Immune Response Testing of Electrospun Polymers: An Important Consideration in the Evaluation of Biomaterials

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ABSTRACT

Due primarily to cell sourcing issues, many in the field of tissue engineering have opted to create scaffolds that promote *in situ* regeneration, using the body as both the bioreactor and the cell source for the remodeling of scaffolds, resulting in the formation of native tissue. This practice raises many concerns, with the body's immune response to such an implant often being neglected as a potential problem in preliminary design and biocompatibility testing. More importantly, what happens over time in terms of the immune responses as the biodegradable scaffold structures being utilized to promote *in situ* regeneration begin to degrade, forming structural fragments and degradation products? In summary, immune response evaluations are critical considerations that must be conducted when evaluating bioresorbable scaffolds. In addition, it is essential that these evaluations analyze materials for their potential dose-response and time-course effects on the various components of innate and acquired immunity.

EDITORIAL BODY

Research into the immune response to biomaterials has been indicated as essential to a complete evaluation of their biocompatibility [1]. Recent work examining the host response to biomaterials has provided some insight into the inflammatory response to these materials. Routine evaluations of biomaterials often consist of co-culturing of macrophages and/or L929 mouse fibroblasts with the material, followed by examination of various indicators of inflammation. To many in the biomedical engineering community, this information is the "gold standard" by which a material is deemed to elicit an acceptable (or undesirable) immune response. Current approaches in biomaterials research that investigate the effects of biomaterials on the immune system lack standardization, and they often only evaluate one small component of the immune response: inflammation. Depending on the objective of the study, this approach may or may not be sufficient for evaluating immunocompetance following exposure to a material. Often not evaluated are effects of biomaterials on acquired (cell-mediated, i.e. T-cell; and humoral, i.e. B-cell) immunity or other innate parameters, such as Natural Killer (NK) cell activity. In addition, the responses of both innate and acquired immunity to electrospun biomaterials have been completely ignored. It is imperative that an overall assessment of immunomodulation be acquired for a given biomaterial before embarking on the more detailed, mechanistic approaches that pervade biomaterial research today. The approach utilized by one of this paper's coauthors (White, [2-9]) to evaluate biomaterials illustrates a comprehensive methodology to evaluate the multiple components of the immune response. In addition, this approach provides a unique opportunity to advance the study of the immune responses to a wide variety of biomaterials and allows for the standardization of a testing hierarchy for evaluating the complete host immune response (both innate and adaptive immunity) following exposure to biomaterials.

The immune response to a biomaterial is not unlike the reaction to any other foreign substance. Highly complex and involving many different cell types, both innate and acquired immune responses are initiated when increased levels of chemokines (chemoattractant signals) result in the initiation of the inflammatory response, eliciting the infiltration of phagocytic cells, including polymorphonuclear cells (PMN) and macrophages. In addition, biomaterial surface contact with the complement protein fragment C3b (produced continuously at low levels)

activates the alternate pathway of the complement cascade. By-products of this cascade (i.e. C5a, C3b, and iC3b) can promote macrophage activation and opsonization [10, 11]; macrophages can be stimulated to release a wide variety of signals (cytokines), including interleukins (IL), interferons (IFN), and tumor necrosis factors (TNF), which in turn mediate other processes, including maintaining inflammation, promoting T- and B-cell development, chemotaxis, and activation, and initiating tissue repair (collagen production) and angiogenesis [12]. Upon activation, T- and B-cells (lymphocytes) respond to foreign antigens, and while these responses are usually slower than innate responses, they are highly antigen-specific, in contrast to innate immune responses. Known as adaptive immunity, these cells are capable of proliferation when appropriately stimulated, and they are highly adept at discriminating self from non-self. Additionally, lymphocytes are capable of developing a memory response, and in the event of a second exposure to the antigen, the immune response is more rapid and of a higher magnitude. In particular, both the secondary antibody response (humoral) and the delayed-type hypersensitivity response (cell-mediated) quickly produce activated effector cells in larger numbers. Delayed-type hypersensitivity (DTH) is also known as Type IV hypersensitivity under the Gell and Coombs classification and by the general population as contact dermatitis. The DTH is mediated by antigen-presenting cells and T-cells, specifically the CD-4+ subset of the T-cell popoulation. Since the immune system is multifaceted, the question arises as to how exposure to a biomaterial affects the various components of the immune system. There will undoubtedly be acute inflammation, resulting from the cut of a scalpel that compromises the integrity of the skin, a major component of innate immunity. Furthermore, should the biomaterial suppress or stimulate the immune system inappropriately, such modulation could be detrimental to the integrity of the biomaterial as well as the immunocompetance of the host.

With regard to biomaterial effects on innate immunity, the primary focus has been on the activity of phagocytic cells. The widely used synthetic vascular prosthetics made of Dacron® and expanded polytetrafluoroethelyne (e-PTFE) have been shown to result in the reduction of phagocyte concentration in human donor blood [13]. It has also been well documented that e-PTFE and other biomedical polymers activate macrophages and increase production of both IL-1 and TNF-α [14-18]. Recently, Brodbeck et al. have demonstrated that the surface chemistry (hydrophilicity and surface charge) of the biomaterial evaluated modulates expression of anti-inflammatory or pro-inflammatory cytokines [19].

Others have focused specifically on the macrophage and its interactions with biomaterials. Greisler et al. have published extensively regarding macrophage interactions with synthetic bioresorbable materials, demonstrating that macrophage contact with these materials promotes activation of the macrophage, followed by biomaterial-specific release of growth factors and various cytokines [20]. In addition, they reported that, when cultured in media preconditioned by macrophage-polymer interactions, endothelial cells demonstrated increased ^{3}H-thymidine incorporation with increasing macrophage-material interaction time [21]. Macrophages have also been shown to promote endothelialization following exposure to vascular prosthetics composed of bioresorbable materials, where the rate of endothelial cell ingrowth is directly related to the rate of resorption of the biomaterial [22].

Perhaps the most extensively investigated biomaterial from an immunological standpoint is collagen. Type II collagen is known for its ability to induce autoimmune arthritis [23], and, while types I and III do not initiate this response, research has indicated that each of these collagen types is immunogenic [24]. The immune responses to collagen types I, II, and III in a rabbit experimental model was reported to be T cell-mediated with minimal humoral response [25]. Others have indicated that T cells play a pivotal role in macrophage recruitment and giant cell formation in reaction to cross-linked dermal sheep collagen [26], suggesting that inflammation in response to collagen may be modulated by controlling T cell activation. In addition, these researchers demonstrated that TNF-α and IFN-γ were not responsible for the onset of the foreign-body reaction to collagen in mice [27, 28]. Recently, it was reported that collagen fragments alone did not modulate innate immunity [29]. Further characterization of the immune response to these collagen fragments, using LPS-activated monocytes, demonstrated that IL-1 levels increased or decreased, depending on the peptide sequences present in the fragments. However, it is impossible to understand the significance and implications of these results without first having some idea as to whether they result in biologically relevant effects on the immune system. A more complete evaluation of the effects on the various components of the immune system is needed, even for this extensively studied biomaterial.

One polymer currently receiving attention is polydioxanone (PDO), a bioresorbable polymer that is completely degraded *in vivo* within six months. PDO vascular prostheses have been shown to be less thrombogenic than both PGA and Dacron® synthetic grafts [30], thus making them desirable to use in vascular tissue engineering applications. However, like with other synthetic polymers, the use of PDO alone does not promote cell infiltration *in vivo* [31]. It has been reported that PDO suture (PDS™-II) produced no sign of acute or chronic toxicological effects in any tissues or organs following a six-month *in vivo* implantation [32], indicating that the long-term effects of this polymer may be negligible. However, studies at time points less than 6 months, during which the polymer is degrading, may indicate effects on the immune system, thus earlier time points must be evaluated in order to determine the specific effects of PDO prior to complete resorption of the polymer. In contrast, PDS™-II suture has been shown to induce fibrosis after 14 days in a rat subcutaneous implantation model [33]. Others have demonstrated that soluble suture fragments of PDO had minimal effects on the macrophage as compared to silk, nylon, and polyglactin [34]. Each of the suture materials examined therein were indicated to release "immunotoxic factors," although no specific factors were identified, nor were any cell types other than the macrophage examined. PDO is believed to be less immunogenic than PGA and PLA [35], yet this too remains to be examined thoroughly. Again, it is imperative that an overall picture of immune responses be obtained prior to assertions regarding a biomaterial's relative effects on the immune system.

Work in our lab focuses on the design and evaluation of tissue engineering scaffolds fabricated by electrospinning. We have successfully electrospun a variety of synthetic polymers [35-38], natural polymers [39-43], and blends of the two [31, 44, 45] for use in a variety of tissue engineering applications. With the sudden increased interest of biomedical engineers in electrospinning, it is important that the effects of electrospun biomaterials upon the immune system be examined. However, only limited published research has examined the immune response to sub-micron diameter fibrous biomaterials.

Sanders, et al. have published regarding the influence of polymer fiber diameter on fibrous capsule formation and thickness [46, 47], demonstrating that implantation of smaller diameter fibers (1.0 μm to 5.9 μm) resulted in thinner or no fibrotic capsule formation when compared to fibers with diameters ranging from 6.0 μm to 15.9 μm. Missing from this puzzle, however, is a determination as to whether smaller diameter fibrous biomaterials generate different immune responses. Our research has suggested that the biocompatibility of a material changes significantly between the micron and the nanometer fiber diameter levels [37], a suggestion that is not difficult to believe given the results reported by Sanders et al. This further emphasizes the need for an examination of the immune response to electrospun sub-micron fibrous biomaterials.

So, if study of the immune responses to biomaterials is truly critical, why is there so little organized focus on such research today? Immune cell interaction with a biomaterial following implantation is unavoidable. Even when bioresorbable materials are used (as temporary scaffolds encouraging formation of native tissue), cells of the immune system have ample opportunity for interaction with these materials; interaction times with permanent materials are likely not much longer, as the formation of a fibrotic capsule around permanent materials is probable, depending on the nature of the biomaterial.

Nearly all published research on immune responses to biomaterials has presented too narrow a focus, a view too mechanistic with little characterization of the overall immune response first. The result is a breadth of knowledge that has no contextual foundation. With the appropriate biological assays as tools, a more complete picture of immunomodulation resulting from exposure to biomaterials can be achieved than is possible from the current approaches. An assortment of assays described by Luster et al. [48] which are typically used in immunotoxicological evaluations of pharmacological compounds can provide a more complete appraisal of acquired and innate immunity than is currently being achieved with the limited assays currently in use by the biomaterial community.

An initial approach for examining immune responses following *in vitro* exposure of a biomaterial could consist of evaluating effects on: cell-mediated immunity by examining T cell proliferation alone or in the presence of anti-CD3 antibody or the T-cell mitogen ConA [49], the humoral immune response by assessing effects on B cell proliferation (LPS-stimulated or $F(ab')_2$ and IL-4 – stimulated [49]) and antibody production (Mishell-Dutton hemolytic AFC assay [50]), and innate responses by examining chemotaxis [51] and activity [52] of phagocytic cells, in addition to NK cell activity [53, 54]. In each of these assays, splenocyte exposure to the biomaterial

occurs *in vitro* by co-culturing spleen cells with the biomaterial for a period of time specific to each individual assay.

Following the evaluation of immunomodulation by *in vitro* biomaterial exposure, a similar array of assays can be used to assess the immune responses following *in vivo* exposure to the biomaterial [48]. Furthermore, additional holistic assays such as the DTH and functional ability of the fixed macrophage of the mononuclear phagocyte system (MPS) can be evaluated. While *in vivo* exposure in laboratory animals can model the use of biomaterials in man (the ultimate objective of this biomaterial research and development), *in vitro* studies have an important role in determining the mechanism of action and/or cell types involved in immune responses to biomaterials.

Using this approach, we have begun to examine changes in murine spleen cells following *in vitro* exposure to electrospun biomaterials. Preliminary results examining the effects of several common biomaterials on humoral immunity have been obtained using the Mishell-Dutton assay, the *in vitro* counterpart to the highly sensitive plaque assay [55]. Polymers electrospun and examined include: nylon, polydioxanone (PDO), poly(glycolic acid) (PGA), poly(lactic acid) (PLA), and a 50:50 (v:v) blend of PDO and polycaprolactone (PCL). Each polymer was dissolved in 1,1,1,3,3,3-hexafluoro-2-propanol (HFP) at a concentration of 100 mg/ml; 3 ml of each solution were electrospun at a rate of 8 ml/hr onto a 303 stainless steel mandrel (7.7 cm x 2 cm x 0.5 cm). Also examined but not electrospun was e-PTFE. Materials were all disinfected in ethanol for a minimum of 10 minutes, followed by three successive rinses in sterile phosphate-buffered saline (PBS) prior to use in the culture system.

As seen in *Figure 1*, results indicating modulation of antibody production following exposure to these synthetic polymers were obtained using a modified version of the *in vitro* hemolytic antibody-forming cell assay as described by Mishell and Dutton [50], in which splenocytes from female B6C3F1 mice were cultured with either 10-mm diameter circular discs of material or positive control (0.3 μM methotrexate, MTX; Sigma Aldrich). In the Mishell-Dutton assay,

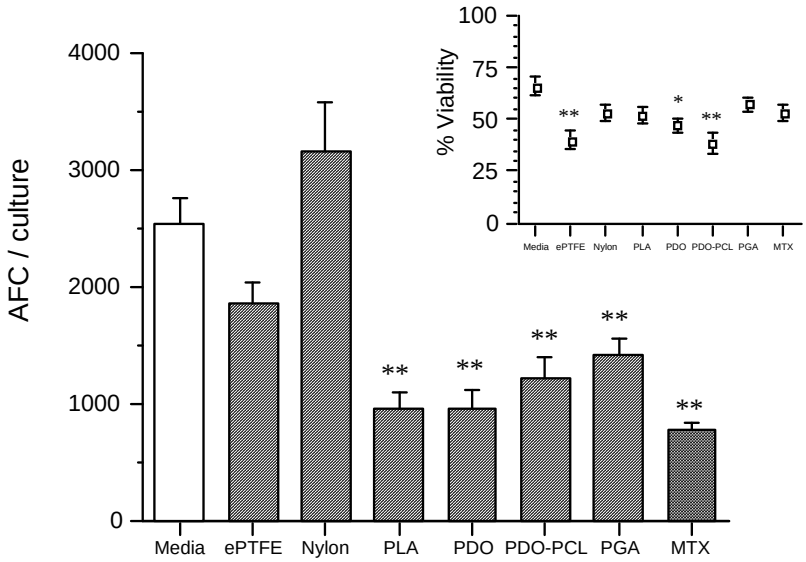

FIGURE 1. ***In vitro*** **Hemolytic Antibody-Forming Cell Response to Sheep Erythrocytes Following Exposure to Synthetic Polymers.** Response to the T-dependent antigen sheep erythrocytes (sRBC) following 4 day *in vitro* co-culture of murine splenocytes and sRBC in the absence ("Media") or presence of 10-mm diameter circular punches of material (ePTFE, nylon, PLA, PDO, PDO-PCL, PGA) or positive control (methotrexate, MTX; 0.3 μM). Following culture, an aliquot of cells was added to a glass dilution tube containing agar, sRBC, and guinea pig complement. The mixture was vortexed, plated on a Petri dish, and covered with a glass cover slip. Following incubation for 3 hours at 37 °C, the number of antibody-forming cells (AFC) were counted using a Bellco plaque viewer. Results were reported as AFC per culture; $n = 6$ for all groups except ePTFE, for which $n = 5$. Also shown are cell viabilities following the 4-day culture period. Results were reported as % Viability, calculated as (Count after pronase incubation) / (Count before pronase incubation) * 100. Statistical analysis testing included Bartlett's test for homogeneity of variances followed by ANOVA and the Tukey-Kramer Multiple Comparison test; asterisks indicate a statistically significant difference from the control group (Media); $*p < 0.05$; $**p < 0.01$.

splenocytes are sensitized *in vitro* with the T-dependent antigen, sheep erythrocytes. Antibodies of the IgM class specific for sheep erythrocytes are produced by mature B cells, known as plasma cells, and the number of these antibody-forming cells (AFC) per culture are enumerated. Several of the polymers tested were found to be immunosuppressive in this assay. Cell counts following culture and removal of polymer discs were performed and demonstrated no differences from Media control, indicating cells were not lost as a result of attachment to the discarded polymer discs (data not shown). Cell viability at the conclusion of the assay was determined for each culture, using the pronase method [56].

The implications of these results are significant, as they indicate that the bioresorbable electrospun polymers (PDO, PGA, PLA, PDO-PCL) are immunosuppressive in the Mishell-Dutton assay, while e-PTFE and electrospun nylon are not. In addition, cell viability results indicate that murine splenocyte exposure *in vitro* to e-PTFE and PDO-PCL each resulted in a significant decrease in % Viability ($p < 0.01$), while PDO also produced a significant decrease ($p < 0.05$). This indicates that the decrease in cell viability contributes, in part, to the immunosuppressive effects of PDO and PDO-PCL seen in this assay.

While no significant differences occurred in cell viability between Media controls and both PGA and PLA, these polymers were also immunosuppressive in the Mishell-Dutton assay. With no effect on viability, these results suggest PGA and PLA affect the functional ability of one or more cell types necessary for the formation of the *in vitro* antibody response. The positive control, MTX is a known immunosuppressive drug and was used in the assay to demonstrate the assay was capable of detecting an effect if one was to occur.

Current work in our laboratory is focused on conducting a more complete assessment of the immune responses to both synthetic and natural electrospun polymers. We aim to achieve this evaluation through assays for cell proliferation, cytokine production, and phagocyte activity, in addition to antibody production capability. In particular, responses of T-cells modulated by the T-cell mitogen concanavalin A (ConA) or by anti-CD3 antibody while in the presence of biomaterials will indicate whether the materials affect T-cell proliferation. Similarly, the responses of B-cells following exposure to the B-cell mitogen lipopolysaccharide (LPS) or following the addition of $F(ab')_2$ antibody fragments in the presence of IL-4 will indicate any effects on B cell proliferation as a possible cause of the immunosuppression presented here.

The holistic assays of humoral and cell-mediated immunity, i.e. the hemolytic AFC assay and Delayed-type Hypersensitivity response, each allow for a sensitive evaluation of overall effects of test material on their respective branches of adaptive immunity. In addition, changes in cytokine production can also be measured on collected supernatants following splenocyte culture with test materials via the conventional enzyme-linked immunosorbent assay (ELISA). The IgM AFC response to sRBC is one of the most sensitive *in vivo* immunotoxicological assays [55, 57]. Any interruption in the functions and interdependence of antigen processing and presentation by macrophages and other antigen presenting cells, T-cell activation, and/or B-cell activation can interfere in the production of antibodies to the sRBC antigen. It remains to be determined which cell type(s) involved in this assay are most affected and the mechanisms by which biomaterials examined in this preliminary study are immunosuppressive.

In addition, recent advances in flow cytometry, with the use of cytometric bead arrays, are enabling the simultaneous detection of multiple cytokines from a single sample. With relative ease, knowledge of changing cytokine levels can point to cell types being affected by the test material. This wide-ranging collection of assays mentioned herein, described in detail by Luster, et al. [48, 55, 58] and in the collective works of White et al., are standards in the immunotoxicological evaluation of pharmaceuticals and other compounds and represent a starting point for conducting more meaningful evaluations of biomaterial-induced immunomodulation.

To summarize, while limited information is being gathered in the study of the immune responses to various biomaterials, there is little standardization in the approaches undertaken by investigators. Current research may be too narrowly focused, without considering effects on the multiple components of the immune system. We believe that the approach presented herein is efficient and effective, and that it will provide focus to this emerging field in the assessment of immune responses to all classes of biomaterials, including those fabricated by electrospinning.

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