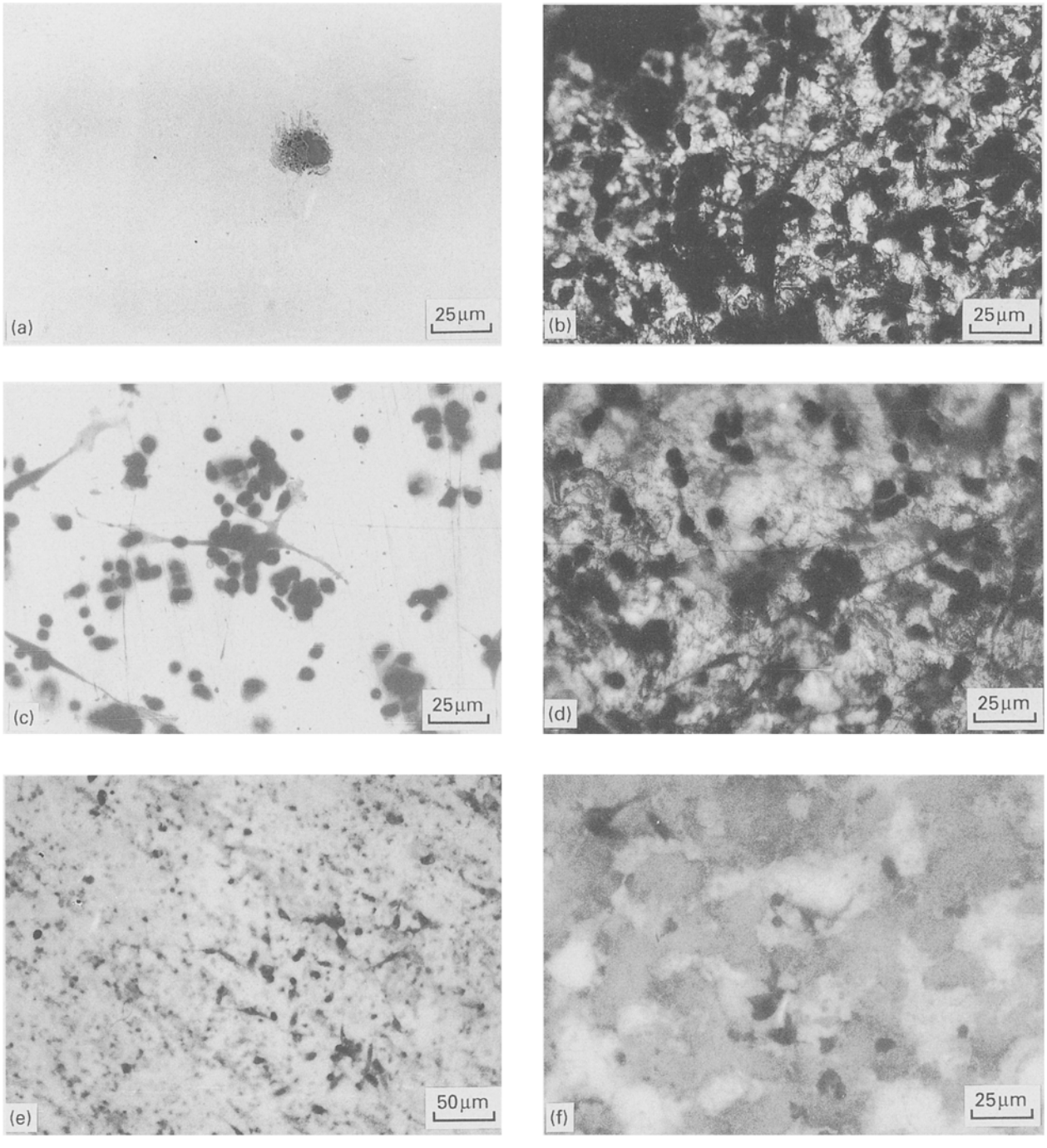

Figure 1 Micrographs showing macrophages cultured on (a) a thermanox disc, (b) polished titanium, (c) cobalt-chrome alloy, (d) stainless steel, (e) HA, (f) TCP.

a Zeiss microscope with a 35 mm camera attached. Representative fields were photographed.

3. Results

1.5 litres of CAPD fluid yielded $5–20 \times 10^6$ cells, 38–45% of which are macrophages as determined by NSE staining, the remainder appearing fibroblastic. Fig. 1a shows a macrophage cultured on a thermanox disc. It can be identified by its lobed nucleus, spread out appearance and black granules in the cytoplasm stained with NSE stain.

Macrophages adhered well to all materials, apart for PVC, where no cells were visible. More cells adhered to the metals and these cells were very dark in appearance. The HA-coated titanium absorbed the stain as did pure HA. The coating has a rough, undulating surface with the majority of cells located in its depressions. The morphology of cells on this material was similar to that on pure HA (Fig. 1e), but could not be photographed due to the roughness of the surface. The surfaces of alumina blast and glass bead blast titanium alloy were also rough and a large number of cells were seen in the indentations. Similarly, these could not be photographed. However, they had a normal morphology but were heavily stained, indicative of some degree of activation. A large number of cells adhered to polished titanium, cobalt–chrome alloy and stainless steel (Fig. 1b, c, d). They appeared both as clusters and individual

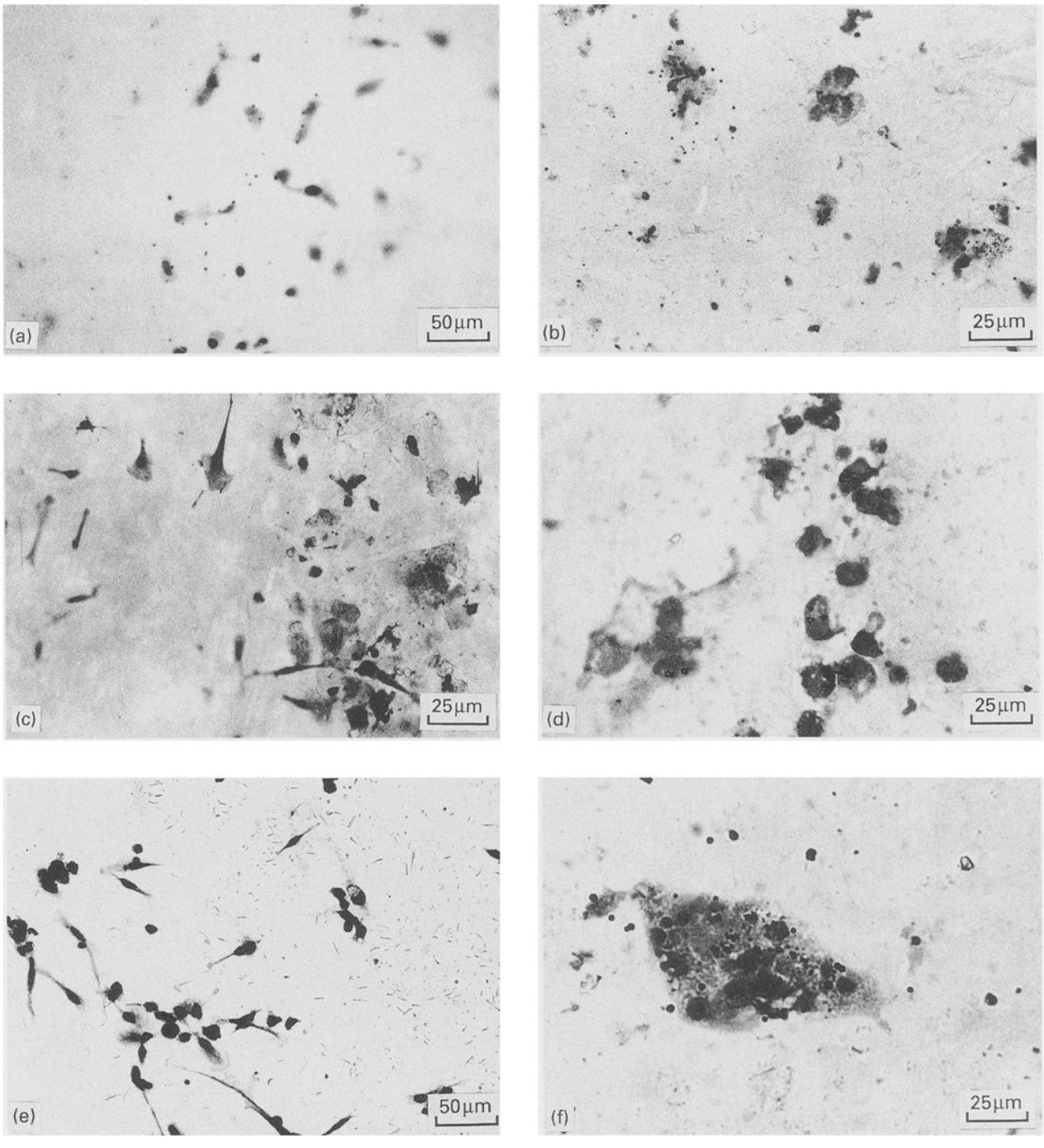

Figure 2 Micrographs showing macrophages cultured on (a) PTFE, (b) PVDF, (c) UHMW-PE, (d) PMMA, (e) PP. (f) A foreign body giant cell on PP.

cells, with normal size and shape. Apart from staining heavily no other signs of activation were evident. Cells on stainless steel showed a preference for the edge of the material where they formed clusters. The cells in the centre adhered singly. Cells could be seen on the metals only when using reflected light. Similarly, reflected light was also required to view cells cultured on HA and TCP (Fig. 1e and f, respectively). Both these materials stained heavily. The cells appeared very dark and granular, indicative of activation.

With the more translucent materials it was necessary to use both reflected and transmitted light in order to view the cells clearly. Likewise, a small amount of reflected light aided in visualizing cells on thick transparent materials. Relatively few cells adhered to PTFE and those that did showed little sign of activation (Fig. 2a). Few cells were visible on PVDF and UHMW-PE and those that were present were found in clusters (Fig. 2b and c). Cells on PVDF appeared to be slightly fragmented and to have jagged edges, which may have been due to activation of the cells. A large amount of peritoneal proteins and debris adhered to this material. Cells on PMMA had a similar appearance to those on PVDF, i.e. fragmented and jagged (Fig. 2d). A higher proportion of the cells on this material were identified as macrophages. Cells cultured on PP were found individually and in close clusters (Fig. 2e and f). Some may have aggregated to form foreign body giant cells (Fig. 2f). The cells were highly activated as seen by the number of darkly stained granules in the cytoplasm.

4. Discussion

Macrophages have been implicated in aseptic loosening which is a common cause of joint replacement failure. The membrane surrounding stable total hip replacements are characterized by the presence of a large population of fibroblasts with relatively few macrophages whereas in the membrane around loose total hip replacements a greater proportion of macrophages were observed [6]. Animal studies have highlighted the presence of inflammatory cells, including macrophages, surrounding implants of PMMA bone cement [7], titanium [8], polyglactin and dacron [9]. Foreign body giant cells, which are formed by the fusion of macrophages if a foreign material is too large for phagocytosis, were also observed in the tissue surrounding some implanted biomaterials [10]. They have increased phagocytic ability and are characteristic of a chronic inflammatory reaction. The mechanism by which both these cell types affect the stability of a prosthesis is by secretion of a large number of different substances – cytokines, inflammatory mediators, growth factors – which can either stimulate or inhibit bone resorption [11]. The presence of a foreign material increases secretion of stimulatory substances, thereby increasing bone resorption, and may result in implant failure. Therefore assessing the reaction of these cells to biomaterials is a very important aspect of biocompatibility testing. The necessity for *in vitro* testing has been thoroughly discussed [12]. Assessing morphological changes is an important initial step in this procedure as morphological changes are indicative of the activation state of the cell. Reflected light microscopy is a very useful technique which can be used for this purpose. More extensive biochemical tests may also be carried out, i.e. testing for production of cytokines; IL-1, TNF; and lysosomal enzymes.

SEM was used by Miller [5] to analyse macrophage activation following attachment to PE and PTFE and similar results to those found in this study were reported. The use of SEM, however, does not allow nuclear and other intracellular structures to be viewed. Here we have assessed macrophage activation microscopically by using staining techniques. Increased phagocytic activity results in the uptake of a larger quantity of dye, thus making activated macrophages appear more heavily stained. This was seen in cell cultured on certain materials. The blackened cytoplasm of the cells cultured on metals may be due to phagocytosis of the metal by the cells. Macrophages have been reported to phagocytose cobalt–chrome [4] and titanium [4, 8]. This reaction to bulk material could only be seen *in vitro* using transmitted light microscopy. It was found that by combining reflected and transmitted light clearer images were obtained on translucent and some transparent materials. This was particularly true with thicker samples. This enabled us to photograph cells on a wide range of materials and make an initial assessment of their degree of activation. Further assessment will be made biochemically by measuring cytokine and lysosomal enzyme release.

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