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Complement C3 participation in monocyte adhesion to different surfaces

(biocompatibility/adhesion mechanisms/leukocyte integrins/fibrinogen)

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ABSTRACT **As part of an ongoing investigation into the role of the monocyte/macrophage in biocompatibility, a major goal is to identify the adhesion mechanisms that initiate and promote the observed *in vivo* morphologic progression of monocyte-to-macrophage-to-foreign body giant cell on biomaterials. We have exploited differently modified polystyrenes, specific component-depleted sera, and monoclonal antibodies (mAbs) to leukocyte integrins to ask what adhesion mechanisms mediate human blood monocyte adhesion to different surfaces *in vitro*. Preliminary findings are that monocyte interactions with fluorinated, siliconized, nitrogenated, and oxygenated surfaces are reduced by 50–100% when complement component C3-depleted serum is used for adsorption; reductions vary with material surface properties. Adhesion is restored on all surfaces when C3-depleted serum is replenished with purified C3. Monocyte adhesion to serum-adsorbed surfaces is inhibited by mAbs to the leukocyte integrin β subunit, CD18 (mAbs 60.3 and MHM23), and partially inhibited by a mAb to the α subunit, CD11b (mAb 60.1), suggesting adhesive interactions between adsorbed C3bi (the hemolytically inactive form of the C3b fragment) and the leukocyte integrin CD11b/CD18. However, adsorbed fibrinogen reduces the effectiveness of these mAbs, indicating that alternative adhesion mechanisms may operate depending on the propensities of critical adhesion-mediating components to be adsorbed onto different surfaces.**

Adherent monocyte (MC)-derived macrophages and foreign-body giant cells (FBGCs), formed by macrophage fusion (1), are prominent and persistent cell types on implanted biomaterials and, through their numerous secretory capacities (2), are believed to exert multiple and complex influences on the inflammatory response at the implant site and on biocompatibility (3, 4). For example, surface cracks were detected directly and only underneath adherent FBGCs on retrieved biomaterial, demonstrating that these multinucleated phagocytic cells can modify their synthetic adhesion substrate and contribute to biomaterial degradation *in vivo* (5). Therefore, MC adhesion to implanted material is critical to biocompatible outcome because it initiates macrophage development and FBGC formation, but it is unknown how MCs recognize biomaterials or how surface properties might influence this event.

Material surface property-dependent blood protein adsorption occurs immediately upon surgical implantation of a medical device (6, 7), and it is the protein-modified biomaterial that inflammatory cells subsequently encounter (3, 4). MCs express receptors for various blood components, but they recognize naturally occurring foreign surfaces—i.e., invasive microorganisms—by receptors for opsonins such as fragments of complement component C3 (C3) (8). Because complement activation by biomaterials has been well documented (9–11), we reasoned that MC interactions with artificial foreign surfaces could occur by similar mechanisms. Exposure to blood during material implantation may permit extensive opsonization with the labile fragment C3b and the rapid conversion of C3b to its hemolytically inactive but nevertheless opsonic and more stable form, C3bi. C3b is bound by CD35 (12), but C3bi is recognized by distinct receptors, CD11b/CD18 (13) and CD11c/CD18 (14). Fibrinogen (FG), a major plasma protein that adsorbs to biomaterials (6, 7, 15), is another described ligand for these molecules (16–21), which together with CD11a/CD18, constitute a subfamily of integrins that is restricted to leukocytes (reviewed in ref. 22). The collective studies with monoclonal antibodies (mAbs) to their common β_2 subunit (CD18) and distinct α chains (CD11a, -b, and -c) have implicated CD11a/CD18 in cell–cell adhesive interactions and CD11b/CD18 and CD11c/CD18 in multiple phagocytic cell responses. Other potential adhesion-mediating proteins that adsorb to biomaterials include IgG (6, 7, 15), which may interact with MCs via receptors for its constant region (8), and fibronectin, for which MCs also express multiple types of receptors (23–25).

Accordingly, we have hypothesized that MC adhesion to biomaterials occurs via specific adhesion mechanisms—i.e., interactions of cell surface receptors with adsorbed proteins—and that material-dependent protein adsorption determines the nature of adhesion molecules engaged. To approach such complexities *in vitro*, four differently modified polystyrene surfaces have been exploited as probes for MC adhesion receptor–material interactions within the context of multiple potential adhesion-mediating proteins. Here, we have utilized specific component-depleted sera and mAbs to leukocyte integrins to ask what adhesion mechanisms mediate the initial adherence of otherwise unactivated human blood MCs to these different surfaces.

MATERIALS AND METHODS

Materials. Donated mAbs were anti-CD18 (60.3) and anti-CD11b (60.1) from Patrick Beatty (University of Utah, Salt Lake City) and John Harlan (University of Washington, Seattle) and anti-CD11a (TS1/22) and anti-CD18 (TS1/18) from Timothy Springer (Harvard University, Boston). Controls, anti-HLA-ABC, monomorphic determinant (40.5), and anti-α-fetoprotein (OM3-1.1) were purified from hybridoma cell line (American Type Culture Collection) supernatants. Other mAbs were purchased: anti-CD11b (OKM1; Ortho Diagnostic Systems), anti-CD11c (Leu-M5; Becton Dickinson), and anti-CD18 (MHM23; Dako). Sterile autologous or pooled serum was prepared from fasting, unmedicated, healthy donors and immediately frozen at −80°C. Fibronec-

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Abbreviations: C3, complement component C3; FBGC, foreign-body giant cell; FG, fibrinogen; mAb, monoclonal antibody; MC, monocyte; PA, Plastek A; PB, Plastek B; PC, Plastek C; PM, Plastek M.

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tin-depleted serum was prepared by a published method (26), and depletion was confirmed by immunoblotting. Other reagents were from Sigma except human fibrinogen (FG; Enzyme Research Laboratories, South Bend, IN), Ficoll-Paque (Pharmacia), IgG-depleted serum (Cappel Laboratories), Sepracell-MN (Sepratech, Oklahoma City, OK), and cell culture media (GIBCO).

Surfaces. Four surface-modified polystyrenes (Mat Tek, Ashland, MA) were obtained in sterile 96-well plate format (Table 1). Two hydrophobic materials, Plastek A (PA) and Plastek B (PB), are fluorinated and siliconized, respectively. Plastek C (PC) and Plastek M (PM) are both hydrophilic but are, respectively, cationic (nitrogenated) and anionic (oxygenated). These surface properties were confirmed by advancing water-contact angle, electron spectroscopic chemical, and infrared spectroscopic analyses (not shown).

Adsorption of Surfaces. Surfaces were adsorbed with undiluted serum or specific component-depleted serum (100 μl per well) for 30 min at 37°C as indicated or were sequentially adsorbed with FG (3 mg/ml) and various sera (FG/serum). Briefly, FG (100 μl per well) was adsorbed for 30 min at 37°C, unadsorbed FG was removed with two washes in phosphate-buffered saline containing Ca^{2+} and Mg^{2+} (PBS), and then undiluted serum was similarly adsorbed. All surfaces were again washed twice and used immediately. The presence of multiple potential adhesion-mediating proteins, including C3bi, FG, fibronectin, and IgG, was confirmed on all four model surfaces by direct ELISA (not shown).

MC Isolation. Human MCs were isolated by modification of a rapid, nonadherent method (27). Briefly, citrated blood (100–150 ml) was diluted 1:2 with PBS/5 mM EDTA (PBSE), layered onto Ficoll-Paque, and centrifuged at 400 × *g* for 30 min (no brake). The resultant mononuclear cells were serially washed three times with 50 volumes of PBSE by centrifugation at 350, 200, and 150 × *g* for 10 min each. These cells were resuspended in 2–3 ml of PBSE, layered onto two 10-ml columns of fetal bovine serum, and centrifuged at 120 × *g* for 9 min. This was repeated, after which cells were resuspended in 3.5 ml of PBSE, added to 6.7 ml of Sepracell-MN, mixed, and centrifuged at 2000 × *g* for 25 min (no brake). The resultant topmost cell layer (1 ml) was washed twice with 50 volumes of cold RPMI 1640 medium, resuspended in cold RPMI 1640, and used immediately. MCs were >99% viable by trypan blue exclusion and >80% pure by nonspecific esterase and peroxidase staining. Lymphocytes were the major contaminant, platelets numbered <2 per 100 cells, and MCs were judged to be otherwise unactivated by their failure to secrete detectable inflammatory cytokines over a 24-hr culture period (not shown).

Adhesion Assay. MCs (1×10^5 per well) were added to adsorbed surfaces in 20% serum or specific component-depleted serum in total volumes of 50 μl per well. For adhesion inhibition experiments, MCs were gently mixed without or with mAbs in 20% (vol/vol) serum for 1 hr in an ice bath and added to adsorbed surfaces. After 90 min in humidified 95% air/5% CO_2 at 37°C, nonadherent cells were removed by washing with warm (37°C) PBS. Adherent cells were fixed with 2.5% glutaraldehyde in PBS for 20 min, washed thrice in distilled water, and air-dried. Relative numbers of adherent cells were measured by Giemsa staining as described (28) except that stain was released with 200 μl of 15% acetic acid per well. Absorbance of released stain was quantified by using a 630-nm filter. Results are expressed as mean ($n \geq 3$) absorbance units (A_{630}) ± SEM or as the percent adhesion of untreated control cells. Figures depict representative data.

Table 1. Modified polystyrene surfaces

Surface	General properties: Hydrophobic	General properties: Hydrophilic	Surface treatment
PA	+	—	Fluorinated
PB	+	—	Siliconized
PC	—	+	Cationic (N^+)
PM	—	+	Anionic (O^-)

RESULTS

Effects of Specific Component-Depleted Sera. A simple approach for determining the importance of C3 for initial MC adhesive interactions with different surfaces was to compare MC adhesion in 20% intact serum or C3-depleted serum. An experiment of this type is depicted in Fig. 1*A*, which shows that MC adhesion is markedly reduced to PA and PM and partially to PB and PC when C3 is not present. Data is shown in Fig. 1*B*, from another experiment from which similar results were obtained when surfaces were treated first with FG and serum, indicating that C3 remains important for adhesion when adsorbed FG is present. In Fig. 2, this requirement for C3 was confirmed by adding back purified C3 to C3-depleted serum; adhesion was restored on all surfaces.

The same protocol was followed with IgG-depleted serum, but significant reductions in MC adhesion were not observed. When using fibronectin-depleted serum, adhesion was reduced by an average (n = 3 MC donors) of 24%, 20%, and 25% to FG/serum-adsorbed PA, PC, and PM surfaces,

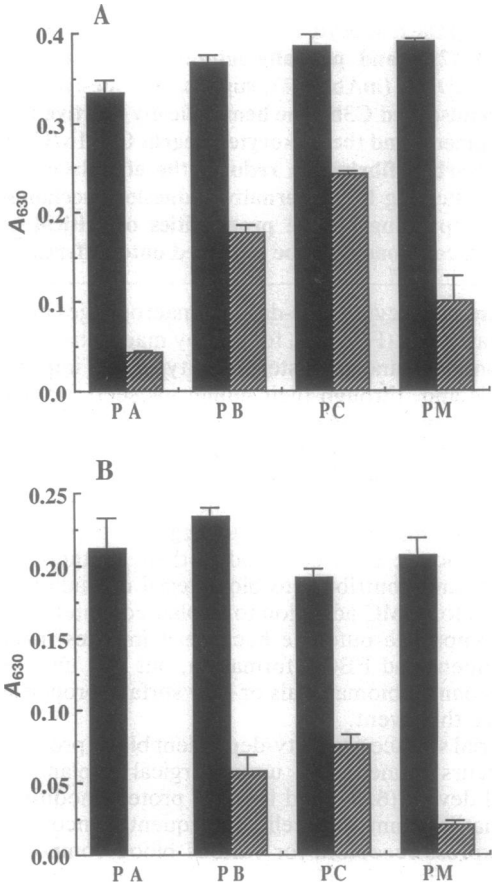

FIG. 1. Effects of C3-depleted serum on MC adhesion to different surfaces. (*A*) MCs were added in the presence of 20% serum (solid bars) or C3-depleted serum (hatched bars). (*B*) Surfaces were treated with FG/serum (solid bars) or FG/C3-depleted serum (hatched bars) for adsorption prior to addition of MCs in the presence of 20% serum or C3-depleted serum, respectively. *A* and *B* represent data from two different MC donors.

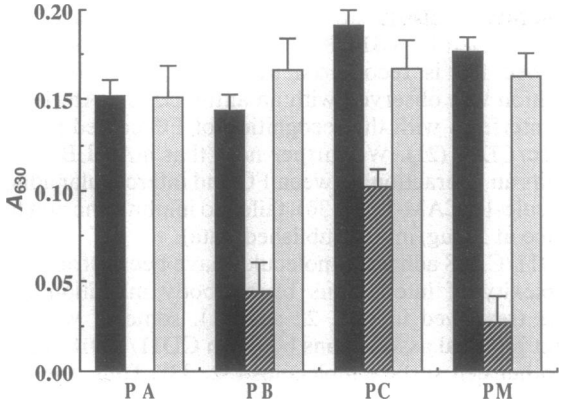

FIG. 2. Effect of repletion of C3-depleted serum with purified C3 on MC adhesion to different surfaces. Surfaces were treated with serum (solid bars), C3-depleted serum (hatched bars), or C3-depleted serum containing 350 μg of C3 per ml (stippled bars), and MCs were added together with 20% serum, C3-depleted serum, or C3-depleted serum containing 350 μg of C3 per ml, respectively.

respectively, but not to the PB surface or any serum-adsorbed surface (not shown).

Effects of mAb to CD11/CD18 Leukocyte Adhesion Receptors. Two anti-CD18 mAbs (60.3 and MHM23) were found to be effective inhibitors of adhesion to all surfaces, and saturating doses of these mAbs were determined, as shown for mAb 60.3 and serum-adsorbed PC in Fig. 3. Control non-cell-binding mAb (anti-α-fetoprotein), cell-binding mAb (anti-HLA-ABC, monomorphic determinant), or functionally defined anti-CD35 did not reduce adhesion (not shown).

Using functionally defined mAbs to leukocyte integrin α chains, we found that anti-CD11b (mAb 60.1) also inhibited MC adhesion (Fig. 3). However, the ability of these mAbs to inhibit adhesion was influenced by adsorption protocol. This is demonstrated in Fig. 4, in which the averaged results from three different MC donors are presented. Again, anti-CD18 (mAb 60.3) was very effective on serum-adsorbed surfaces, but adhesion inhibition was reduced when adsorbed FG was present together with other adsorbed serum components. Similarly, anti-CD11b inhibited adhesion to all serum-adsorbed surfaces; adhesion inhibition by this antibody was consistent, but degrees of inhibition varied between different MC donors. On FG/serum-adsorbed surfaces, however, its effectiveness was greatly diminished or abolished. This was

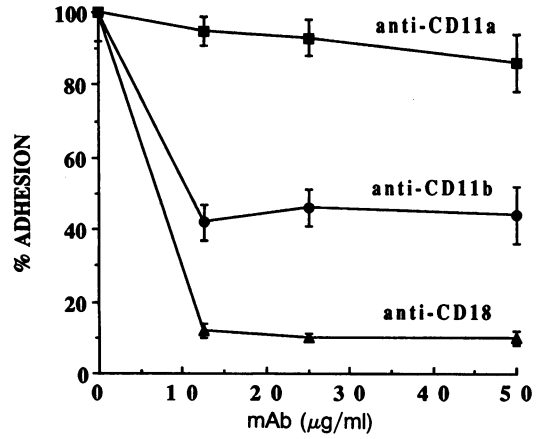

FIG. 3. Effects of mAb to CD11/CD18 adhesion receptors on MC adhesion to serum-adsorbed PC. MC were treated with the indicated concentrations of anti-CD18 (mAb 60.3), anti-CD11b (mAb 60.1), or anti-CD11a (mAb TS1/22) in the presence of 20% serum and were added to serum-adsorbed PC. Results are expressed as the % adhesion of untreated control cells.

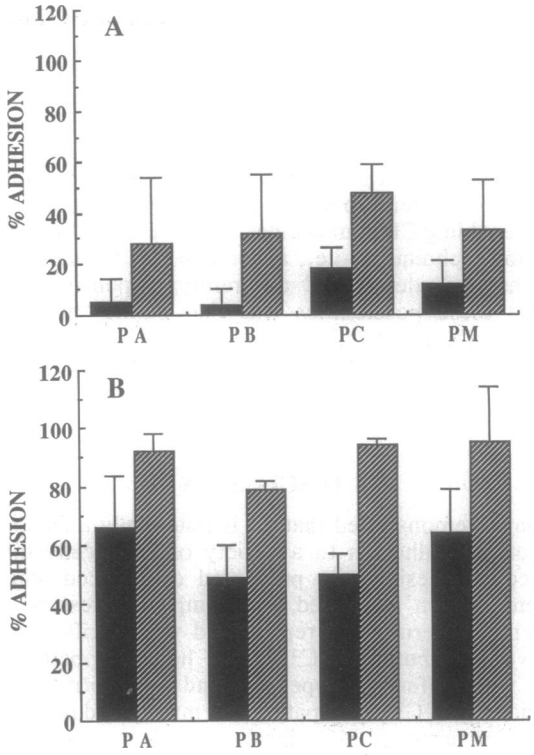

FIG. 4. Effect of mAb to CD18 (mAb 60.3) or CD11b (mAb 60.1) on MC adhesion to different surfaces adsorbed with autologous serum (*A*) or FG/autologous serum (*B*). MC were treated with 15 μg of mAb to CD18 (solid bars) or to CD11b (hatched bars) per ml in the presence of 20% autologous serum and were added to adsorbed surfaces. Results shown depict averaged data from three different MC donors and are expressed as the % adhesion of untreated control cells.

not due to decreases in overall levels of adhesion because relative numbers of adherent MCs from the same donor were always similar for the two adsorption protocols (not shown). mAb 60.1 did not inhibit adhesion to surfaces adsorbed with FG alone in the absence of added serum, confirming that it does not interfere with adhesion to FG (not shown). Anti-CD11c (mAb Leu-M5) was nonblocking (Table 2), as was anti-CD11a (mAb TS1/22) at all concentrations tested (Fig. 3) on all surfaces (not shown).

Inasmuch as mAb 60.1 inhibits rosetting of C3bi-coated, but not C3b-coated, erythrocytes to neutrophils (29), these collective data suggest that the interaction of adsorbed C3bi with MC CD11b/CD18 mediates a substantial degree of adhesion to each serum-adsorbed surface. When adsorbed FG is present together with other components, this apparent C3bi-mediated adhesion is diminished, suggesting alternative

Table 2. Effect of mAb on MC adhesion to different FG/serum-adsorbed surfaces

	MC adhesion, % of values obtained without mAb		
Surface	TS1/18	OKM1	LeuM5
PA	102 ± 3	96 ± 8	96 ± 3
PB	107 ± 6	85 ± 7	114 ± 2
PC	93 ± 8	98 ± 7	103 ± 3
PM	94 ± 12	91 ± 3	109 ± 17

MC were added together with 20% autologous serum to surfaces previously exposed to FG/autologous serum for adsorption. The following antibodies were added at 40 μg/ml: TS1/18 (anti-CD18), OKM1 (anti-CD11b), and Leu-M5 (anti-CD11c).

interactions between adsorbed FG and CD11/CD18 molecules, which are unaffected by these mAbs.

However, MC CD11b/CD18 express binding sites for FG only upon cellular activation with select agonists such as ADP (16), which is not consistent with a role for adsorbed FG in providing an alternative ligand for CD11b/CD18 on otherwise unactivated MCs. Nevertheless, the presentation of solid-phase proteins to MC may have supplied an activational signal enabling CD18 molecules to bind FG, or differences in isolation techniques—i.e., adherence (16) versus density—may have rendered MC differently responsive. Arguing against these possibilities was that neither mAb OKM1 (anti-CD11b) nor mAb TS1/18 (anti-CD18), which inhibit the CD11b/CD18-mediated interaction of ADP-activated MC with FG in solution (16), was able to block adhesion to FG/serum-adsorbed surfaces (Table 2).

DISCUSSION

We have demonstrated that C3 is potentially a major mediator of MC adhesion to a variety of chemically different surfaces. Adhesion was prevented or reduced when C3-depleted serum was used and completely restored when C3-depleted serum was replenished with purified C3. The relative contribution of C3 varied, however, between surfaces with different properties, and a pattern of surface dependency of C3 participation was maintained regardless of adsorption protocol. MC adhesion to fluorinated PA appears to be strikingly dependent on the presence of C3. On oxygenated PM, C3 also plays a major role, whereas on siliconized PB and nitrogenated PC, MC adhesion is partially dependent on C3. Thus, on the latter surfaces, additional adhesion mechanisms that do not largely involve adsorbed fibronectin or IgG operate and remain to be elucidated. The participation of C3 in MC adhesion was clearly extended to surfaces with adsorbed FG, because the presence of adsorbed FG did not compensate for the absence of C3. Therefore, regardless of adsorption protocol, C3 appears to contribute to adhesion to all surfaces, suggesting that in the context of multiple components, either MCs adhere preferentially to adsorbed C3 or indirectly require C3 to interact with other adsorbed proteins.

Antibody inhibition experiments extended these findings by suggesting that the interaction of MC CD11b/CD18 with adsorbed C3bi is an important adhesion mechanism on all serum-adsorbed surfaces. In the presence of adsorbed FG, this major mechanism appears to be considerably replaced on all surfaces because adhesion inhibition by both anti-CD18 and anti-CD11b was diminished. As noted above, however, the presence of adsorbed FG did not compensate for the absence of C3 in overall degrees of adhesion, and when adsorbed FG is present, C3 may still participate in adhesion, but indirectly, by facilitating MC interactions with other components. If so, adsorbed FG may promote these interactions, possibly by restricting opsonization with C3bi (unpublished observations). Thus, different adhesion mechanisms may operate depending on the intrinsic abilities of different surfaces to adsorb and present pertinent adhesion-mediating components to MCs.

Although it is reasonable that such an alternative interaction could be adhesion to adsorbed FG, the present study has not determined this. The binding of soluble FG to MC CD11b/CD18 requires selective cellular activation, and although mAbs TS1/18 and OKM1 are reported to block this binding (16), we found that they did not inhibit otherwise unactivated MC adhesion to FG/serum-adsorbed surfaces (Table 2). We note, however, that OKM1 does not inhibit adhesion of phorbol ester-stimulated neutrophils to FG-coated plastic (17). Thus, from these preliminary data, it cannot be determined whether interactions take place between MC CD11b/CD18 and regions of solid-phase FG that are unaffected by TS1/18 or OKM1. It is also unlikely that adsorbed FG is recognized by CD11c/CD18 because no inhibition was observed with an anti-CD11c mAb (Leu-M5) that interferes with the recognition of FG-coated plastic by CD11c/CD18 (21). We further note that mAb LB-2, which inhibits an interaction between FG and intercellular adhesion molecule-1 (ICAM-1; ref. 30), failed to inhibit adhesion to any surface at 50 μg/ml (unpublished data).

CD11/CD18 adhesion molecules have been attributed to a complexity of interactions by antibody inhibition experiments (reviewed in refs. 22 and 31), some of which may reflect physical associations between CD11/CD18 receptors and other cell surface molecules (32, 33). Thus, the overall lesser levels of adhesion inhibition observed on serum-adsorbed surfaces with anti-CD11b may represent CD11b/CD18 ligand-dependent (C3bi-mediated) adhesion, while a portion of anti-CD18-inhibitable adhesion is attributed to CD11/CD18 ligand-independent adhesive interactions for which functional CD18 molecules are still required. A further and intriguing possibility, which may be particularly relevant on certain hydrophobic materials, is that CD11b/CD18 and CD11c/CD18 recognize as yet undefined protein domains that have been denatured by adsorption (34).

The engagement of biomaterial surface-dependent MC adhesion mechanisms may have important consequences for the inflammatory response at the implant site *in vivo*. Adhesion to different adsorbed proteins selectively stimulates transcription of genes for MC inflammatory mediators, effects that are inhibited by anti-CD18 (35, 36). Similarly, the cytokine-induced secretion of reactive oxygen intermediates by neutrophils occurs only if these cells are adherent to certain proteins and is blocked by anti-CD18 (37). MC/macrophage CD11b/CD18 interactions with solid-phase FG but not fibronectin increase the activation-induced cell surface expression of tissue factor (38) and secretion of tumor necrosis factor α (39). Of interest, the latter response is not merely contact-dependent because cellular spreading occurred on fibronectin. Macrophage phagocytic activity (40, 41), migration (42), and macrophage fusion to form FBGCs (40, 43) are also differently influenced by adhesion to different proteins, further emphasizing the importance of adhesive substrate in determining phenotype and function.

Finally, we raise two issues. First, MC–material interactions of the type we have measured—i.e., the initial adhesion of otherwise unactivated MC—may not reflect those adhesion mechanisms that operate *in vivo* at the inflammatory site created by biomaterial implantation. Here, we have attempted to address how MC might adhere to different surfaces in the absence of activation by inflammatory mediators but when presented with multiple adsorbed and soluble protein components. Second, we question whether the adhesion mechanisms that mediate initial interactions of otherwise unactivated or inflammatory mediator-activated MC with different surfaces will lead to macrophage development and FBGC formation and how surface properties will affect MC/macrophage/FBGC functions that are relevant to biocompatible outcome. Answers to these questions could be useful for the design of biomaterials that either minimize MC adhesion or promote MC adhesion that is unproductive in terms of leading to macrophage development and fusion.

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