Macrophage subpopulation differentiation by stimulation with biomaterials

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Abstract: Macrophages were elicited by the subcutaneous implantation of ultra high molecular weight polyethylene (UHMWPE) for periods of 2, 7, and 14 days in rats. Exudates of varying volumes were produced that was comprised of granulocytes, monocytes, immature and mature macrophages, and T-lymphocytes. No B-lymphocytes were observed at any time periods. Cell types were identified by their granularity and positivity to the following antibodies: leucocyte common antigen (LCA, pan leucocyte); CD11b/c (macrophage/monocyte); CD5 (T-lymphocyte); CD45RA (B-lymphocyte); HIS48 (granulocyte); ED2 (mature macrophage); and MCP-1 (monocyte chemoattractant protein 1). Monocytes isolated from control rat blood demonstrated a size slightly larger than that of granulocytes but with less granularity. Their size and granularity were followed over increasing time periods. The macrophages elicited by UHMWPE showed a similar pattern, with the exception of an apparently highly granular subpopulation with volumes similar to that of granulocytes but significantly more granular. The granular macrophage subset had a very high degree of ED2 and MCP-1 positivity, and their proportion, compared with other macrophages, was greatest at 2 days. The high MCP-1 expression was accounted for by MCP-1 molecules bound to the surface of a small proportion of macrophages that were activated. It is postulated that this subpopulation was responsible for the synthesis of the MCP-1 and could indicate a mechanism by which monocytes are attracted to the site of an implanted material. © 1997 John Wiley & Sons, Inc. *J Biomed Mater Res,* **37,** 481–488, 1997.

Key words: MCP-1; biomaterials; flow cytometry; macrophages

INTRODUCTION

Characterization of the host response to implanted biomaterials can be achieved in a number of ways. Histological methods usually are preferred since advances in histochemical and immunocytochemical stains allow information to be gained about both the morphological and the biochemical status of the tissue.[1–3] We recently have shown[4,5] that flow cytometry may add an additional dimension to host response analysis since it may be possible to harvest the exudate that forms in response to implantation and characterize the cells it contains. In particular, the changing numbers of lymphocytes, granulocytes, and macrophages may be observed over different time periods and with different materials. More important, it is possible to probe the receptor expression of these cells because the presence of subpopulations of different cell types is likely to be as significant as the general classes of cells. It is only recently that the possible role of cell subpopulations in the host response to biomaterials, particularly in the rat model, has been explored.[6]

Macrophages are derived from monocytes in the blood stream. They are elicited at the site of infection or trauma and have a variety of different functions once they arrive.[7–9] There are various functional subpopulations of macrophages,[10–12] and it is possible that they exhibit a differential functionality with respect to their response to biomaterials. In the context of inflammation due to lung injury induced in rats, it has been shown that different subpopulations of macrophages are responsible for initial monocyte infiltration compared to subsequent macrophage accumulation.[13] Additionally, monocyte chemoattractant protein 1 (MCP-1), a chemotactic factor specific for monocytes,[14,15] was shown to have an involvement in this infiltration.[13] The mechanism of monocyte infiltration due to the presence of polymeric biomaterials, however, has not been studied. The synthesis and release of MCP-1 has been observed in a variety of different cell types in humans, baboons, rabbits, rats, and mice (endothelial cells,[16] fibroblasts,[17] macrophages,[18] lymphocytes,[19] smooth muscle cells,[20] and

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epithelial cells,[21] but not neutrophils[22]) and has been implicated in a variety of immunological and inflammatory processes.

It is a widely held belief that the cellular response to the main groups of biomaterials is relatively nonspecific; that is, the presence of cells at the implantation site, and their individual characteristics, is not purely dependent upon the exact type of material. Thus in the aftermath of implantation surgery, the number and type of inflammatory cells often are more related to surgical trauma and other factors than to the material, especially at early time periods.[23,24] The exudates obtained from our laboratory[4,5] suggest that this may not be so, and it is necessary to explore the question of whether the details of the cellular response are, to some extent, material specific[25–27] even at the earlier time periods. As a first step, the possibility that different subpopulations of cells are seen at the site of implantation has been investigated. In this study, flow cytometry has been used in an attempt to define these subpopulations of macrophages as characterized by size, granularity, and the expression of specific antigens. Blood monocytes were cultured into macrophages in order to make an assessment of the differences between transformations *in vitro* and those observed in biomaterial-stimulated inflammation. This reference was, however, not intended to be a *direct* comparison. The subcutaneous rat implantation model was chosen because cells that have received stimulation only from the test material, other than that from surgery, can be harvested from an inflammatory exudate with minimal manipulation. It has been used in the past to gain useful information about inflammation caused by a variety of materials.[4,5]

Flow cytometry has been developed to assess the fluorescence exhibited by stained cells in response to the light of a particular wavelength. Cells have a large number of different antigens expressed on their surface, some specific to that cell type and others whose expression is dependent upon the activation state of the cell and its prior environment. Monoclonal antibodies specific for a particular antigen and conjugated to a fluorochrome (for example fluorescein isothiocyanate, FITC, or phycoerythrin, PE) will bind very strongly to these surface proteins and render the cell fluorescent to the appropriate wavelength of light. A flow cytometer passes single cells in a hydrodynamically focused stream of biologically balanced, isotonic saline through a beam of laser light. The shadow caused by the presence of the cell is used to assess cell volume (forward light scatter) while light of the laser's wavelength detected at right angles to the direction of illumination is used to assess intracellular granularity (90° light scatter). A series of dichroic filters and mirrors is used to select fluorescence of varying wavelengths to determine the intensity of fluorescence emanating from a number of different fluorochromes. The intensity of fluorescence is proportional to the concentration of fluorochrome/antibody conjugate and therefore to antigenic expression.

MATERIALS AND METHODS

Materials

The implantation material was ultra high molecular weight polyethylene (UHMWPE), obtained from the Welding Institute (Abbington, Cambridge, U.K.). Samples were cut into rectangular-shaped blocks 35 mm × 12 mm × 4 mm, two blocks being bonded together, overlapping along their longest dimension by 10 mm.

Implantation

Samples were implanted subcutaneously into the back of six-month-old black and white hooded Lister rats of the Liverpool strain, all weighing in the range of 300–310 g. A single sample was implanted into each rat per time period, on one side of the spine, lying on top of the dorso–lumbar muscle. U.K. Home Office guidelines for the care and use of laboratory animals were observed. The rats were sacrificed after 2, 7, and 14 days. The implants were carefully retrieved and the exudate that had collected around the implant harvested by suction. The exudates were diluted with a buffered and filtered (to 0.22 μm) saline solution so as to have equal cell concentrations of approximately 5×10^6 cells/mL.

Cell culture

Cell populations within the exudates were compared to cells derived from the blood of the same strain of rat. Fresh rat blood was collected by cardiac puncture and anticoagulated with 0.2 IU/mL heparin (Multiparin, Wrexham, U.K.) final concentration. Five mL were layered onto an equal volume of Ficoll–Paque (Pharmacia, Piscataway, NJ) and centrifuged at 400 g for 25 min. The resultant lymphocyte/monocyte layer was retrieved from the plasma/Ficoll interface. The cells were suspended in RPMI and centrifuged at 400 g for 5 min. The pellet then was resuspended in RPMI (supplemented with 10% heat-inactivated fetal calf serum, gentomycin, streptomycin, and L-glutamine, Gibco, Paisley, U.K.) and then layered onto a cell culture flask (Falcon, Lincoln Park, NJ). After 3 h incubation at 37°C in 5% CO_2, the nonadherent cells

(lymphocytes) were removed by changing the tissue culture medium with fresh RPMI. At 6 h, 2, 6, and 10 days, maturing macrophages were removed by cold shock (chilled RPMI) and gentle scraping with a rubber policeman. These cells were concentrated by centrifugation and diluted to the same concentration as the exudates with filtered, buffered saline.

Flow cytometry

Twenty μL diluted exudate or cell culture were incubated with 10 μL of each of various monoclonal antibodies, all directly conjugated with FITC, to determine specific cell types. The following mouse monoclonal antibodies specific for rat antigens were obtained from Serotec (Oxford, U.K.): leucocyte common antigen (LCA), a pan leucocyte marker (M.ab MRC-OX1); CD11b/c, a macrophage and monocyte marker (M.ab MRC-OX42); CD5 (M.ab MRC-OX19); granulocyte (HIS48); and mature macrophage (ED2). The B-lymphocyte marker CD45RA (M.ab MRC-OX33) was obtained from Sera Lab (Crawley Down, Sussex, U.K.). The monocyte chemoattractant protein 1 (MCP-1) marker was obtained from internal development. Red blood cells, when present in the exudate samples, were lysed prior to flow cytometry. In this case, 300 μL FACS lysing solution (Becton Dickinson, San José, CA) at recommended concentration were added to the stained cell suspensions (30 μL) and incubated at room temperature in the dark for 12 min. The lysing action was stopped by the addition of 2.2 mL filtered saline (FACSFlow, Becton Dickinson, Oxford, U.K.). Non-lysed samples were diluted with 300 μL FACSFlow prior to analysis. Flow cytometry was performed using a Becton Dickinson FACSort (San José, CA).

Volume and granularity distributions were obtained by comparing cell forward light scatter with 90° light scatter. Relative fluorescence was measured for all samples and positivity tested by reference to a negative control. This was provided by incubating both cultured cells and exudates with an antibody of the same subclass as the test antibodies and also directly conjugated with FITC. All incubations were performed in the dark at 4°C to limit the degree of nonspecific binding. The minimum antibody concentration that gave the highest fluorescence reading in a tube of positive cell types (at a concentration of 5×10^6 cells/mL) was used. Specifically, the antibodies were diluted to achieve the following concentrations: LCA – 0.01 μg/mL; CD11b/c – 0.1 μg/mL; CD5 – 0.1 μg/mL; CD45RA – 0.1 μg/mL; HIS48 – 0.05 μg/mL; ED2 – 0.05 μg/mL; MCP-1 – 0.05 μg/mL. Cells (30,000) were counted for each exudate or cell culture/antibody combination, and cells with a diameter of less than approximately 5 μm were thresholded-out electronically.

Cell sorting

Cell identity was double checked by sorting discrete volume/granularity peaks and positively fluorescent cells into separate tubes using the piezo-electric sorter facility on the flow cytometer. The sorting tubes were coated with 5% bovine serum albumin (Sigma, Poole, Dorset, U.K.) and incubated at 4°C for at least 4 h prior to collection to prevent the loss of cells to the walls of the tubes. These were concentrated by centrifugation and viewed directly by light and laser-scanning confocal microscopy.

RESULTS

Measures of volume and granularity of the monocyte population of leucocytes in whole rat blood after lysis of red blood cells are shown in Figure 1 and the various populations of other cells as found in the inflammatory exudates in Figure 2. Figure 3 shows the comparative distribution of cultured macrophages, harvested at different time periods. The discrete population of monocytes spread into a continuum of increasing size and apparent granularity as they matured into macrophages. This shift in size and granularity was more pronounced with increasing time, an observation consistent with expectations.

The macrophages in the implant exudates (Fig. 4) did not show grossly dissimilar behavior in comparison to the cultured monocytes (Fig. 3), volume and apparent granularity increasing with time. The overall number of monocytes/macrophages increased over

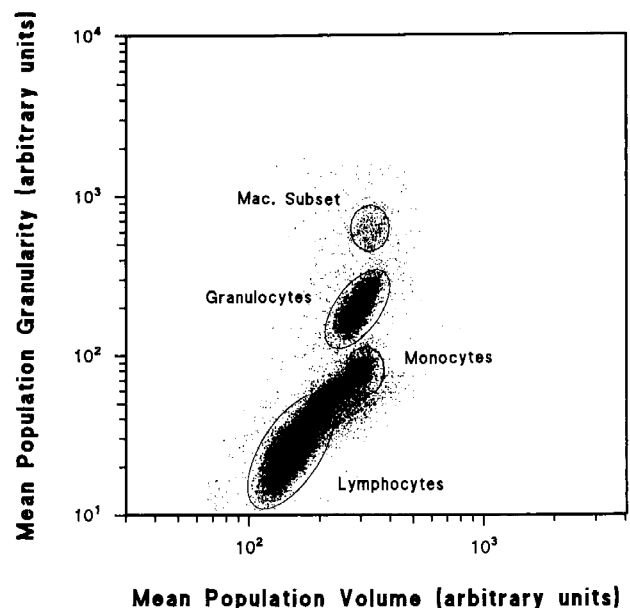

Figure 1. Volume (x axis) and granularity (y axis) distribution of monocytes in normal rat whole blood.

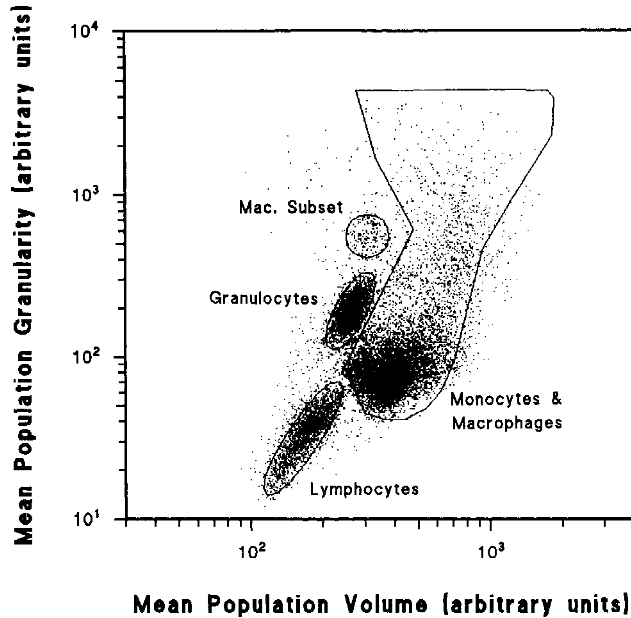

Figure 2. Volume (x axis) and granularity (y axis) distribution of cells present in an inflammatory exudate after 7 days, retrieved from implanted UHMWPE.

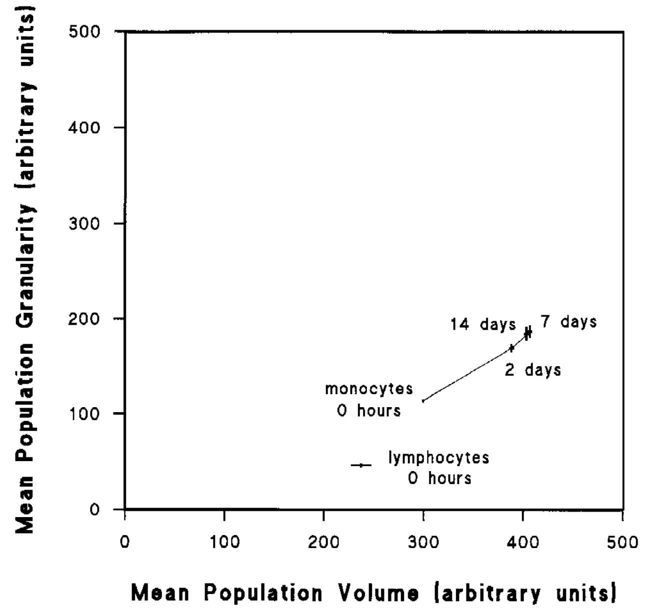

Figure 4. Change in volume and granularity of monocytes appearing in exudates derived from UHMWPE. Mean ± Standard Error (SE).

time. There was a greater number of larger and more mature macrophages, which appeared to be more granular, at longer time periods (7 and 14 days), and a greater number of immature, monocyte-sized macrophages at the earliest time period. There appeared to be a constant infiltration of monocytes during the implantation period, which influenced the mean volume, granularity, and expression of ED2 of the monocyte/macrophage population.

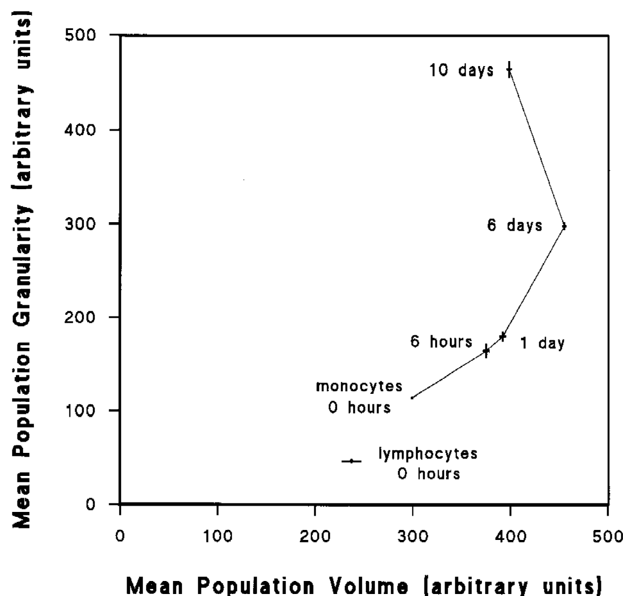

Figure 3. Change in volume and granularity of cultured blood monocytes over time compared to lymphocytes. Mean ± Standard Error (SE).

At day 2 there was evidence of a very small, highly granular peak of macrophages, which declined thereafter. This subpopulation had a mean volume similar to that of granulocytes and smaller than monocytes. They were, however, even more granular than granulocytes. Their identity was confirmed as a monocyte/macrophage subpopulation by viewing by light and laser-scanning confocal microscopy after direct sorting into a separate tube, and concentration by centrifugation.

The relative quantities of each type of monocyte/macrophage population in culture are shown in Table I. In the exudates from implanted UHMWPE, there were granulocytes and lymphocytes present in addition to monocytes and macrophages. The number of monocytes infiltrating increased with time, with the percentages of the small, granular macrophage subset declining after its peak at day 2.

The expression of the LCA, CD5, granulocyte, and CD11b/c antigens was largely as expected: all macro-

TABLE I
Proportion of monocytes and macrophages present in culture medium as a percentage of the total number of monocytes and macrophages

	Percentage of Cells Present in Culture	
Time Period	Monocytes	Macrophages
Isolation	97.7%	2.3%
6 Hours	74.1%	25.9%
2 Days	46.0%	54.0%
6 Days	1.4%	98.6%
10 Days	1.0%	99.0%

phages, monocytes, lymphocytes, and granulocytes expressing LCA; T-cells expressing CD5; granulocytes expressing HIS48; and monocytes and macrophages expressing CD11b/c. The major macrophage population expressed little ED2 activity at any time period (Fig. 5): the mean fluorescence at day 14 was approximately only 1.23 times greater than that at day 2, indicating that the infiltrating macrophage population was still relatively immature. The smaller, granular macrophage subpopulation expressed vastly more fluorescence at all time periods, differences that were highly significant (Fig. 5): at day 2 the subset mean fluorescence was approximately 29 times greater than the major macrophage population.

The expression of MCP-1 molecules on the surface of macrophages (Fig. 6) showed a similar pattern: very high MCP-1 positivity on the macrophage subset that slowly declined over the period of the study while the main monocyte/macrophage population had a mean fluorescence that was significantly smaller (a factor of between 10 and 40) at all time periods, the greatest difference being at 2 days, the smallest at 14 days.

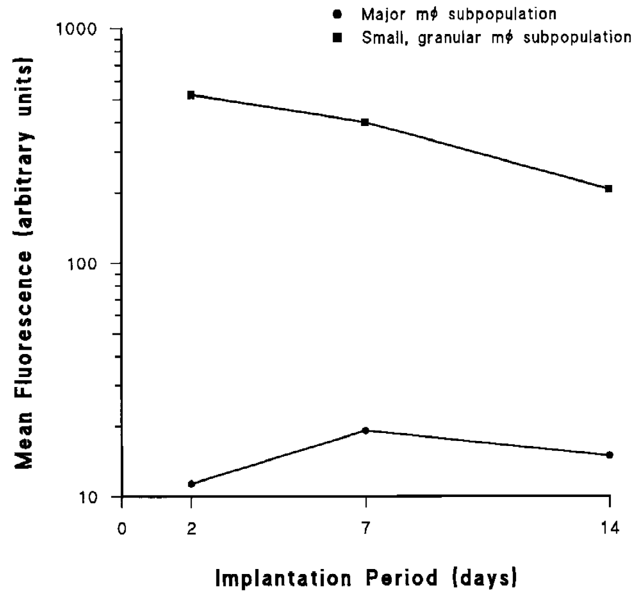

Figure 6. Relative fluorescence of exudate macrophages stained with anti-MCP-1 (monocyte chemoattractant protein 1). The differences were significant at the 95% confidence level.

DISCUSSION

A variety of biomaterials had been shown previously to elicit the accumulation of a large number of macrophages in this subcutaneous rat implantation model.[4,5] In this study, a comparison has been made between the macrophages elicited in this environment and those maturing in an *in vitro* environment free from the inflammatory stimuli associated with implantation. The *in vitro* macrophages do, however, receive stimulation from contact with the culture dish.

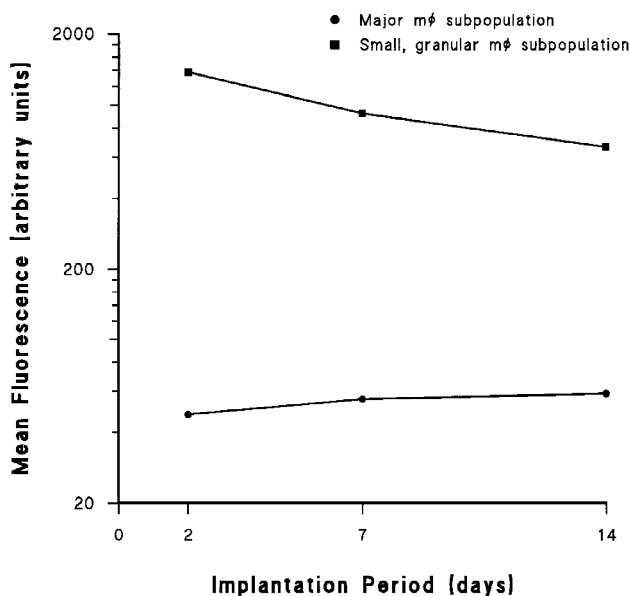

Figure 5. Relative fluorescence of exudate macrophages stained with antibody ED2 (mature macrophage). The differences were significant at the 95% confidence level.

In some respects, the observed populations of rat macrophages are the same, whether elicited by the implantation of a biomaterial or matured in a culture flask. The cells in each population increased in volume and granularity in a similar fashion. There was, however, an apparently highly granular, yet relatively small, macrophage subpopulation present in the exudates retrieved from implanted polyethylene but never visible in populations of cultured blood monocytes or in exudates retrieved from other types of material in other experiments.[4,5]

It is known that macrophages have a variety of functions in many homeostatic, immune, and inflammatory reactions.[7,8,28,29] They are of course well known as phagocytic cells,[30,31] but they do have many more significant roles, including the activation and subsequent modulation of lymphocytes.[32] They also can kill tumor cells[33] and certain micro-organisms,[34] for example bacteria,[35] viruses,[36] and fungi.[37] It also is known that macrophages have specific functional subpopulations,[10–12] the heterogeneity arising despite the fact the origin of virtually all macrophages is the blood monocyte. Macrophages have a fairly long life span (up to several weeks in the peritoneal cavity) but exist as a monocyte for only one or two days. They normally exit the blood stream after this time and become resident macrophages in a variety of tissues and cavities, for example lung, liver, spleen, and peritoneum, displaying specific characteristics appropriate for these locations. A small percentage of these macrophages are, however, produced locally (≈5%), being

derived directly from other macrophages[38] and apparently being morphologically similar to them.

An inflammatory stimulus will initiate diapedesis, the movement of granulocytes and monocytes from the blood, and these monocytes will mature into macrophages. These are different from resident macrophages in that they have not matured to full macrophage status (in terms of size and granularity) and so possess virtually all the characteristics of a blood monocyte, but to varying degrees have become activated. It has been reported that resident macrophages can be observed at the start of an inflammatory reaction as a small subpopulation.[38] Mature macrophages are known, however, to be larger and more granular than their immature counterparts.[39]

The subpopulation of small macrophages in our exudate samples would appear to be neither self-replicating nor resident macrophages, from this evidence. The ED2 staining data, however, would suggest otherwise. This subpopulation is extremely positive for the ED2 antibody, which has been shown previously to stain exclusively mature macrophages.[40] There also is a small number of them present in normal rat whole blood (with a mean of 0.6% of total leucocytes), and one would expect them to infiltrate into the exudate in the same way as monocytes and neutrophils. They are not derived from monocytes in the classically described manner—slowly maturing by increasing in size. They were never observed in our monocyte cultures and were not present in other material implant experiments,[4,5] at least not in such numbers as to be detectable.

These experiments were carried out on pieces of UHMWPE that were cut into an irregular shape for which there is no absolute control. It is possible that implants of this shape and size cause the production of exudates in a material-independent manner into which are elicited macrophages of the observed phenotype. While microscopical examination of the exudate reveals a moderately severe inflammatory response, one could suggest that this is not due to the chemistry of the implant. Previous studies[4,5] have shown that materials of different chemistries but with the same physical dimensions do, in fact, produce a host response that spans a spectrum of responses from the almost totally acellular and nonexistent inflammation to an extremely severe, highly cellular, chronic response. Other experiments (unpublished results) involving materials larger than those in this study have not produced macrophages similar to those observed here. Surface topography also may contribute, but the PTFE implanted previously[4] had very similar surface features to the UHMWPE when viewed under scanning electron microscopy. It is therefore concluded that the observed cell types and phenotypes are, in fact, controlled by material chemistry-dependant phenomena.

It is possible that the observed differences in 90° light scatter in the macrophage population were not due to differential maturity. Macrophages increase in their 90° light scatter in culture and *in vivo* after diapedesis, largely due to the production of enzymatic species enclosed in granules. The number of curved surfaces within a cell has a direct bearing on the quantity of light reflected orthogonally rather than being transmitted through it. While granules make up the majority of these surfaces in most cell types, macrophages are different in that they have the ability to phagocytose. The materials retrieved after implantation did not show any sign of degradation on their surfaces, suggesting that phagocytosis was not occurring. It is possible, however, that the inflammatory response elicited by UHMWPE had induced the macrophages into frustrated phagocytosis, causing the production of membranous elements in otherwise small, immature cells. However, since these cells also were observed in whole blood from control rats, it would appear that this is unlikely.

The data, therefore, suggest that different materials may cause differential responses in terms of their macrophage infiltration or activation. The response to UHMWPE was moderately severe, as assessed using immunohistochemical techniques. It is possible that the small macrophage subpopulation controls the severity of the subsequent inflammatory reaction. One must ask the question whether there are monocytes or macrophages within blood that exhibit surface antigens previously seen only in resident macrophages, or does the stimulus caused by the implantation of UHMWPE quickly induce ED2 expression in these infiltrating cells? ED2 expression previously has been shown to be inducible in adhering, nonproliferating conditions[40] or reversible in the invasion of a porous biomaterial.[25]

Expression of MCP-1 molecules on the surface of this macrophage subpopulation indicates that this cytokine is present within the exudate since it is not normally present within noninflammatory tissue. It is interesting that MCP-1 adhesion is almost exclusively to or on the surface of the small macrophage subset. This does not determine the original site of MCP-1 release, but the fact that it is present would indicate a mechanism by which MCP-1 plays a role in the induction or modulation of the inflammatory response to certain implanted biomaterials, as has been demonstrated in certain disease states (e.g., pulmonary granuloma formation) in rats.[13,41] One would expect that large quantities of MCP-1 at the implant site would attract monocytes to the general area. The release of MCP-1 might not be from this macrophage subpopulation (MCP-1 release has been demonstrated from a variety of cell types, including granulocytes and endothelial cells), but they are very strong candidates considering their activation status. The expres-

sion of MCP-1 molecules on the surface of these macrophages might create a focal point for the accumulation and retention of monocytes, thus propagating and modulating the inflammatory response.

CONCLUSIONS

The data from this study would suggest that there exists a small subpopulation of submonocyte-sized macrophages with high granularity in the exudate of an inflammatory reaction to UHMWPE in this rat model. These cells are present in whole rat blood, can be seen in the exudates produced at 2 days and at decreasing concentrations thereafter. Further analysis will be necessary to determine any functional differences between these cells and blood monocytes and mature macrophages. Flow cytometry data would suggest that the macrophage subpopulation comprises mature macrophages. They appear to have a strong affinity to MCP-1 molecules, the presence of which would implicate MCP-1 in the initiation, modulation, or propagation of an inflammatory response in relation to the implantation of a biomaterial.

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