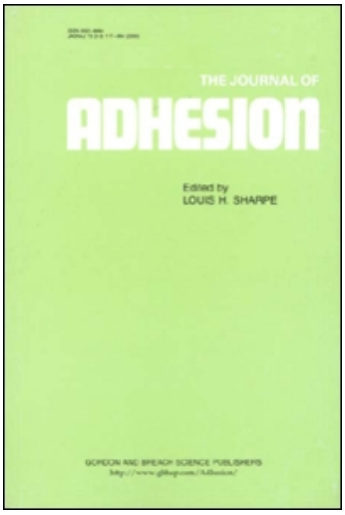

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Cell Adhesion and Interactions with Biomaterials

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Cell Adhesion and Interactions with Biomaterials

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The related phenomena of biocompatibility and biodegradation are of major concern in the selection of synthetic polymers for use as implantable biomedical devices. These properties are largely determined by the consequences of the cellular interactions that occur at the polymer/tissue interface during inflammation. Using an *in vivo* cage implant system, in conjunction with surface analysis techniques, we have investigated the variation in the cellular events that occurred on a polyetherurethaneurea and a cytotoxic poly(vinyl chloride). Quantitative and qualitative information that describe the cellular response to polymer implantation will be presented. From the results, a chronological sequence has been established which suggests that the important events follow cellular adhesion, and include cell spreading over a polymer surface accompanied by lysosomal degranulation of the adherent cells.

KEY WORDS Biomedical polymers; soft tissue implants; cellular adhesion; biocompatibility; inflammatory response to polyetherurethaneurea; cytotoxicity of poly(vinyl chloride).

INTRODUCTION

The use of synthetic materials for biomedical devices has increased considerably in recent years. The principal reason for this proliferation has been the increasing availability of materials, particularly polymers, that possess the appropriate mechanical and physical properties. A wide range of polymeric materials have been pro-

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posed, tested and actually used for biomedical applications.[1] These materials range from delicate hydrogels for soft contact lenses to tough, high density polyethylene for components of joint prostheses. Recently, attempts have been made to design materials specifically for intended clinical applications, although progress remains inhibited by the lack of understanding of the complex biological reactions that can occur with foreign body implants.

The fundamental objective in the analysis of a material's biocompatibility is an appreciation of the relationship between the implanted material and the host. This generally is not an easy task, as the physical, morphological and chemical properties of a material may be involved in provoking one or more biological reactions.

Factors related to the biological response that have found a consensus among researchers state that a biomaterial should be nontoxic, noninflammatory, noncarcinogenic and should not induce immunologic responses or have any detrimental systemic effects.[2,3] An important aspect of the biological criteria is the possibility that a biomaterial may contain extractable additives. Typical additives include stabilizers, antioxidants, plasticizers and perhaps some unreacted monomer. Since the migration of extractable components to the adjacent tissue may promote one of the undesirable reactions, it appears essential that biomaterials should be as "pure" as possible. Many materials intended for use are required to be biologically resistant for long periods and consequently, the possibility of environmental degradation has to be considered.

The *in vivo* tissue compatibility of materials has been the subject of numerous investigations.[4–7] Most studies are based on histologic observations in the area adjacent to the implant. The biocompatibility of a given material with tissue is then described in terms of the acute and chronic inflammatory responses and the fibrous capsule formation. The degree and duration of the response to a subcutaneously or intramuscularly implanted material is then correlated to a qualitative assessment of compatibility.

The implantation of any foreign material in soft tissue initiates an inflammatory response. The cellular intensity and duration of the response is controlled by a variety of mediators and determined by the size and nature of the implanted material, site of implantation and reactive capacity of the host. The response is characterized by an acute phase in which polymorphonuclear leukocytes (PMNs) predominate, and followed by a chronic phase in which mono-

nuclear cells (macrophages and lymphocytes) predominate. A chronic inflammatory response implies the continued presence of the injurious agent, which may be represented even by a relatively inert biomaterial. Factors which can provoke greater levels of chronic inflammation include: an implant that causes physical irritation to neighboring tissues, extensive surgical injury, bacterial infection or host factors such as poor blood supply or nutrition. During the chronic phase, fibrous tissue begins to form around the mass of leukocytes (white cells). Eventually, this process encapsulates the implanted material in a dense capsule of connective tissue known as a foreign body granuloma, the objective being to isolate the implant, and therefore its effect on the host, from the rest of the body. Accumulation of blood pigments, lipids and calcium salts is often observed within the granuloma and is indicative of a continuing chronic inflammatory reaction.

While the general pathologic sequence of inflammation is well-known, it is the intensity and duration of the reaction that will vary according to the nature of the implant. Typical results of tissue compatibility studies have generally shown that polyetherurethaneureas like the clinically used Biomer do not provoke an intense host reaction,[8-9] and appear suitable for short-term and possibly long-term biomedical applications.[10] The study of Pollock and coworkers[9] showed that porous Biomer is well tolerated when intramuscularly implanted in the rat for up to 3 months. Tissue ingrowth appeared to be a function of porosity; a pore size of greater than 15 microns was required to permit ingrowth. In a comparative study of tissue ingrowth in porous vascular graft materials, Hiratzka and coworkers[11] found large areas of granulomatous inflammation in response to polyurethane grafts compared to the minimal response to Silastic grafts. Some fragmentation of the polyurethane and fat deposits were also observed at 32 weeks, although little difference was observed in the development of the respective pseudoneointimas. A similar granulomatous inflammatory response also has been reported with clinical breast prostheses.[12] Discoloration and some fragmentation of the prostheses were observed, which suggests a degradation phenomenon may have stimulated the inflammatory reactions. It should be noted, however, that no descriptive information of the type of polyurethane used was provided in the study of Cocke, *et al.*[12]

The ultimate safety of materials used for biomedical applications

depends upon its compatibility with the biological system. Toxicological testing has generated much information on the relative toxicity of a wide range of chemicals that is a considerable resource in the analysis of biomaterial toxicity. However, the toxicological testing of potential biomaterials has unique constraints that limit the applicability of classical protocols. The fundamental problem in the application of these protocols is that a synthetic material is a complex entity, the toxicity of which is mediated by both the physical and chemical properties of the material. The approach to such evaluation of biomaterial toxicity is therefore often highly empirical.

Our approach to the investigation of cellular biocompatibility has been to utilize a novel implant system[13,14] that enables quantitative as well as qualitative evaluation of the inflammatory response to implanted materials. This system can be used to determine the cellular and enzymic components of inflammation and permits access to study the adhesion behavior of leukocytes with a biomaterial surface. The various studies that can be performed are shown in Figure 1. From a materials perspective, it can be seen that

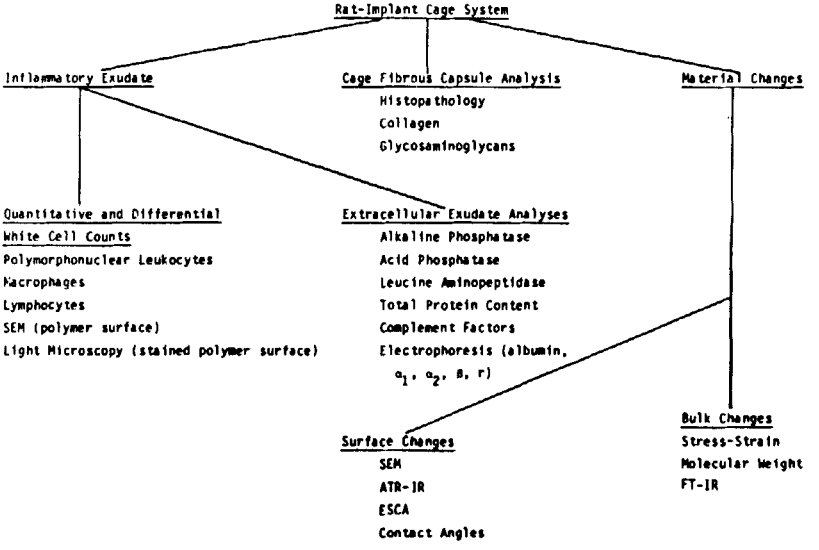

FIGURE 1 Experimental approach to the study of biocompatibility and biodegradation phenomena.

microscopic and spectroscopic techniques such as SEM, ATR-FT-IR and ESCA can be, and have been, employed to investigate surface changes and possible degradation effects.[15] This report concentrates on the area of inflammatory cell adhesion and interactions with two contrasting polymeric materials: Biomer, which is a clinically used polyetherurethaneurea, and a cytotoxic poly(vinyl chloride) (PVC).

EXPERIMENTAL

Films of Biomer (0.014 cm thick) were obtained from Dr. B. D. Ratner of the University of Washington, Seattle. In a dust-free class 100 laminar flow hood, a 20 wt% Biomer/dimethylacetamide (DMAC) solution was used to cast films on chromic cleaned glass. Films were air dried for 2 weeks and then under vacuum at less than 1 mm Hg for 24 h. On receipt, film samples were sonicated in double-distilled water for 30 min, dried to constant weight and stored in a vacuum desiccator. Immediately before implantation, polymer films are steam sterilized at 120°C for 15 min. ATR-FT-IR and XPS studies[14,15] indicated the absence of any residual solvent in the films, and that the cleaning and sterilization procedures have little effect on polymer surface composition. Preliminary SEM studies showed that the substrate surface (that surface cast against glass) was smooth on the submicron level, while the air-dried surface was smooth to the micron level.

Films of cytotoxic PVC (0.05 cm thick) were obtained from Dr. E. O. Dillingham of the University of Tennessee, Memphis. PVC sheet was prepared using a dryblend that was compounded and pelletized using a twin-screw extruder and then a single screw extruder for preparing sheets. Geon-103 EP PVC, diethylexylthalate, stearic acid and Thermolite-31 (dioctyltinbisoctylmercaptoacetate) were used in the dryblend formulation. Previous studies at the University of Tennessee, using a comprehensive *in vitro* toxicity screen,[2] have shown that the organotin stabilizer is principally responsible for the material's high toxicity. The PVC contained approximately 1.8% by weight of the cytotoxic agent.

The cage implant system has been described in detail.[13] Briefly, the system involved the subcutaneous implantation of stainless steel

wire mesh cylinders, 3.5 cm in length and 1 cm in diameter. Each implant contained a film sample of either Biomer or PVC measuring 1.6 cm by 0.5 cm. Each animal received one implant. Polymer samples were retrieved for surface analysis at 4, 7 and 21 days after implantation.

The procedures for the determination and evaluation of cells in the inflammatory exudate and on the polymer surfaces have been described.[14] The analysis included manual determination of the cell concentration in the exudate using a hemacytometer with differential cell counts from Wright's stained and non-specific esterase stained microscope slide preparations, and determination of the population density and differential analysis of cells adherent to polymer samples from microscopic examination of Wright's stained and acid phosphatase stained samples. Morphologic evaluation of adherent cells was carried out using scanning electron microscopy of samples which had been fixed in glutaraldehyde and critical point dried.

RESULTS AND DISCUSSION

One of the most important factors in determining the biocompatibility of biomedical polymers with tissue is the consequence of the cellular interactions that occur at the material interface. The use of the cage implant system containing polymer specimens is based on the concept that inflammnatory cells in the exudate can interact with the polymer or agents released from the polymer. These interactions may then modify the inflammatory response and the extent of the perturbation correlated to the cellular biocompatibility of the implanted polymer. In studies where the intensity of the cellular response is quantified, the results may be statistically compared to a control system that does not contain any polymer sample.[13] Thus, the trauma of implantation provokes the initial inflammatory response, which is then modified by the presence of the cage and the polymer.

After the stimulus of implantation, inflammatory cells actively migrate from the vasculature in response to chemical signals (chemotaxins) at the source of injury. The cellular migration is accompanied by the transport of protein-rich fluid or exudate. Once

TABLE I
The leukocyte concentration and differential counts in the exudates around biomer and cytotoxic PVC with implantation time

Implantation time	Biomer			Poly(vinyl chloride)		
	4	7	21	4	7	21
Cell Counts:						
Total leukocytes/μL	5310 ± 1560	1070 ± 130	300 ± 90	5800 ± 1850	8940 ± 1620	12300 ± 3400
PMNs (%)	76.7 ± 6.0	39.5 ± 10.9	3.6 ± 2.6	88.1 ± 6.4	87.0 ± 6.2	44.4 ± 12.3
Macrophages (%)	10.2 ± 2.7	29.5 ± 6.7	30.9 ± 5.0	10.3 ± 6.6	9.1 ± 4.8	53.2 ± 12.0
Lymphocytes (%)	13.1 ± 4.8	30.9 ± 9.2	65.5 ± 2.8	1.9 ± 1.2	3.9 ± 2.5	2.4 ± 1.1

Mean value ± standard deviation. $n = 5$ for all values.
Leukocytes (white cells) = PMNs + Macrophages + Lymphocytes.

at the wound site, phagocytic cells (PMNs and macrophages) attempt to clear away and digest any soluble and particulate matter which may include implant materials, tissue debris, bacteria, fibrin and erythrocytes, so that the healing phase can commence.

Over the 21-day implantation period which we have investigated, the intensity of inflammation will either decrease or increase depending on the implanted material. Table I compares the results for Biomer and the cytotoxic PVC. The table shows that the total leukocyte concentration in exudate around Biomer decreased over the implantation period, while the corresponding level of cellular intensity increased in the PVC wound area. The respective total leukocyte concentrations are a manifestation of cumulative cellular and biochemical events occurring in response to the implanted material which may be described as a measure of biocompatibility.

PMNs are indicative of the acute phase response that should be resolving in favor of a low intensity chronic phase dominated by macrophages and lymphocytes. This type of behavior is shown for Biomer in Table I. The percent PMNs steadily decrease with implantation time and by 21 days, the concentration is negligible. Macrophages and lymphocytes make up the remaining small concentration of leukocytes. In the case of the PVC two concurrent events are indicated. The acute phase is sustained at a very high level throughout the implantation period, and the macrophage concentration steadily increases. The acute phase has not resolved and an intense chronic phase also has been established. This is a clear indication of continued cellular migration from the vasculature

as PMNs normally have only a short lifetime (48 hrs). By itself, this result classifies the PVC as having a high toxicity level.

Each leukocyte, particularly macrophages, has the ability to release mediators which can modify the response. The release of interleukin I from macrophages promotes fibroblast proliferation with subsequent connective tissue synthesis and healing. The release of other mediators such as complement factors will lead to the generation of chemotactic signals for cellular migration from the cell reservoir of the circulation. Any cytotoxic agent in the polymer implant will prematurely lyse the leukocytes automatically leading to the extracellular release of mediators that provoke further inflammation.

The interactions between the cells and the implanted material or agents released from the material provides the focal point for directing the level and duration of the response. Figure 2a shows a low magnification SEM illustrating the adhesion of numerous cells to the Biomer surface at 4 days after implantation. The surfaces contained areas of high and low cell population density including an occasional aggregate of cells.

The Wright stained polymer samples were used in determining the number and type of adhering cells as well as an appreciation of their morphologic condition. An example is shown in Figure 2b. The results of the quantitative analysis are given in Table II. The table shows that the number of adhering cells decreases with implantation time, although at a much slower rate than the corresponding decrease in the exudate. The reasons for this may be

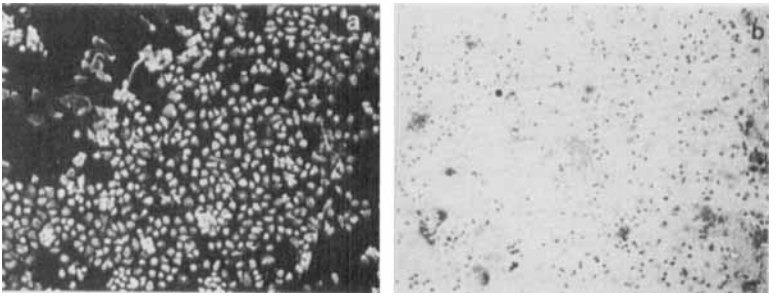

FIGURE 2a–b Low magnification of cellular adhesion to Biomer implants after 4 days implantation. (a) SEM, original magnification: 200×; (b) Optical micrograph (Wright's stain), original magnification: 100×.

TABLE II
The variation in the leukocyte population density, differential counts and intracellular acid phosphatase staining on biomer surfaces with implantation time

Implantation Time (days)	4	7	21
Cell counts:			
Total leukocytes/mm^2	155 ± 45	88 ± 31	27 ± 11
Single cells/mm^2	152 ± 45	84 ± 31	25 ± 10
FBGCs/mm^2	3.0 ± 1.5	3.9 ± 0.5	2.7 ± 1.9
PMNs (%)	24.2 ± 11.4	0.4 ± 0.4	0.1 ± 0.2
Macrophages (%)	73.4 ± 10.9	95.0 ± 1.6	89.7 ± 5.8
FBGCs (%)	2.4 ± 2.3	4.6 ± 7.6	10.2 ± 5.7
Acid phosphatase staining:			
Exudate (% + ve)	74.4 ± 9.8	77.3 ± 7.1	72.0 ± 7.0
Biomer surface (% + ve)	79.4 ± 1.4	74.8 ± 4.8	48.8 ± 4.0

Mean value ± standard deviation. $n = 5$ for all values.

found in the differential analysis of the cells. Comparing the percent PMNs and macrophages on the Biomer surface to the exudate values shows marked differences. On the polymer surface the macrophage dominates even at 4 days, while in the exudate the PMN is the predominant cell type. These results suggest that the macrophage preferentially adheres to the Biomer surface compared to either PMNs or lymphocytes. The preferential adherence of macrophages to an implanted synthetic polymer also has been observed by Matlaga and Salthouse using TEM techniques.[16] The slower rate of cell disappearance from the surface compared to the exudate was therefore a result of the relatively smaller percent of PMNs on the surface.

No data are presented for cells adhering to the PVC for the simple reason that a negligible number of cells were observed. The few cells that were observed demonstrated disrupted morphology and topography, which suggested that the cells were no longer viable. An example of such a cell is shown in Figure 3. The release of the cytotoxic agent from the polymer inhibited cellular attachment.

In the absence of any released cytotoxic agent, what factors determine a cell's ability to attach to an implanted synthetic polymer? Certainly, the polymer's surface properties, such as surface charge and wettability, are probably influential in determin-

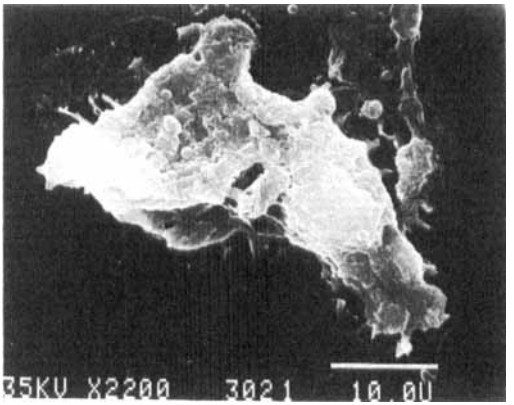

FIGURE 3 SEM, original magnification 2200×. The figure illustrates the typical disrupted morphologic conditions of the rare leukocyte on the PVC surface.

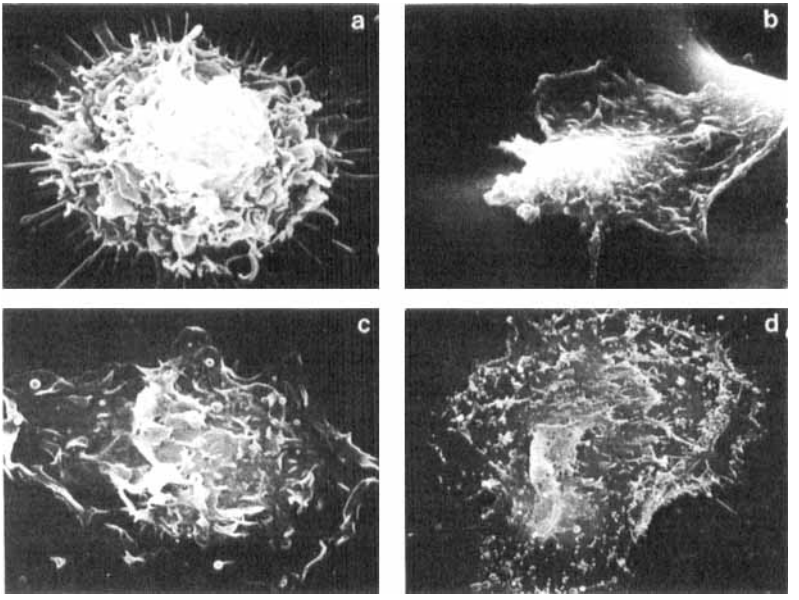

FIGURE 4a–d The phenomena of cellular attachment and spreading. (a) SEM, original magnification: 7200×, the cell has attained secured attachment to the surface; (b) SEM, original magnification: 2800×, cytoplasmic spreading of the cell over the surface; (c) SEM, original magnification: 5400×, further spreading to the point where the nucleus also has flattened; (d) SEM, original magnification: 2400×, cellular disintegration.

ing cellular adhesion.[17] However, the surface properties also will affect the nature of the modification of the surface by adsorbed proteins, which, in turn, will affect cellular attachment. Phagocytic cells can attach to polymer surfaces in the absence of pre-adsorbed protein, but certain proteins such as the complement factor C3b will provide a recognizable receptor for the incoming cell and increase the adhesion probability. It is this aspect of the uncertain role of the different protein intermediaries that make a quantitative analysis of cellular adhesion and accurate predictions of compatibility very difficult *in vitro,* and often confusing *in vivo.*

During the initial interaction between a leukocyte and the polymer surface, the cell may or may not attach. The situation of secured attachment is illustrated in Figure 4a. This cell is typical of many that were observed on 4 day Biomer implants. The cell topography includes cytoplasmic processes (filopodia), membrane ruffles and a little cytoplasmic spreading. The filopodial extensions provide points of attachment or anchor sites for the cell. This mechanism increases the cell's probability of attachment to a surface, because the initial points of direct interaction are minimized. The membrane ruffles are indicative of cellular activation, and enable a leukocyte to increase its surface area and therefore the chance of interacting with foreign matter or wound debris.

For the Biomer implants, cellular migration to the wound site was significantly diminished from 4 days. This enabled the chronolkogical sequence of cellular events to be documented, without the complication of large numbers of newly arrived cells. The changes that occurred to the cells on the Biomer surface from 4 to 7 days were considerable with a much higher proportion of the adherent cells having extensive cytoplasmic spreading. The phenomenon of cell spreading across the polymer surface is illustrated by Figures 4a–d. With time, the adherent cells spread their cytoplasm over the foreign body surface in an attempt to engulf the material, so that digestion might be achieved more easily.

The polymer surface is infinitely large compared to an adherent cell which makes the task of engulfment virtually impossible. A mechanism by which macrophages can increase their coverage of the polymer is to form multinucleated foreign body giant cells (FBGC). These cells are generated through the cytoplasmic fusion of two or more macrophages after they have simultaneously

attempted to phagocytize (digest) the same particle,[18] a polymer implant for example. It appears inevitable that FBGCs will eventually form on the surface of any large noncytotoxic biomaterial. On Biomer implants, FBGCs have been observed as early as 4 days after implantation. No giant cells were observed on any of the PVC implants, because adhering cells were lysed before they had sufficient time to fuse. Table II shows that the number of FBGCs remains relatively constant at each time period. With implantation time more adhering cells are incorporated into each giant cell, so that by 21 days giant cells having more than 50 nuclei were commonly observed. FBGCs represented over 10% of the adhering cell population by the final observation period. Examples of FBGCs are illustrated in Figures 5a–b. Our observations suggested that giant cells pass through the same morphologic and cytochemical phases as single cells which ultimately leads to cellular disintegration.

In wound healing the phagocytic cells will use their considerable enzyme arsenal to digest engulfed particles. Many of these lysosomal enzymes have broad specificity and normally are effective in degrading most ingested particles. The enzymes are stored in granules and then released into intracellular zones (phagolysosomes) around ingested material. Acid phosphatase is an abundant lysosomal enzyme which can be utilized as a cytochemical marker. Figure 6a shows an activated macrophage that was observed in the Biomer exudate at 4 days. The cell shows intense positive staining with numerous stained granules. On the polymer surface macro-

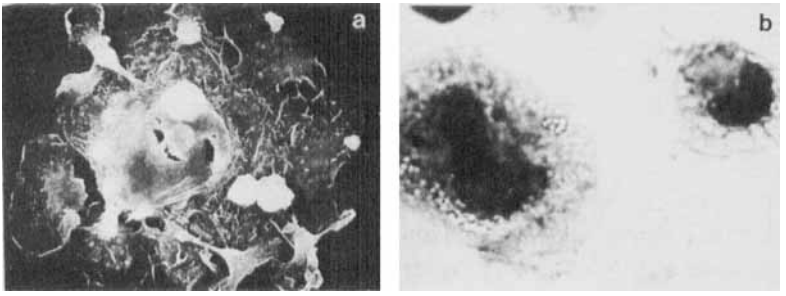

FIGURE 5a–b Foreign body giant cells on the implanted Biomer surface. (a) SEM original magnification: 780×; (b) optical micrograph (Wright's stain) original magnification: 250×. A binuclear FBGC.

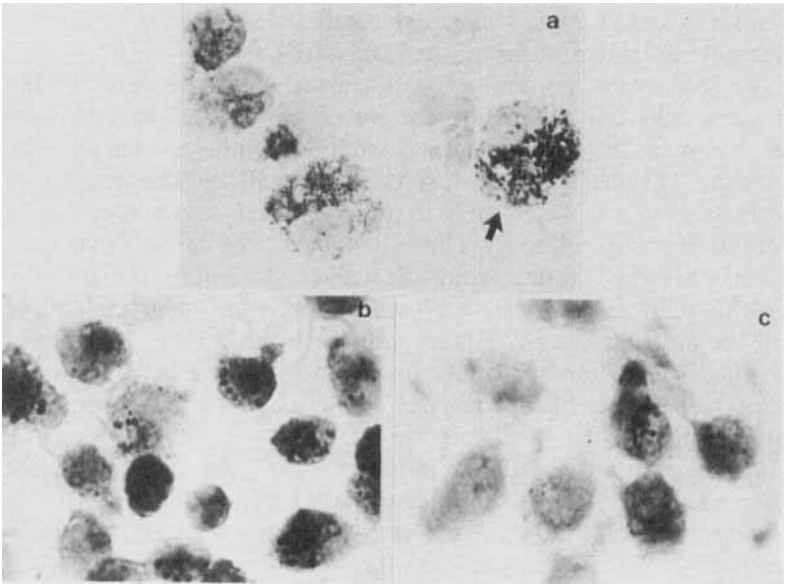

FIGURE 6a–c Optical micrograph (acid phosphatase stain). (a) Original magnification: 500×, demonstrates the intense staining behavior observed in exudate macrophages (arrow); (b) original magnification: 500×, at 4 days implantation time, numerous positively stained cells on the Biomer surface; (c) original magnification: 500×, at 7 days implantation time, much more diffuse and weak staining characteristics in the adherent cells.

phages rarely achieved such staining intensity because the cell tends to release enzymes extracellularly. The extracellular release of lysosomal enzymes is a consequence of incomplete cytoplasmic fusion, since the implant material is never completely engulfed by the cells. The result of this process is illustrated by comparing Figures 6b and 6c. The macrophages in Figure 6b were observed at 4 days after implantation and show positive staining characteristics. By 7 days (Fig. 6b) however, the adherent macrophages show only weak, if any, positive staining behavior.

The staining behavior of cells in the exudate compared to cells on the polymer surface is shown quantitatively in Table II. The percentage of cells demonstrating positive staining remains relatively constant in the exudate where most of the non-staining cells are lymphocytes and PMNs. On the surface, the percentage of positively stained cells decreases with time and markedly so

between 7 and 21 days. Non-staining cells on the surface at the two later time points are macrophages and FBGCs.

The observed morphologic and cytochemical events of the adhering cells can be directly correlated. As the cell spreads over the polymer surface, it unsuccessfully attempts to engulf the polymer. The cell's failure results in the extracellular release of enzymes that manifests itself in the observed degranulation and reduced staining behavior. The reduction in the intracellular content of enzymes will ultimately affect the cell's ability to digest any engulfed matter. Thus, the cell loses its phagocytic abilities. If these cells are not replaced by cells recently arrived from the vasculature, then the whole process of inflammation will resolve in favor of the connective tissue healing phase. This sequence of events was observed for the Biomer implants.

In the case of the PVC implants, few adherent cells were observed. Here, most of the reaction to the implant occurred in the surrounding exudate where leukocytes experienced the cytotoxic effects of the released additive. Cell lysis, followed by the release of inflammatory mediators caused further inflammation. The whole process of inflammation was intensified and prolonged with a concomitant inhibition of connective tissue wound healing.

Clearly, the reaction to the PVC represents an extreme example of what can occur if an inappropriate biomaterial is utilized and illustrates the potential damaging effect of leachable additives. Ideally, a biocompatible material is one that does not initiate any cellular or biochemical reactions that are detrimental to the host. Such a benign response is unlikely to be achieved with any synthetic material. Consequently, biocompatibility has come to mean minimal host response and is represented in soft tissue by a characterization of the foreign body reaction. The cellular events described for Biomer will, in general, apply to other nontoxic materials, but may vary in degree and time dependence. The physical and chemical properties of a biomaterial will affect the intensity and duration of the response, including the nature of the cellular interactions, the formation of giant cells and the fibrous capsule. A greater understanding of the cell and molecular interactions will eventually lead to an appreciation of the phenomena which ultimately determine the relative biocompatibility of a material.

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