CHAPTER

4

Host Reactions to Biomaterials and Their Evaluation

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4.1 INTRODUCTION

Frederick J. Schoen

Biomaterials and medical devices are now commonly used as prostheses in cardiovascular, orthopedic, dental, ophthalmological, and reconstructive surgery, in interventions such as angioplasty (stents) and hemodialysis (membranes), in surgical sutures or bioadhesives, and as controlled drug release devices. Most implants serve their recipients well for extended periods by alleviating the conditions for which they were implanted. However, some implants and extracorporeal devices ultimately develop complications—adverse interactions of the patient with the device, or vice versa—which constitute device failure and thereby may cause harm to or death of the patient. Complications result largely as a consequence of biomaterial–tissue interactions, which all implants have with the environment into which they are placed. Effects of both the implant on the host tissues and the host on the implant are important in mediating complications and device failure (Fig. 1).

Chapter 4 contains overview discussions of the most important host reactions to biomaterials and their evaluation, including nonspecific inflammation and specific immunological reactions, systemic effects, blood–materials interactions, tumor formation, and infection. To a great extent, these interactions arise from alterations of physiological (normal) processes (e.g., immunity, inflammation, blood coagulation) comprising host defense mechanisms that function to protect an organism from the deleterious external threats (such as bacteria and other microbiologic organisms, injury and foreign materials). Chapter 6 addresses degradation mechanisms in biomaterials (i.e., the effect of the host on biomaterials). Several key concepts of biomaterials–tissue interactions are emphasized here in an effort to guide the reader and facilitate the use of this chapter.

THE INFLAMMATORY REACTION TO BIOMATERIALS

In their respective chapters, Anderson, Mitchell, and Johnson describe the inflammatory and potential immunological interactions that occur with biomaterials. In contrast to living organ transplants, biomaterials are not generally "rejected." The process of organ rejection denotes an inflammatory process that results from a specific immune response and which causes tissue death, which synthetic biomaterials typically do not generate. The usual response to biomaterials comprises nonspecific inflammation (see Chapters 4.2, 4.3, and 4.4).

However, as summarized by Mitchell (2001), tissue-derived biomaterials (such as bioprosthetic heart valves) may express foreign histocompatibility antigens and be antigenic and capable of eliciting an immune response, including antibodies and antigen-specific T cells. Nevertheless, it is important to understand the following:

1. Tissue immunogenicity does not necessarily induce immunologically mediated device dysfunction.
2. Specific immunological responses can be not only a cause of but can result from device failure.
3. Although mononuclear inflammatory cell infiltrates (containing macrophages and lymphocytes) are characteristically associated with organ/tissue rejection on histological examination, mononuclear inflammatory infiltrates are themselves nonspecific and comprise a largely stereotyped and generic response to tissue injury. Therefore, the presence of mononuclear cells does not necessarily denote a rejection pathogenesis.

In order to invoke an immunological reaction to a biomaterial as the cause of a device failure, an immunological variant of the classical Koch's postulates, which are the objective criteria for concluding that a disease is infectious and caused by a specific microbiologic agent, would be appropriate. The classic Koch's postulates state that:

1. A suspected infectious agent should be recoverable from the pathologic lesions of the human host.
2. The agent should cause the pathologic lesions when inoculated into an animal host.
3. The agent should then be recoverable from the pathologic lesions in the animal.

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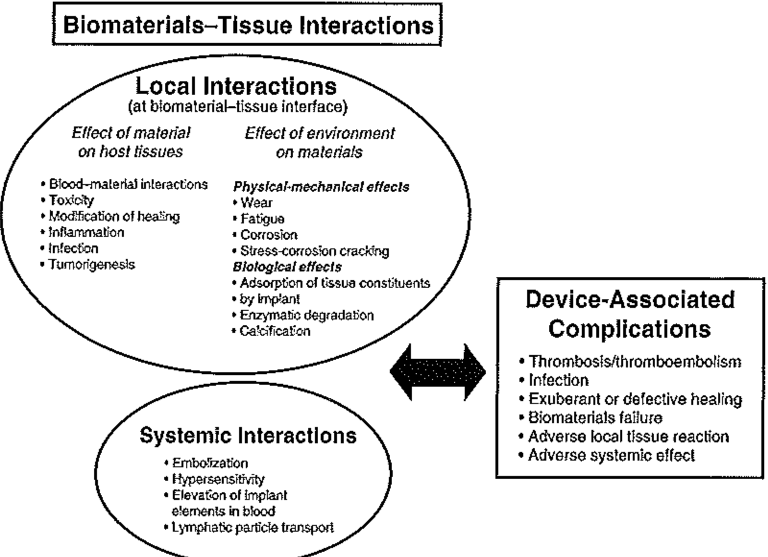

FIG. 1. Biomaterials–tissue interactions (reproduced from Schoen FJ). In: *Advances in Cardiovascular Medicine* (Harvey 1602–2002 Symposium, on the 4th Centenary of William Harvey's Graduation at the University of Pauda), Thiene G, Pessina AC (eds.), Universita degli Studi di Padova, 2002; 289–307.

Mitchell describes an immunological variant of Koch's postulates to test an immunological hypothesis for calcific and noncalcific bioprosthetic valve failure (Schoen, 1999) as follows:

1. Antigen-specific elements (antibodies or cells) should be directly associated with failing valves. Moreover, control experiments should be performed to demonstrate that any antibodies or cells on implanted valves are not simply present because of surgical manipulation or aberrant flow conditions.
2. Antibodies or cells from experimental animals that have dysfunctional implanted valves transferred into an appropriate second host (immunologically matched to the original valve donor) should cause valve failure.
3. Adoptively transferred cells or antibodies should be detectable on a failed valve in the second recipient.

Although some evidence for these criteria can be obtained in humans, carefully designed animal investigations provide the only rigorous way to satisfy them. With respect to tissue heart valves, although some investigators have demonstrated that such tissues can be immunogenic, there exists no evidence that valve destruction or loss of function is mediated by immune elements, or that blockade of immune mechanisms by immunosuppression prevents that outcome.

Most biomaterials of potential clinical interest typically elicit the foreign body reaction (FBR), a special form of nonspecific inflammation. The most prominent cells in the FBR are macrophages, which attempt to phagocytose the material and are variably successful, but complete engulfment and degradation are often difficult. The macrophages, activated in the process of interacting with a biomaterial, may elaborate cytokines that stimulate inflammation or fibrosis. Multinucleated giant cells in the vicinity of a foreign body are generally considered evidence of a more severe FBR. The more "biocompatible" the implant, the more quiescent (less inflammation in) the ultimate response. When the implant is a source of particles not easily controlled, such as wear debris from articulating joint surfaces (Jacobs *et al.*, 2001), the inability of inflammatory cells to adhere to but not phagocytose particles larger than a critical size ("frustrated phagocytosis") can lead to release of enzymes (exocytosis) and cytokines and other chemical mediators (e.g., prostaglanding, tumor necrosis factor-alpha, and interleukin-1) and cause harm to the extracellular environment. Thus, inflammatory cell products that are critical in killing microorganisms in typical inflammation can damage tissue adjacent to foreign bodies.

The nature of the reaction is largely dependent on the chemical and physical characteristics of the implant. For most inert biomaterials, the late tissue reaction is encapsulation by a relatively thin fibrous tissue capsule (composed of collagen and fibroblasts). Tissue interactions can be modified by changing the chemistry of the surface (e.g., by adding specific chemical groupings to stimulate adhesion or bone formation in orthopedic implants), inducing roughness or porosity to enhance physical binding to the surrounding tissues, incorporating a surface-active agent to chemically bond the tissue, or using a bioresorbable component to allow slow replacement by tissue to simulate natural healing properties.

SYSTEMIC AND REMOTE EFFECTS

Hensten-Pettersen and Jacobsen summarize biomaterials-related systemic toxicity and hypersensitivity reaction (through lymphatics and the bloodstream) in animals and patients with either stainless steel or cobalt-base orthopedic total joint replacement components, elevations of metallic components occur in tissue (at both local and remote sites) and in serum

and urine. Transport of particulates over large distances by macrophages to regional lymph nodes and to the lungs has been considered a systemic and remote effect. As a consequence of silicone migrated through lymphatic vessels to lymph nodes, an enlarged, hard axillary lymph node in a woman who received a silicone-gel breast prosthesis for reconstruction following mastectomy for a carcinoma can be misdiagnosed as tumor.

"Metal allergy" is well-recognized and is frequently associated in women with the use of cheap, high-nickel-alloy costume jewelry or earrings and can occur in association with metallic implants (Hallab *et al.*, 2001). By themselves, metal ions lack the structural complexity required to challenge the immune system. However, when combined with proteins, such as those available in the skin, connective tissues, and blood, a wide variety of metals induce immune responses and this can have clinical effects. Cobalt, chromium, and nickel are included in this category, with nickel perhaps the most potent; at least 10% of a normal population will be sensitive by skin test to one or more of these metals, at some threshold level.

THROMBOEMBOLIC COMPLICATIONS

Hanson emphasizes that exposure of blood to an artificial surface can induce thrombosis, embolization, and consumption of platelets and plasma coagulation factors, as well as the systemic effects of activated coagulation and complement products, and platelet activation. It is clear that no synthetic or modified biological surface generated by man is as resistant to thrombosis (thromboresistant) as normal unperturbed endothelium (the cellular lining of the circulatory system). However, it is important to understand that under some circumstances endothelial cells can be "dysfunctional" and although physically intact can express prothrombotic molecules that can induce thrombosis (Bonetti *et al.*, 2003).

Thromboembolic complications are a major cause of mortality and morbidity with cardiovascular devices. Both fibrin (red) thrombus and platelet (white) thrombus form in association with valves and other cardiovascular devices. As in the cardiovascular system in general, Virchow's triad (i.e., the conditions of surface thrombogenicity, hypercoagulability, and locally static blood flow) largely predicts the relative propensity toward thrombus formation and often the location of thrombotic deposits with cardiovascular prostheses (Anderson and Schoen, 1992). However, despite over a quarter century of intense research, the physical and chemical characteristics of materials that control the outcome of blood–surface interaction are incompletely understood.

When non-physiologic surfaces contact blood, three events comprise thrombotic interactions: 1) plasma protein deposition, 2) adhesion of platelets and leukocytes, and 3) bulk fibrin formation (blood coagulation). All foreign materials exposed to blood spontaneously and rapidly (seconds) absorb a film of plasma protein, largely fibrinogen. This is followed by cellular thrombogenesis (beginning with platelet adhesion to the first adsorbed plasma proteins). If conditions of relatively static flow are present, the fiber-forming steps of the coagulation process occur, and macroscopic thrombus ensues.

Considerable evidence implicates a primary regulatory role for blood platelets in the thrombogenic response to artificial surfaces. Platelet adhesion to artificial surfaces strongly resembles that of adhesion to the vascular subendothelium that has been exposed by injury. Nevertheless, the major clinical approach to controlling thrombosis in cardiovascular devices is the use of systemic anticoagulants, particularly coumadin (warfarin), which inhibits thrombin and fibrin formation but does not inhibit platelet-mediated thrombosis.

TUMORIGENESIS

Schoen emphasizes that although animals frequently have sarcomas at the site of an experimental biomaterial implant, neoplasms in humans occurring at the site of implanted medical devices are rare, despite the large numbers of implants used clinically over an extended duration. Moreover, the presence of a neoplasm at an implant site does not prove that the implant had a causal role. Cancers associated with foreign bodies can appear at any postoperative interval but tend to occur many years postoperatively. The pathogenesis of implant-induced tumors is not well understood; most experimental data indicate that the physical rather than chemical characteristics of the foreign body primarily determine tumorigenicity.

INFECTION

Infection occurs in as many as 5 to 10% of patients with implanted prosthetic devices and is a major source of morbidity and mortality (Jansen and Peters, 1993; Klug *et al.*, 1997; Kunin *et al.*, 1988; Mulcahy, 1999; Schierholz and Beuth, 2001; Tanner *et al.*, 1997; Vlessis *et al.*, 1997). Infections associated with medical devices are often resistant to antibiotics and host defenses, often persisting until the devices are removed. Early implant infections (less than approximately 1 to 2 months postoperatively) are most likely due to intraoperative contamination from airborne sources or nonsterile surgical technique, or to early postoperative complications such as wound infection. In contrast, late infections likely occur by a hematogenous (blood-borne) route and are often initiated by bacteremia induced by therapeutic dental or genitourinary procedures. Perioperative prophylactic antibiotics and periodic antibiotic prophylaxis given shortly before diagnostic and therapeutic procedures protect against implant infection. Infections associated with foreign bodies are characterized microbiologically by a high prevalence of *Staphylococcus epidermidis* and other staphylococci, especially *S. aureus*. Ordinarily, *S. epidermidis* is an organism with low virulence and thus an infrequent cause of non-prosthesis-associated deep infections. This emphasizes the unique environment in the vicinity of a foreign body.

The presence of a foreign body per se potentiates infection. A classic experiment indicated that the staphylococcal bacterial inoculum required to cause infection in the presence of a foreign implant was 10,000 less than that when no foreign body was present (Elek and Conen, 1957). Devices could facilitate infection in several ways. Microorganisms are provided access to the circulation and to deeper tissue by damage to natural barriers

against infection during implantation or subsequent function of a prosthetic device. Moreover, an implanted foreign body could (1) limit phagocyte migration into infected tissue or (2) interfere with inflammatory cell phagocytic mechanisms, through release of soluble implant components or surface-mediated interactions, thus allowing bacteria to survive adjacent to the implant. As Costerton *et al.* emphasize, adhesion of bacteria to the prosthetic surface and the formation of microcolonies within an adherent biofilm are fundamental steps in the pathogenesis of clinical and experimental infections associated with foreign bodies.

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4.2 Inflammation, Wound Healing, and the Foreign-Body Response

James M. Anderson

Inflammation, wound healing, and foreign body reaction are generally considered as parts of the tissue or cellular host responses to injury. Table 1 lists the sequence/continuum of these events following injury. Overlap and simultaneous occurrence of these events should be considered; e.g., the foreign body reaction at the implant interface may be initiated with the onset of acute and chronic inflammation. From a biomaterials perspective, placing a biomaterial in the *in vivo* environment requires injection, insertion, or surgical implantation, all of which injure the tissues or organs involved.

TABLE 1 Sequence/Continuum of Host Reactions Following Implantation of Medical Devices

Injury
Blood–material interactions
Provisional matrix formation
Acute inflammation
Chronic inflammation
Granulation tissue
Foreign-body reaction
Fibrosis/fibrous capsule development

The placement procedure initiates a response to injury by the tissue, organ, or body and mechanisms are activated to maintain homeostasis. The degrees to which the homeostatic mechanisms are perturbed and the extent to which pathophysiologic conditions are created and undergo resolution are a measure of the host reactions to the biomaterial and may ultimately determine its biocompatibility. Although it is convenient to separate homeostatic mechanisms into blood–material or tissue–material interactions, it must be remembered that the various components or mechanisms involved in homeostasis are present in both blood and tissue and are a part of the physiologic continuum. Furthermore, it must be noted that host reactions may be tissue-dependent, organ-dependent, and species-dependent. Obviously, the extent of injury varies with the implantation procedure.

OVERVIEW

Inflammation is generally defined as the reaction of vascularized living tissue to local injury. Inflammation serves to contain, neutralize, dilute, or wall off the injurious agent or process. In addition, it sets into motion a series of events that may heal and reconstitute the implant site through replacement of the injured tissue by regeneration of native parenchymal cells, formation of fibroblastic scar tissue, or a combination of these two processes.

Immediately following injury, there are changes in vascular flow, caliber, and permeability. Fluid, proteins, and blood cells escape from the vascular system into the injured tissue in a process called exudation. Following changes in the vascular system, which also include changes induced in blood and its components, cellular events occur and characterize the inflammatory response. The effect of the injury and/or biomaterial *in situ* on plasma or cells can produce chemical factors

TABLE 2 Cells and Components of Vascularized Connective Tissue

Intravascular (blood) cells
Erythrocytes (RBC)
Neutrophils (PMNs, polymorphonuclear leukocytes)
Monocytes
Eosinophils
Lymphocytes
Plasma cells
Basophils
Platelets
Connective tissue cells
Mast cells
Fibroblasts
Macrophages
Lymphocytes
Extracellular matrix components
Collagens
Elastin
Proteoglycans
Fibronectin
Laminin

that mediate many of the vascular and cellular responses of inflammation.

Blood–material interactions and the inflammatory response are intimately linked, and in fact, early responses to injury involve mainly blood and vasculature. Regardless of the tissue or organ into which a biomaterial is implanted, the initial inflammatory response is activated by injury to vascularized connective tissue (Table 2). Since blood and its components are involved in the initial inflammatory responses, blood clot formation and/or thrombosis also occur. Blood coagulation and thrombosis are generally considered humoral responses and may be influenced by other homeostatic mechanisms such as the extrinsic and intrinsic coagulation systems, the complement system, the fibrinolytic system, the kinin-generating system, and platelets. Thrombus or blood clot formation on the surface of a biomaterial is related to the well-known Vroman effect (see Chapter 3.2), in which a hierarchical and dynamic series of collision, absorption, and exchange processes, determined by protein mobility and concentration, regulate early time-dependent changes in blood protein adsorption. From a wound-healing perspective, blood protein deposition on a biomaterial surface is described as provisional matrix formation. Blood interactions with biomaterials are generally considered under the category of hematocompatibility and are discussed elsewhere in this book.

Injury to vascularized tissue in the implantation procedure leads to immediate development of the provisional matrix at the implant site. This provisional matrix consists of fibrin, produced by activation of the coagulation and thrombosis systems, and inflammatory products released by the complement system, activated platelets, inflammatory cells, and endothelial cells. These events occur early, within minutes to hours following implantation of a medical device. Components within or released from the provisional matrix, i.e., fibrin network (thrombosis or clot), initiate the resolution, reorganization, and repair processes such as inflammatory cell and fibroblast recruitment. The provisional matrix appears to provide both structural and biochemical components to the process of wound healing. The complex three-dimensional structure of the fibrin network with attached adhesive proteins provides a substrate for cell adhesion and migration. The presence of mitogens, chemoattractants, cytokines, and growth factors within the provisional matrix provides for a rich milieu of activating and inhibiting substances for various cellular proliferative and synthetic processes. The provisional matrix may be viewed as a naturally derived, biodegradable, sustained release system in which mitogens, chemoattractants, cytokines, and growth factors are released to control subsequent wound-healing processes. In spite of the increase in our knowledge of the provisional matrix and its capabilities, our knowledge of the control of the formation of the provisional matrix and its effect on subsequent wound healing events is poor. In part, this lack of knowledge is due to the fact that much of our knowledge regarding the provisional matrix has been derived from *in vitro* studies, and there is a paucity of *in vivo* studies that provide for a more complex perspective. Little is known regarding the provisional matrix which forms at biomaterial and medical device interfaces *in vivo*, although attractive hypotheses have been presented regarding the presumed ability of materials and protein adsorbed materials to modulate cellular interactions through their interactions with adhesive molecules and cells.

The predominant cell type present in the inflammatory response varies with the age of the inflammatory injury (Fig. 1). In general, neutrophils predominate during the first several days following injury and then are replaced by monocytes as the predominant cell type. Three factors account for this change in cell type: neutrophils are short lived and disintegrate and disappear after 24–48 hour; neutrophil emigration from the vasculature to the tissues is of short duration; and chemotactic factors for neutrophil migration are activated early in the inflammatory response. Following emigration from the vasculature, monocytes differentiate into macrophages and these cells are very long-lived (up to months). Monocyte emigration

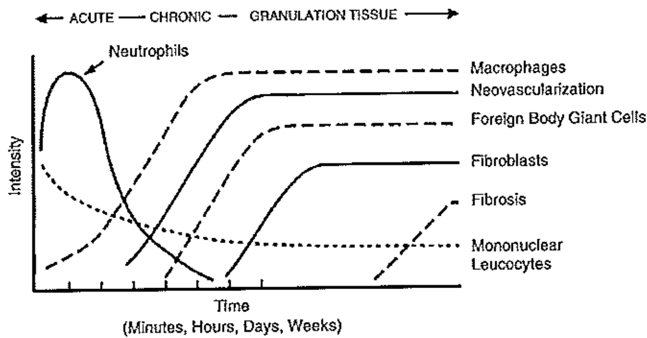

FIG. 1. The temporal variation in the acute inflammatory response, chronic inflammatory response, granulation tissue development, and foreign-body reaction to implanted biomaterials. The intensity and time variables are dependent upon the extent of injury created in the implantation and the size, shape, topography, and chemical and physical properties of the biomaterial.

may continue for day to weeks, depending on the injury and implanted biomaterial, and chemotactic factors for monocytes are activated over longer periods of time.

The temporal sequence of events following implantation of a biomaterial is illustrated in Fig. 1. The size, shape, and chemical and physical properties of the biomaterial may be responsible for variations in the intensity and duration of the inflammatory or wound-healing process. Thus, intensity and/or time duration of the inflammatory reaction may characterize the biocompatibility of a biomaterial.

While injury initiates the inflammatory response, the chemicals released from plasma, cells, or injured tissue mediate the inflammatory response. Important classes of chemical mediators of inflammation are presented in Table 3. Several points must be noted in order to understand the inflammatory response and how it relates to biomaterials. First, although chemical mediators are classified on a structural or functional basis, different mediator systems interact and provide a system of checks and balances regarding their respective activities and functions. Second, chemical mediators are quickly inactivated or destroyed, suggesting that their action is predominantly local (i.e., at the implant site). Third, generally the lysosomal proteases and the oxygen-derived free radicals produce the most significant damage or injury. These chemical mediators are also important in the degradation of biomaterials.

TABLE 3 Important Chemical Mediators of Inflammation Derived from Plasma, Cells, or Injured Tissue

Mediators	Examples
Vasoactive agents	Histamines, serotonin, adenosine, endothelial-derived relaxing factor (EDRF), prostacyclin, endothelin, thromboxane α_2
Plasma proteases	
Kinin system	Bradykinin, kallikrein
Complement system	C3a, C5a, C3b, C5b-C9
Coagulation/fibrinolytic system	Fibrin degradation products, activated Hageman factor (FXIIA), tissue plasminogen activator (tPA)
Leukotrienes	Leukotriene B_4 (LTB_4), hydroxyeicosatetranoic acid (HETE)
Lysosomal proteases	Collagenase, elastase
Oxygen-derived free radicals	H_2O_2, superoxide anion
Platelet activating factors	Cell membrane lipids
Cytokines	Interleukin 1 (IL-1), tumor necrosis factor (TNF)
Growth factors	Platelet-derived growth factor (PDGF), fibroblast growth Factor (FGF), transforming growth factor TGF-α or (TGF-β), epithelial growth factor (EGF)

ACUTE INFLAMMATION

Acute inflammation is of relatively short duration, lasting for minutes to hours to days, depending on the extent of injury. Its main characteristics are the exudation of fluid and plasma proteins (edema) and the emigration of leukocytes (predominantly neutrophils). Neutrophils (polymorphonuclear leukocytes, PMNs) and other motile white cells emigrate or move from the blood vessels to the perivascular tissues and the injury (implant) site. Leukocyte emigration is assisted by "adhesion molecules" present on leukocyte and endothelial surfaces. The surface expression of these adhesion molecules can be induced, enhanced, or altered by inflammatory agents and chemical mediators. White cell emigration is controlled, in part, by chemotaxis, which is the unidirectional migration of cells along a chemical gradient. A wide variety of exogenous and endogenous substances have been identified as chemotactic agents. Specific receptors for chemotactic agents on the cell membranes of leukocytes are important in the emigration or movement of leukocytes. These and other receptors also play a role in the transmigration of white cells across the endothelial lining of vessels and activation of leukocytes. Following localization of leukocytes at the injury (implant) site, phagocytosis and the release of enzymes occur following activation of neutrophils and macrophages. The major role of the neutrophil in acute inflammation is to phagocytose microorganisms and foreign materials. Phagocytosis is seen as a three-step process in which the injurious agent undergoes recognition and neutrophil attachment, engulfment, and killing or degradation. In regard to biomaterials, engulfment and degradation may or may not occur, depending on the properties of the biomaterial.

Although biomaterials are not generally phagocytosed by neutrophils or macrophages because of the disparity in size (i.e., the surface of the biomaterial is greater than the size of the cell), certain events in phagocytosis may occur. The process of recognition and attachment is expedited when the injurious agent is coated by naturally occurring serum factors called "opsonins." The two major opsonins are immunoglobulin G (IgG) and the complement-activated fragment, C3b. Both of these plasma-derived proteins are known to adsorb to biomaterials, and neutrophils and macrophages have corresponding cell-membrane receptors for these opsonization proteins. These receptors may also play a role in the activation of the attached neutrophil or macrophage. Other blood proteins such as fibrinogen, fibronectin, and vitronectin may also facilitate cell adhesion to biomaterial surfaces. Owing to the disparity in size between the biomaterial surface and the attached cell, frustrated phagocytosis may occur. This process does not involve engulfment of the biomaterial but does cause the extracellular release of leukocyte products in an attempt to degrade the biomaterial.

Henson has shown that neutrophils adherent to complement-coated and immunoglobulin-coated nonphagocytosable surfaces may release enzymes by direct extrusion or exocytosis from the cell. The amount of enzyme released during this process depends on the size of the polymer particle, with larger particles inducing greater amounts of enzyme release. This suggests that the specific mode of cell activation in the inflammatory response in tissue depends upon the size of the implant

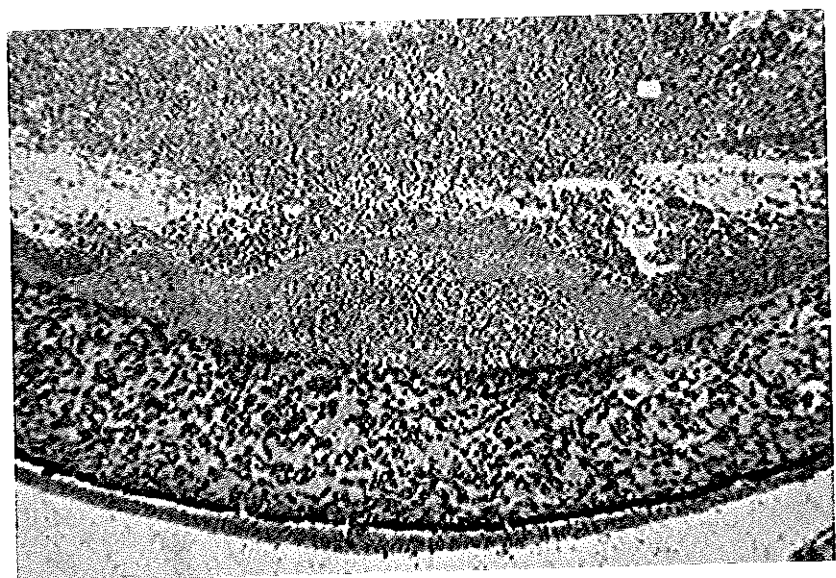

FIG. 2. Acute inflammation, secondary to infection, of an ePTFE vascular graft. A focal zone of polymorphonuclear leukocytes is present at the lumenal surface of the vascular graft, surrounded by a fibrin cap, on the blood-contacting surface of the ePTFE vascular graft. Hematoxylin and eosin stain. Original magnification 4×. (See color plate)

and that a material in a phagocytosable form (i.e., powder or particulate) may provoke a different degree of inflammatory response than the same material in a nonphagocytosable form (i.e., film).

Acute inflammation normally resolves quickly, usually less than 1 week, depending on the extent of injury at the implant site. However, the presence of acute inflammation (i.e., PMNs) at the tissue/implant interface at time periods beyond 1 week (i.e., weeks, months, or years) suggests the presence of an infection (Fig. 2).

CHRONIC INFLAMMATION

Chronic inflammation is less uniform histologically than acute inflammation. In general, chronic inflammation is characterized by the presence of macrophages, monocytes, and lymphocytes, with the proliferation of blood vessels and connective tissue. Many factors can modify the course and histologic appearance of chronic inflammation.

Persistent inflammatory stimuli lead to chronic inflammation. While the chemical and physical properties of the biomaterial in themselves may lead to chronic inflammation, motion in the implant site by the biomaterial or infection may also produce chronic inflammation. The chronic inflammatory response to biomaterials is usually of short duration and is confined to the implant site. The presence of mononuclear cells, including lymphocytes and plasma cells, is considered chronic inflammation, whereas the foreign-body reaction with the development of granulation tissue is considered the normal wound healing response to implanted biomaterials (i.e., the normal foreign-body reaction). Chronic inflammation with the presence of collections of lymphocytes and monocytes

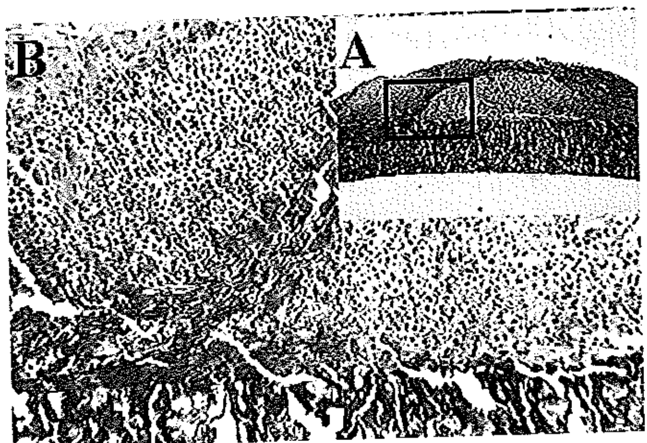

FIG. 3. Chronic inflammation, secondary to infection, of an ePTFE arteriovenous shunt for renal dialysis. (A) Low-magnification view of a focal zone of chronic inflammation. (B) High-magnification view of the outer surface with the presence of monocytes and lymphocytes at an area where the outer PTFE wrap had peeled away from the vascular graft. Hematoxylin and eosin stain. Original magnification (A) 4×, (B) 20×. (See color plate)

at extended implant times (weeks, months, years) also may suggest the presence of a long-standing infection (Fig. 3A, B).

Lymphocytes and plasma cells are involved principally in immune reactions and are key mediators of antibody production and delayed hypersensitivity responses. Although they may be present in nonimmunologic injuries and inflammation their roles in such circumstances are largely unknown. Little is known regarding humoral immune responses and cell-mediated immunity to synthetic biomaterials. The role of macrophages must be considered in the possible development

materials, particulate, or microspheres will have higher ratios of macrophages and foreign-body giant cells in the implant site than smooth-surface implants, which will have fibrosis as a significant component of the implant site.

The foreign-body reaction consisting mainly of macrophages and/or foreign-body giant cells may persist at the tissue–implant interface for the lifetime of the implant (Fig. 1). Generally, fibrosis (i.e., fibrous encapsulation) surrounds the biomaterial or implant with its interfacial foreign-body reaction, isolating the implant and foreign-body reaction from the local tissue environment. Early in the inflammatory and wound-healing response, the macrophages are activated upon adherence to the material surface.

Although it is generally considered that the chemical and physical properties of the biomaterial are responsible for macrophage activation, the subsequent events regarding the activity of macrophages at the surface are not clear. Tissue macrophages, derived from circulating blood monocytes, may coalesce to form multinucleated foreign-body giant cells. It is not uncommon to see very large foreign-body giant cells containing large numbers of nuclei on the surface of biomaterials. While these foreign-body giant cells may persist for the lifetime of the implant, it is not known if they remain activated, releasing their lysosomal constituents, or become quiescent.

Figure 5 demonstrates the progression from circulating blood monocyte to tissue macrophage to foreign-body giant cell development that is most commonly observed. Indicated in the figure are important biological responses that are considered to play an important role in FBGC development. Material surface chemistry may control adherent macrophage apoptosis (i.e., programmed cell death) (see Chapter 3.3) that renders potentially harmful macrophages nonfunctional, while the surrounding environment of the implant remains unaffected. The level of adherent macrophage apoptosis appears to be inversely related to the surface's ability to promote diffusion of macrophages into FBGCs, suggesting a mechanism for macrophages to escape apoptosis.

Figure 8 demonstrates the sequence of events involved in inflammation and wound healing when medical devices are implanted. In general, the PMN predominant acute inflammatory response and the lymphocyte/monocyte predominant chronic inflammatory response resolve quickly (i.e., within 2 weeks) depending on the type and location of the implant. Studies using IL-4 or IL-13, respectively, demonstrate the role for Th2 helper lymphocytes in the development of the foreign body reaction at the tissue/material interface. Th2 helper lymphocytes have been described as "antiinflammatory" based on their cytokine profile, of which IL-4 is a significant component.

FIBROSIS/FIBROUS ENCAPSULATION

The end-stage healing response to biomaterials is generally fibrosis or fibrous encapsulation (Fig. 9). However, there may be exceptions to this general statement (e.g., porous materials inoculated with parenchymal cells or porous materials implanted into bone) (Fig. 10). As previously stated, the tissue response to implants is in part dependent upon the extent of injury or defect created in the implantation procedure and the amount of provisional matrix.

Repair of implant sites can involve two distinct processes: regeneration, which is the replacement of injured tissue by parenchymal cells of the same type, or replacement by connective tissue that constitutes the fibrous capsule. These processes are generally controlled by either (1) the proliferative capacity of the cells in the tissue or organ receiving the implant and the extent of injury as it relates to the destruction, or (2) persistence of the tissue framework of the implant site. See Chapter 3.4 for a more complete discussion of the types of cells present in the organ parenchyma and stroma, respectively.

The regenerative capacity of cells allows them to be classified into three groups: labile, stable (or expanding), and permanent (or static) cells (see Chapter 3.3). Labile cells continue to proliferate throughout life; stable cells retain this capacity but do not normally replicate; and permanent cells cannot reproduce themselves after birth. Perfect repair with restitution of normal structure can theoretically occur only in tissues consisting of stable and labile cells, whereas all injuries to tissues composed of permanent cells may give rise to fibrosis and fibrous capsule formation with very little restitution of the normal tissue or organ structure. Tissues composed of permanent cells (e.g., nerve cells and cardiac muscle cells) most commonly undergo an organization of the inflammatory exudate, leading to fibrosis. Tissues of stable cells (e.g., parenchymal cells of the liver, kidney, and pancreas); mesenchymal cells (e.g., fibroblasts, smooth muscle cells, osteoblasts, and chondroblasts); and vascular endothelial and labile cells (e.g., epithelial cells and lymphoid and hematopoietic cells) may also follow this pathway to fibrosis or may undergo resolution of the inflammatory exudate, leading to restitution of the normal tissue structure.

The condition of the underlying framework or supporting stroma of the parenchymal cells following an injury plays an important role in the restoration of normal tissue structure. Retention of the framework with injury may lead to restitution of the normal tissue structure, whereas destruction of the framework most commonly leads to fibrosis. It is important to consider the species-dependent nature of the regenerative capacity of cells. For example, cells from the same organ or tissue but from different species may exhibit different regenerative capacities and/or connective tissue repair.

Following injury, cells may undergo adaptations of growth and differentiation. Important cellular adaptations are atrophy (decrease in cell size or function), hypertrophy (increase in cell size), hyperplasia (increase in cell number), and metaplasia (change in cell type). Other adaptations include a change by cells from producing one family of proteins to another (phenotypic change), or marked overproduction of protein. This may be the case in cells producing various types of collagens and extracellular matrix proteins in chronic inflammation and fibrosis. Causes of atrophy may include decreased workload (e.g., stress-shielding by implants), and diminished blood supply and inadequate nutrition (e.g., fibrous capsules surrounding implants).

Local and systemic factors may play a role in the wound-healing response to biomaterials or implants. Local factors include the site (tissue or organ) of implantation, the adequacy

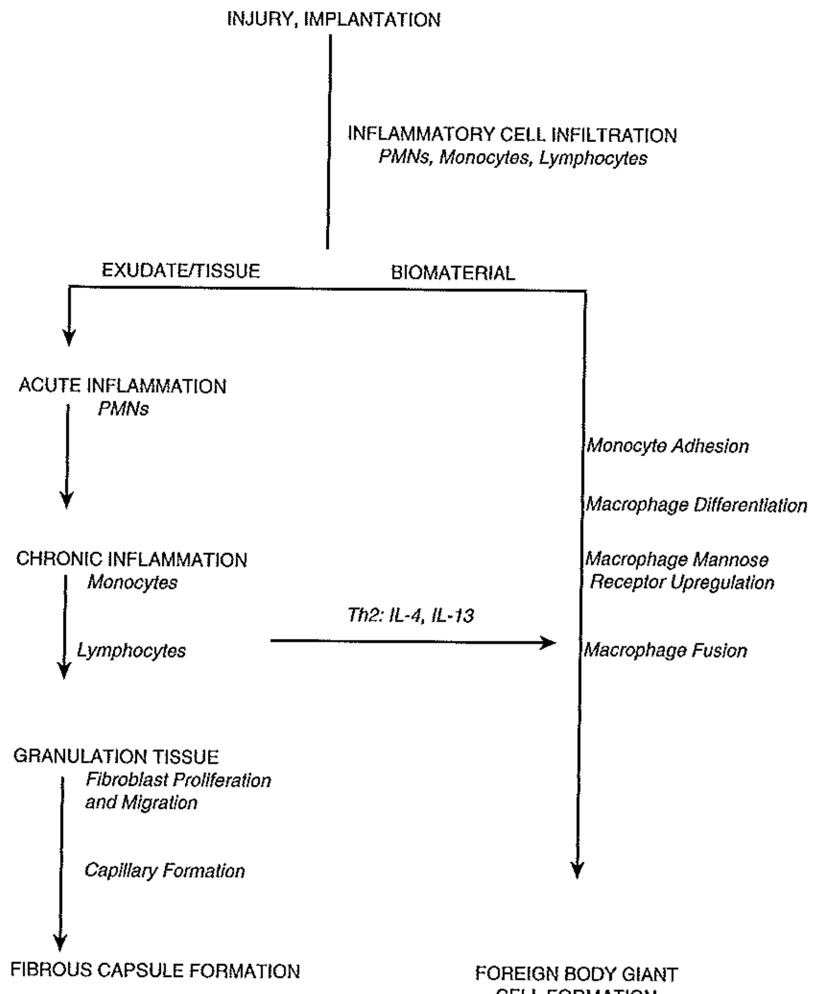

FIG. 8. Sequence of events involved in inflammatory and wound-healing responses leading to foreign-body giant cell formation. This shows the important of Th2 lymphocytes in the transient chronic inflammatory phase with the production of IL-4 and IL-13, which can induce monocyte/macrophage fusion to form foreign-body giant cells.

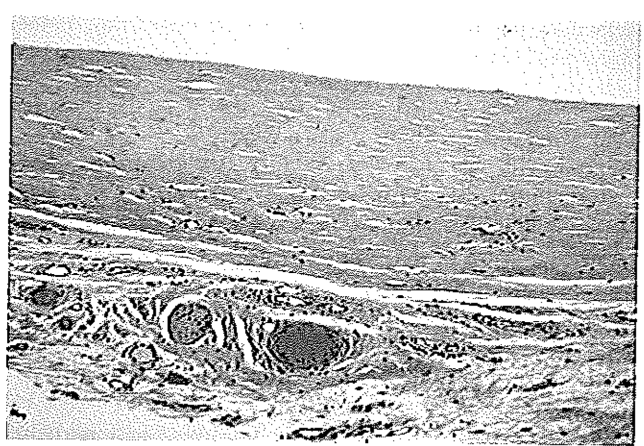

FIG. 9. Fibrous capsule composed of dense, compacted collagen. This fibrous capsule had formed around a Mediport catheter reservoir. Loose connective tissue with small arteries, veins, and a nerve is identified below the acellular fibrous capsule. (See color plate)

of blood supply, and the potential for infection. Systemic factors may include nutrition, hematologic derangements, glucocortical steroids, and preexisting diseases such as atherosclerosis, diabetes, and infection.

Finally, the implantation of biomaterials or medical devices may be best viewed at present from the perspective that the implant provides an impediment or hindrance to appropriate tissue or organ regeneration and healing. Given our current inability to control the sequence of events following injury in the implantation procedure, restitution of normal tissue structures with function is rare. Current studies directed toward developing a better understanding of the modification of the inflammatory response, stimuli providing for appropriate proliferation of permanent and stable cells, and the appropriate application of growth factors may provide keys to the control of inflammation, wound healing, and fibrous encapsulation of biomaterials.

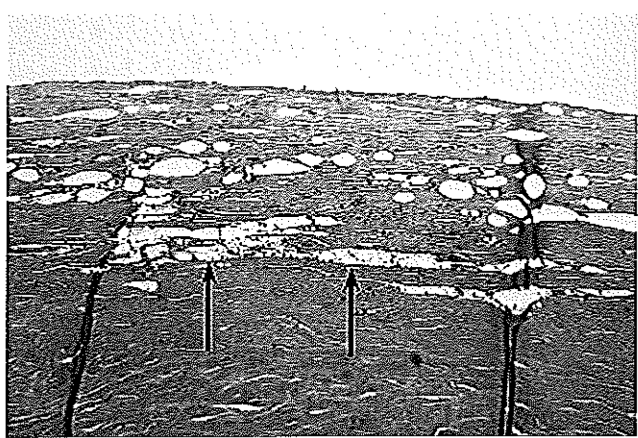

FIG. 10. Fibrous capsule with a focal foreign-body reaction to silicone gel from a silicone gel–filled silicone-rubber breast prosthesis. The breast prosthesis–tissue interface is at the top of the photomicrograph. Oval void spaces lined by macrophages and a few giant cells are identified and a focal area of foamy macrophages (arrows) indicating macrophage phagocytosis of silicone gel is identified. Hematoxylin and eosin stain. Original magnification 10×. (See color plate)

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4.3 Innate and Adaptive Immunity: The Immune Response to Foreign Materials

Richard N. Mitchell

This is a fairly extensive topic, typically encompassing an entire course (with its own introductory text) called "Immunology." Thus, an overview chapter can only begin to acquaint the reader with the complexities of innate and adaptive immunity. Nevertheless, the goal here is to understand the broad organization of the immune system (specific and nonspecific components), how the different elements recognize perceived "invaders," and what effector responses are elicited. The end result is to understand the response of the body to the insertion of a foreign device, and to predict the potential outcome. For more extensive discussion of some aspect of the immune system, you are encouraged to refer to any of a number of excellent basic immunology texts (Abbas and Lichtman, 2003; Benjamini *et al.*, 2000; Janeway, 2001).

OVERVIEW

The function of the immune system is ultimately to defend the host against infectious organisms. The immune system is triggered into action whenever the host perceives tissue injury, anticipating that with that injury, microbial agents either have been causal or will become secondarily involved. The immune system accomplishes its protective role by stratifying the plethora of molecules it may ultimately contact as either "self" or "non-self." In general, the immune system does not react to self molecules or injure host tissues. However, when a particular molecule is perceived as non-self, the full gamut of immune responses are brought to bear in an attempt to remove or isolate it. In most cases, the immune response is so exquisitely specific and well-regulated that host tissues are not significantly affected. However, severe infections, persistent injury, or autoimmunity (inappropriate immune response to self) can lead to substantial tissue injury directly attributable to the host immune system. Thus, although the system evolved primarily to identify and eliminate infectious agents, noninfectious foreign materials also elicit immune responses, occasionally culminating (even if not infected) in severe tissue injury. Consequently, a more inclusive definition of immunity is a reaction to any foreign substance (microbes, proteins, polysaccharides, Silastic implants, etc.) regardless of the pathologic consequences. In order to understand the basics of the immune response, we will initially focus in this chapter on the physiologic pathways of immune responses to infectious agents. Once we understand those pathways, th

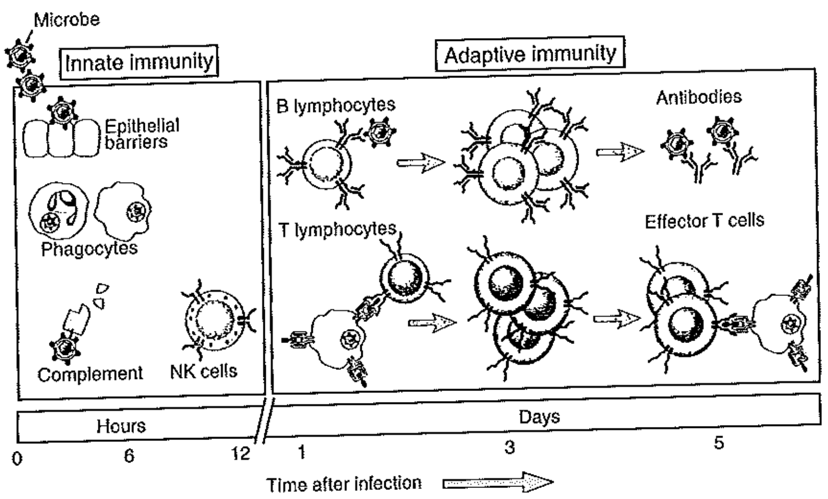


FIG. 1. Innate and adaptive immunity. Although with a limited ability to recognize invading organisms, the relatively primitive (evolutionarily speaking) components of innate immunity provide the first line of defense against microbial infections. Adaptive immunity, with exquisite specificity to any particular infectious agent, develops sometime later after innate components have responded. Notably, the elements of innate immunity not only respond first, but direct subsequent adaptive immunity; in turn, elements of the adaptive immune responses orchestrate a more efficient and vigorous response by the components of innate immunity. The specific kinetics of the responses shown are approximations and may vary depending on the inciting agent. Figure reprinted with permission from Abbas and Lichtman (2003).

mechanisms underlying a response to a foreign body will be briefly examined.

INNATE AND ADAPTIVE IMMUNITY

Defense against microbes is a two-stage process, beginning with a relatively nonspecific innate response to "injury," followed by a targeted adaptive response more uniquely focused on the specific causal agent (Fig. 1, Table 1).

INNATE IMMUNITY

Rapid (hours) response to infection is accomplished by the components of innate immunity (also called "natural" or "native" immunity) (Medzhitov and Janeway, 2000). Innate immunity is an evolutionarily primitive system found even in invertebrates and to some extent in plants. Cells and proteins of this system constitute the first line of defense, and in many cases, can also quite capably eliminate infections on their own. The components of innate immunity are also critical in mobilizing all subsequent effectors—including elements of adaptive immunity—to clear invading microorganisms (Fig. 2). Innate immunity is triggered by molecular structures that are common to groups of related microbes (Fig. 3) (Janeway and Medzhitov, 2002). The receptors for recognition therefore have a fairly limited diversity (fewer than 20 different types of molecules) and have no capacity to make fine distinctions between different substances; each cell of the innate system also expresses essentially the same cohort of receptors. The components of innate immunity react in essentially the same way each time they encounter the same infectious agent, so that there is no functional memory to allow more rapid or specific responses upon second encounter with the same agent. The principal components of innate immunity are:

- Physical and chemical barriers such as epithelia and antimicrobial proteins (e.g., defensins)
- Phagocytic cells (neutrophils and macrophages) that ingest (phagocytize) and destroy microbes (Fig. 4) (Underhill and Ozinsky, 2002)
- Natural killer (NK) cells that kill non-self targets
- Circulating proteins (complement, coagulation factors, C-reactive protein, etc.) that either directly insert pore-forming proteins in microbes that lead to cell death

TABLE 1 Components of Innate versus Adaptive Immunity

	Innate	Adaptive
Physical/chemical barriers	Skin, mucosal epithelium, antimicrobial proteins	Lymphocytes in epithelia, secreted antibodies
Blood proteins	Complement	Antibodies
Cells	Phagocytes (macrophages, neutrophils), natural killer cells	Lymphocytes

Adapted from Abbas and Lichtman (2003).

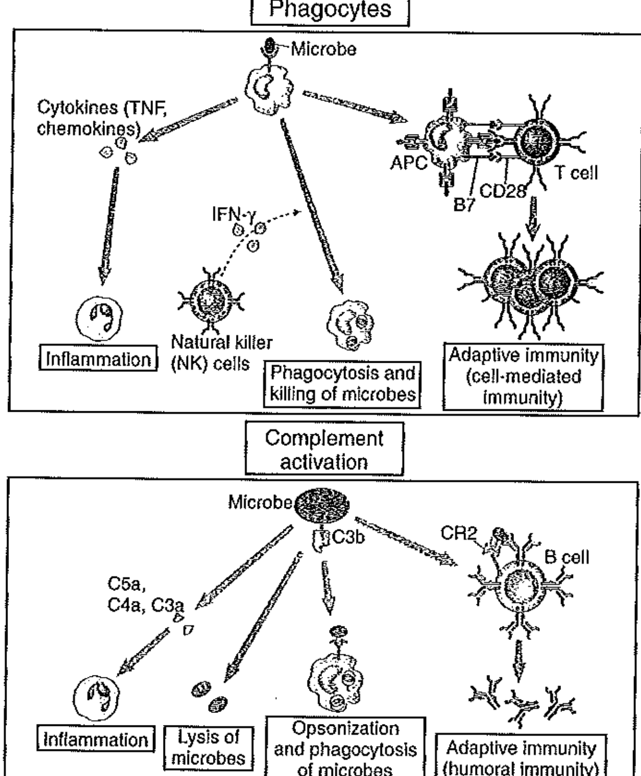


FIG. 2. Basic mechanisms of innate immunity. The two principal components of innate immunity in defense against microbial infection are phagocytes (cells) and complement (a collection of proteins, see also Chapter 4.3). Phagocytes (primarily the macrophage cell type shown here) will directly bind, ingest, and intracellularly degrade various microbes; in addition, macrophages can secrete cytokines to recruit and activate additional inflammatory cell types (e.g., neutrophils) to sites of infection and will help drive the activation of the T lymphocytes of the adaptive immune response. Note that macrophages may also require the production of cytokines by other cell types (in the figure, "IFN-γ" is interferon-γ, a cytokine with a variety of activities) in order to be most active. Complement proteins form pores in the membranes of microbes to cause direct cell lysis; in addition, complement components will incite inflammatory cell recruitment, augment phagocytosis (opsonize microbes), and participate in the activation of B lymphocytes in the adaptive immune response. Figure reprinted with permission from Abbas *et al.* (2000).

(e.g., complement, see Chapter 4.3), or that opsonize microbes (rendering them more "attractive" and readily phagocytized) (Fig. 5) (Barrington *et al.*, 2001; Walport, 2001a, b).

- Cytokines: proteins secreted by cells of innate or adaptive immunity that regulate and coordinate the cellular response (Seder and Gazzinelli, 1998).

ADAPTIVE IMMUNITY

Cellular and circulating protein (also called humoral) mediators that temporally follow innate immunity in recognizing and resolving infections (also called "specific" or "acquired" immunity) are termed adaptive immunity. Adaptive immunity is more evolutionarily advanced, first seen in phylogenic development with the jawed vertebrates. It has the cardinal feature of exquisite specificity for distinct macromolecules and memory, the latter being the ability to respond more vigorously to subsequent exposures of the same microorganism. Adaptive immunity also has a virtually limitless diversity, with the capacity to recognize 10^9–10^{11} distinct antigenic determinants (Fig. 3). Each cell of the adaptive immune system can recognize and respond to only a single antigenic determinant, so that the immense diversity of the system requires an equally large number of different cells. Foreign substances that induce these specific immune responses are called antigens and each antigen will typically activate only one (or a small set) of cells. The principal components of adaptive immunity (see also later discussion) are:

- T lymphocytes (also known as T cells), functionally divided into helper T cells (Th cells), which provide signals and soluble protein mediators (cytokines) to orchestrate the activity of other cell types, and cytotoxic T cells (Tc cells) which kill selected target cells
- B lymphocytes (also known as B cells), responsible for making antibodies
- Antibodies: proteins secreted by B lymphocytes with specificity for a specific antigen
- Cytokines: proteins secreted by cells of innate or adaptive immunity that regulate and coordinate the cellular response.

Innate and adaptive immunity are integrated components in the host defense response; the cells and proteins of each system function cooperatively. Thus, the initial innate response to microbes stimulates adaptive immune responses and influences how the adaptive immune system will respond (e.g., antibodies versus cellular mediators). Moreover, adaptive immunity directs and utilizes the effector mechanisms of innate immunity to clear infectious agents.

RECOGNITION AND EFFECTOR PATHWAYS IN INNATE IMMUNITY

The components of the innate system recognize structures that are characteristic of microbial pathogens and are not present on mammalian tissues; thus recognition via this pathway can distinguish self and non-self (Fig. 3) (Janeway and Medzhitov, 2002). Because the microbial products that are recognized are usually essential for survival of the microorganism, they cannot be discarded or mutated. These structures may be recognized by the cells or by humoral elements of the innate system and include:

- Double-stranded RNA found in cells containing replicating viruses. This induces cytokine production by infected cells leading to destruction of the intracellular virus.
- Unmethylated CpG DNA sequences characteristic of bacterial infections. These induce autocrine macrophage activation and more effective intracellular killing of phagocytosed organisms.

	Innate immunity	Adaptive immunity
Specificity	For structures shared by classes of microbes ("molecular patterns") Different microbes Identical mannose receptors	For structural detail of microbial molecules (antigens): may recognize nonmicrobial antigens Different microbes Distinct antibody molecules
Receptors	Encoded in germline; limited diversity LPS receptor *N*-formyl-methionyl receptor Mannose receptor Scavenger receptor	Encoded by genes produced by somatic recombination of gene segments; greater diversity TCR Ig
Distribution of receptors	Nonclonal: identical receptors on all cells of the same lineage	Clonal: clones of lymphocytes with distinct specificities express different receptors
Discrimination of self and nonself	Yes; host cells are not recognized or they may express molecules that prevent innate immune reactions	Yes; based on selection against self-reactive lymphocytes; may be imperfect (giving rise to autoimmunity)

FIG. 3. Characteristics of innate and adaptive immunity. Cells of innate immunity have a limited number of receptors for foreign molecular structures; the same receptors are present on all cells, and because the number of different receptors is relatively small, they are all encoded in the germline. In comparison, the recognition of specific antigens by the adaptive immune system is specific and unique for each potential antigen. In order to encode such a huge diversity (10^9–10^{11} variations), antibodies and T cell receptors (TCR) are formed by somatic recombination of different gene segments. Moreover, each T or B cell will express only a single receptor type and can therefore recognize only one antigen. Lack of response to self is controlled by a number of mechanisms (not always perfect, hence the development of autoimmune disease), including destruction of self-reactive clones, or induction of specific "unresponsiveness." Figure reprinted with permission from Abbas and Lichtman (2003).

- *N*-Formylmethionine peptides from bacterial protein synthesis. Binding to receptors on neutrophils and macrophages causes chemotaxis (movement up a concentration gradient) and activation. Similar chemotaxis can be engendered by protein fragments released during complement activation, lipid mediators of inflammation, and chemokine proteins released by "stressed" cells.
- Mannose-rich oligosaccharides from bacterial or fungal cell walls. Engagement of receptors on macrophages induce phagocytosis; soluble mannose-binding protein in the plasma opsonizes or enhances phagocytosis of microbes bearing mannose.
- Bacterial or fungal wall oligosaccharides directly activate complement and induce either direct microbial lysis or microbial coating with complement that markedly enhance phagocytosis.
- Phosphorylcholine in bacterial cell walls binds to circulating C-reactive protein (CRP; also called pentraxin); CRP induces opsonization and also activates complement.
- Lipopolysaccharide (LPS) from certain (gram-negative) bacteria binds to circulating LPS-binding protein which in turn binds to CD14 surface molecules on macrophages. This binding elicits a wide range of cytokine responses from the macrophages including tumor necrosis factor (TNF) and interleukin-12, which recruit and activate neutrophils and NK cells, respectively. By the same pathways, LPS induces severe systemic responses that culminate in septic shock (Hack *et al.*, 1997).
- Teichoic acid from gram-positive bacteria elicits responses comparable to LPS.

Components of the innate system also recognize sites of injury, anticipating that these either may be primarily caused by infection or may be subject to subsequent infection. Thus, components of the coagulation cascade or denatured connective tissue elements (such as might occur at sites of trauma) may bind to macrophage cell surface receptors and induce activation. These become especially important in the context of the implantation of foreign bodies where otherwise minor trauma and the presence of denatured ECM bound on "inert" surfaces lead to macrophage activation (Tang and Eaton, 1999).

The function of all these recruiting and activating factors is to attract phagocytes to ingest and destroy microbes. The primary responding cell type in the earliest stages of injury or infection is the neutrophil, a short-lived (hours) phagocytic cell capable of ingesting and destroying microbes, as well

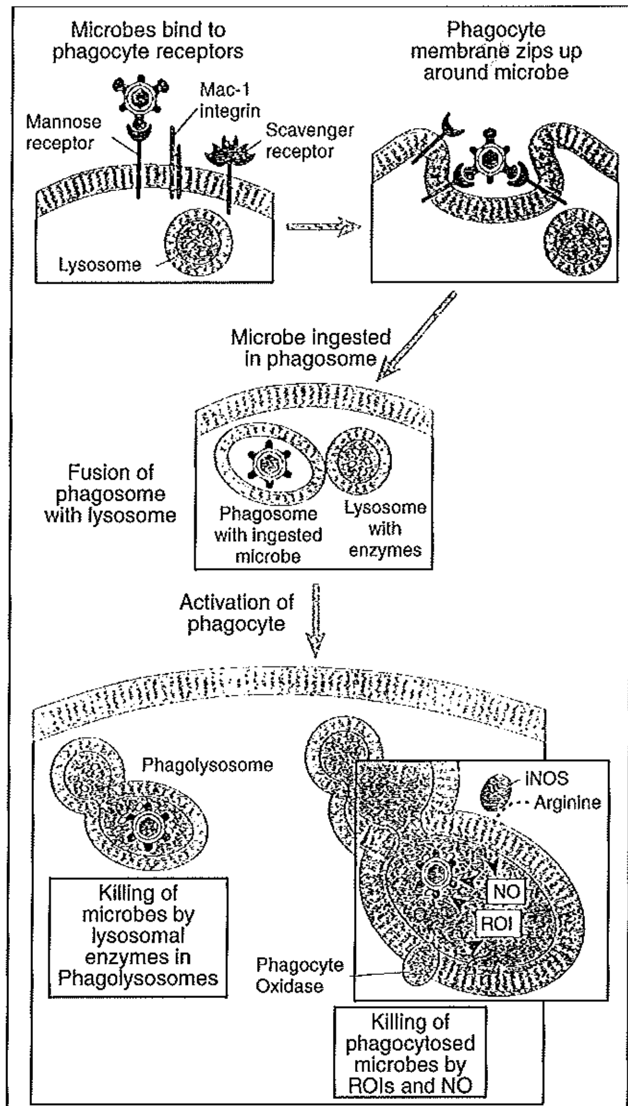


FIG. 4. Phagocytosis and intracellular destruction of microorganisms. Surface receptors on phagocytes either can bind microbes directly or may bind opsonized microbes (for example, Mac-1 integrin binds microorganisms after they have been coated with complement proteins). After binding to one (or more) of the variety of surface receptors, microbes are internalized into phagosomes, which subsequently fuse with intracellular lysosomes to form phagolysosomes. Fusion results in generation of reactive oxygen intermediates (ROI) and nitric oxide (NO) which kill the microbes largely via free radical injury; fusion with lysosomes also results in release of lysosomal enzymes that will also digest the microbes. Figure reprinted with permission from Abbas and Lichtman (2003).

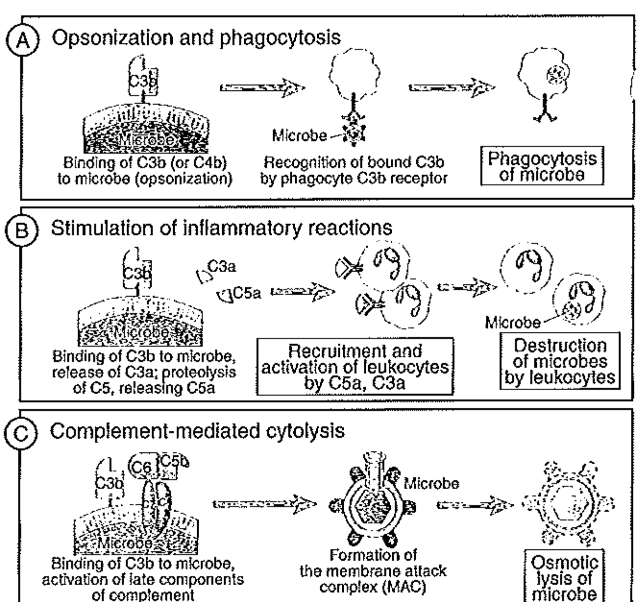


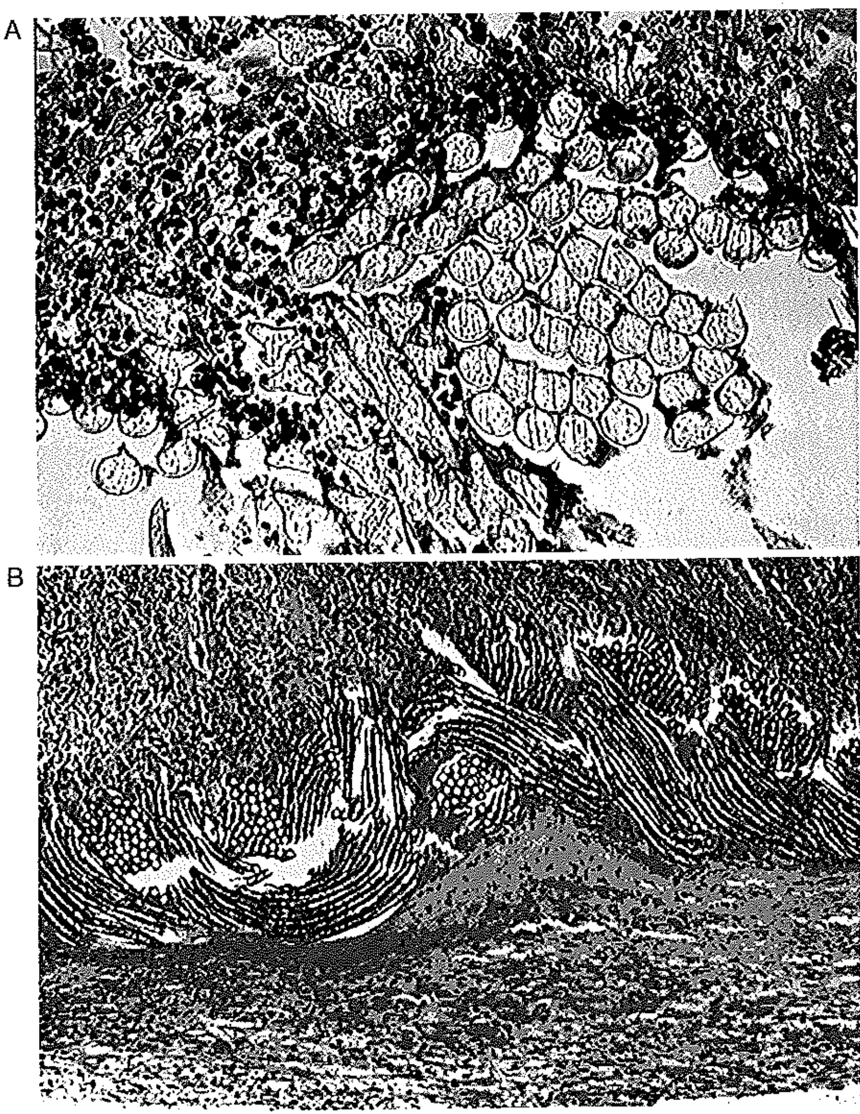
FIG. 5. Functions of complement. (A) Complement components will bind to microbe surfaces and render them more readily internalized by phagocytes (opsonization). (B) Fragments resulting from proteolytic activation of complement will recruit and activate inflammatory cells (shown here are neutrophils). (C) Complement also forms pores in the microbial membrane (so-called membrane attack complex or MAC) that result in osmotic rupture of microorganisms. Figure reprinted with permission from Abbas and Lichtman (2003).

as releasing a variety of potent proteases. Macrophages are secondarily recruited to sites of injury but are much longer-lived (they can last the lifetime of the host!) and persist at sites of inflammation, making them the dominant effector cell type in late-stage innate immunity. These phagocytes are recruited to sites of injury by changes in adhesion molecule expression on endothelial cells in the vicinity, and by chemotactic signals (acting, e.g., through G-protein-coupled receptors on neutrophils) delivered by injured cells (i.e., chemokines), by complement components (generated during complement activation), and by microorganisms themselves (see the preceding list) (Fig. 6). The phagocytic cells clear opsonized microorganisms, kill them with reactive oxygen intermediates (superoxide, oxyhalide molecules, nitric oxide, and hydrogen peroxide), and degrade them with proteases (Fig. 4). However, release of such cytotoxic and degradative molecules into the neighboring environment can also cause local tissue injury. Severe local injury due to excessive neutrophil activation results in an abscess with total destruction of parenchyma and stroma.

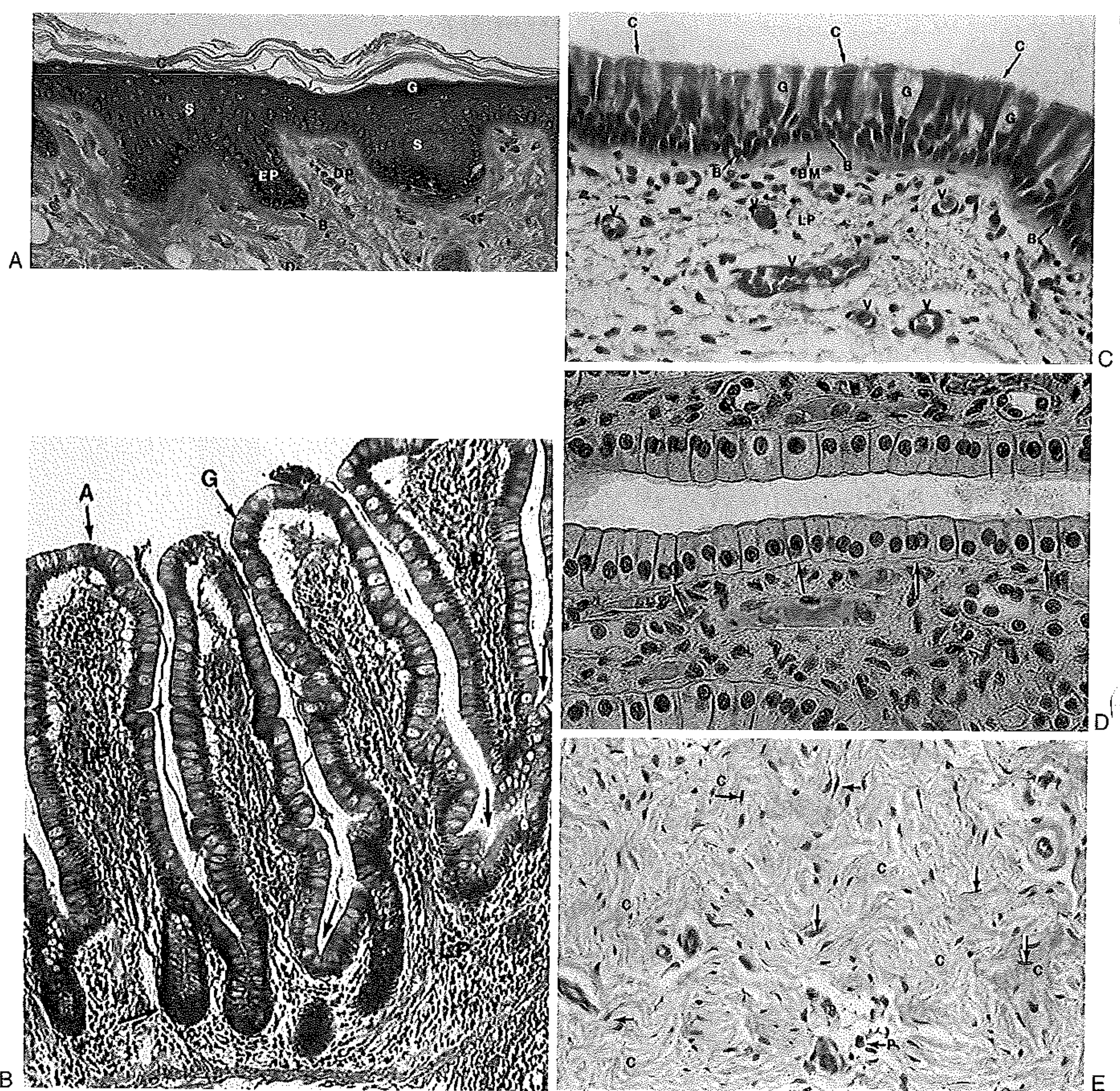
In addition, activated macrophages release a variety of cytokines and other factors that can have both local and systemic effects (Fig. 6) (Seder and Gazzinelli, 1998):

- Tumor necrosis factor (TNF) recruits and activates neutrophils
- Interleukin-12 (IL-12) activates T cells and NK cells
- Coagulation pathways (tissue factor elaboration)
- Angiogenic factors (new blood vessel formation)
- Fibroblast activating factors (e.g., platelet-derived growth factor; PDGF) that induce fibroblast proliferation
- Transforming growth factor-β(TGF-β) expression increasing extracellular matrix (ECM) synthesis
- Matrix metalloproteinases that remodel the ECM

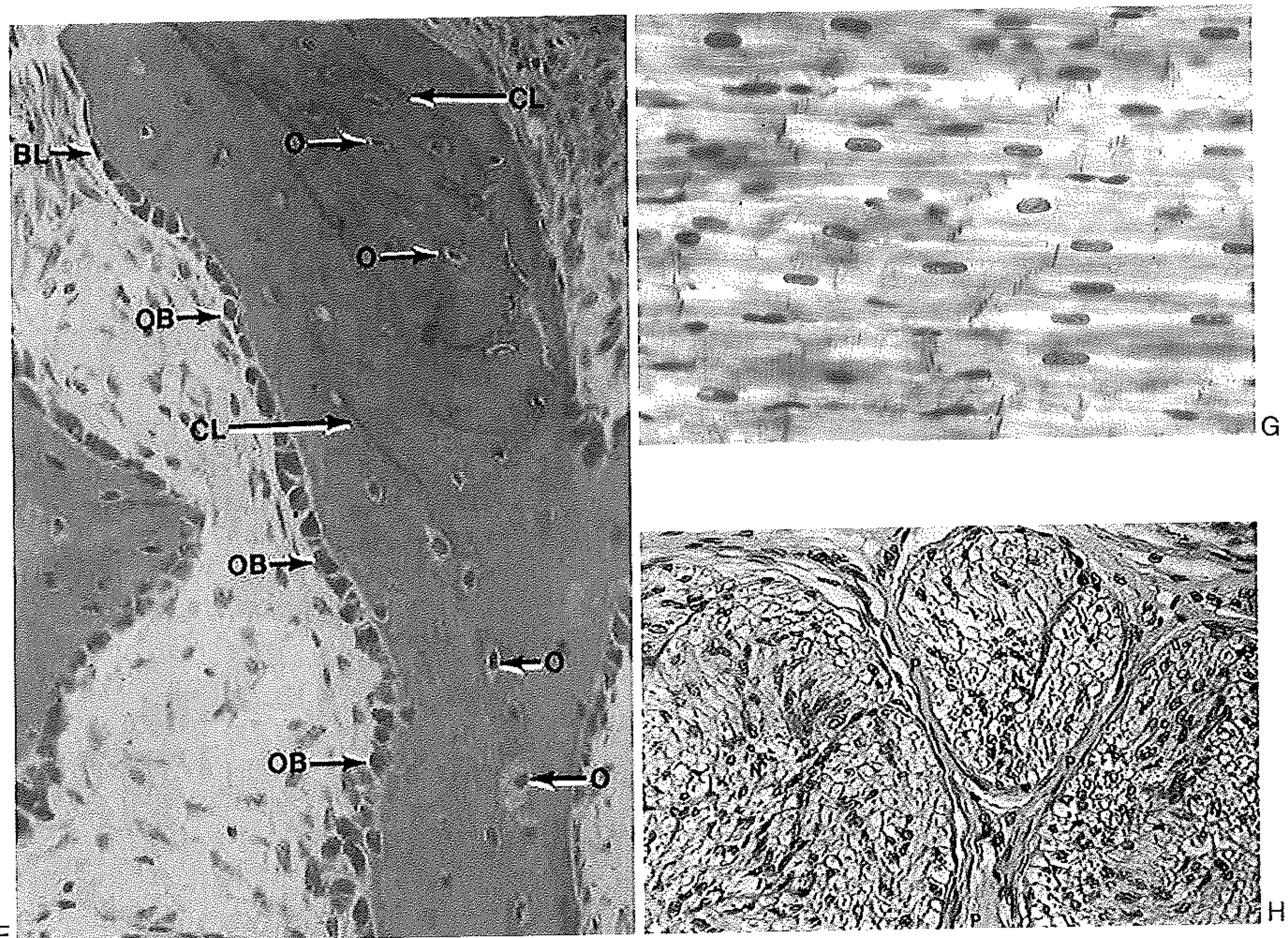
Thus, in the setting of prolonged activation, macrophages will ultimately mediate tissue fibrosis and scarring (Fig. 6).



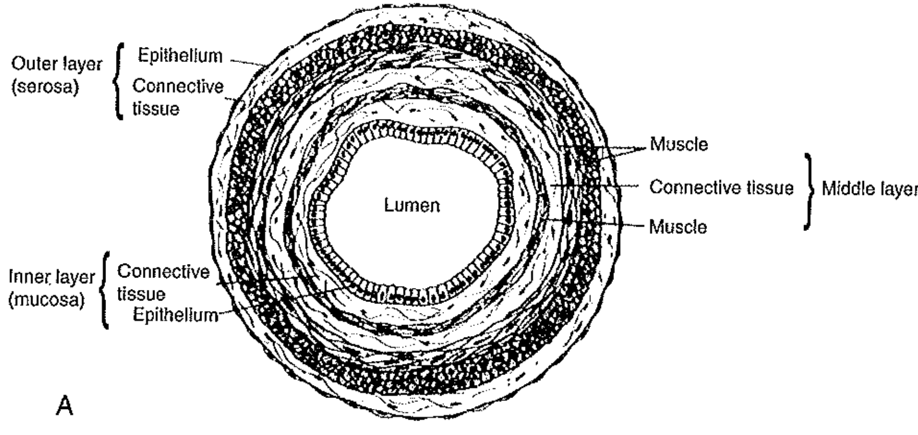
Chapter 2.4, Fig. 9 (A) Weft knit inflammatory response at 4 weeks (Golaski Microkit); (B) Warp knit inflammatory response at 3 days (Microvel).

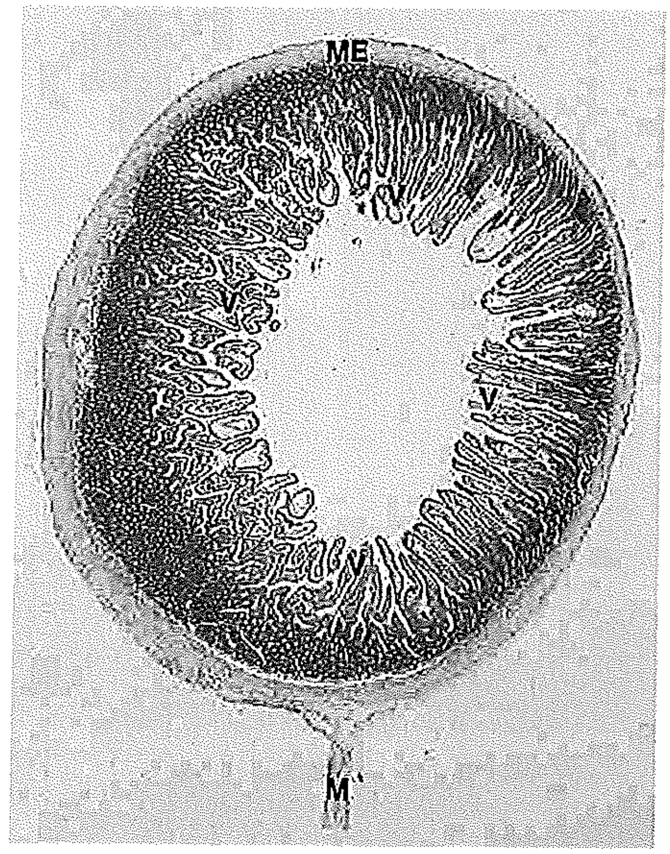


Chapter 3.4, Fig. 5 Photomicrographs of basic tissues, emphasizing key structural features. (A–D) Epithelium; (E, F) connective tissue; (G) muscle; and (H) nervous tissue. (A) Skin. Note the thin stratum corneum (c) and stratum granulosum (g). Also shown are the stratum spinosum (s), stratum basale (b), epidermal pegs (ep), dermal papilla (dp), and dermis (d). (B) Trachea, showing goblet cells (g), ciliated columnar cells (C), and basal cells (B). Note the thick basement membrane (bm) and numerous blood vessels (v) in the lamina propria (lp). (C) Mucosa of the small intestine (ileum). Note the goblet (g) and columnar absorbing (a) cells, the lamina propria (lp), muscularis mucosae (mm), and crypts (arrows). (D) Epithelium of a kidney collecting duct resting on a thin basement membrane (arrows). (E) Dense irregular connective tissue. Note the wavy unorientated collagen bundles (c) and fibroblasts (arrows). p, plasma cells. (F) Cancellous bone clearly illustrating the morphologic difference between inactive bone lining (endosteal, osteoprogenitor) cells (bl) and active osteoblasts (ob). The clear area between the osteoblasts and calcified bone represents unmineralized matrix or osteoid. cl, cement lines; o, osteocycles. H) Small nerve fascicles (n) with perineurium (p) separating it from two other fascicles (n). (A–F and H reproduced by permission from Berman, I., 1993. *Color Atlas of Basic Histology.* Appleton and Lange, 1993. G reproduced by permission from Schoen F. J., The heart. in *Robbins Pathologic Basis of Disease,* 7th ed., R. S. Cotran, V. Kumar and T. Collins, eds. Saunders, Philadelphia, in press.)

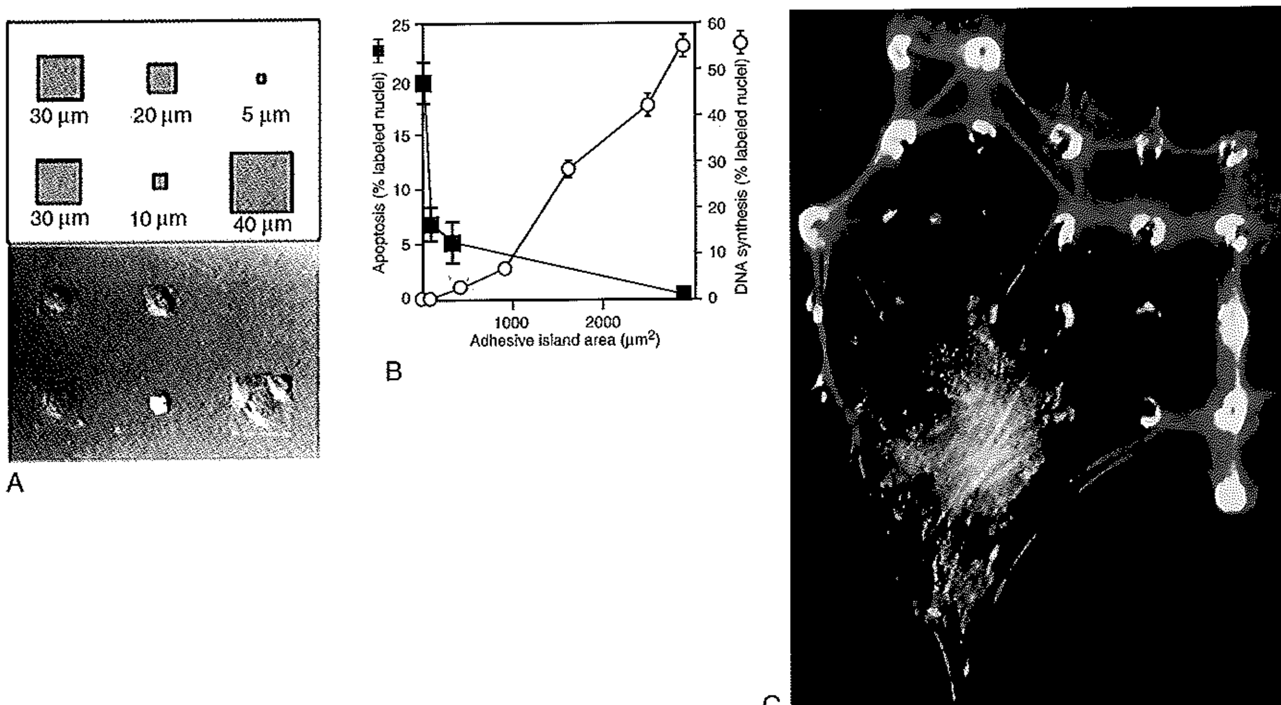


Chapter 3.4, Fig. 5—continued





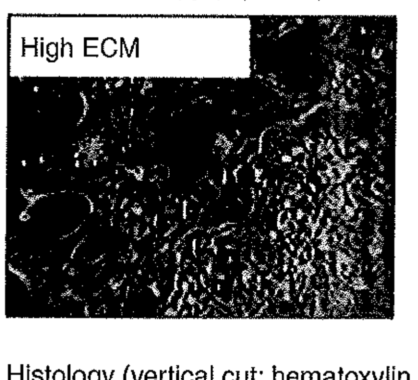
Chapter 3.4, Fig. 7 (A) Organization of tissue layers in the digestive tract (e.g., stomach or intestines). (B) Photomicrograph of the dog jejunum illustrating villi (v), the muscularis external (me), and mesentery (m). In this organ the epithelium is organized into folds (the villi) in order to increase the surface area for absorption. (A, Reproduced by permission from Borysenko, M., and Beringer, T., *Functional Histology*, 3rd ed. Copyright 1989 Little, Brown, and Co. B, Reproduced by permission from Berman, I., 1993. *Color Atlas of Basic Histology*, Appleton and Lange.)

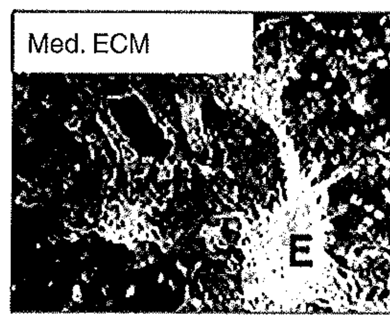


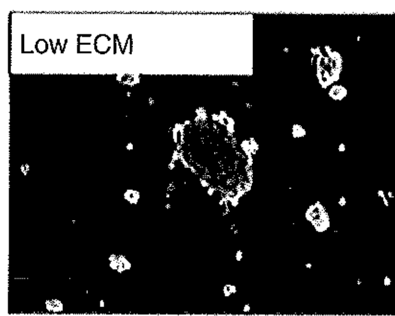
Chapter 3.4, Fig. 13 Effect of spreading on cell growth and apoptosis. (A) Schematic diagram showing the initial pattern design containing different-sized square adhesive islands and Nomarski views of the final shapes of bovine adrenal capillary endothelial cells adherent to the fabricated substrate. Distances indicate lengths of the squares' sides. (B) Apoptotic index (percentage of cells exhibiting positive TUNEL staining) and DNA synthesis index (percentage of nuclei labeled with 5-bromodeoxyuridine) after 24 hours, plotted as a function of the projected cell area. Data were obtained only from islands that contained single adherent cells; similar results were obtained with circular or square islands and with human or bovine endothelial cells. (C) Fluorescence micrograph of an endothelial cell spread over a substrate containing a regular array of small (5-µm-diameter) circular ECM islands separated by nonadhesive regions created with a microcontact printing technique. Yellow rings and crescents indicate colocalization of vinculin (green) and F-actin (red) within focal adhesions that form only on the regulatory spaced circular ECM islands. (A, B, Reproduced by permission from Chen, C. S., *et al.*, 1997. Geometric control of cell life and death. *Science* 276: 1425. C, Reproduced by permission from Ingber, D. E., 2003. Mechanosensation through integrins: Cells act locally but think globally. *Proc. Natl. Acad. Sci. USA* 100: 1472.)

Hepatocyte/Endothelial Cell Sorting

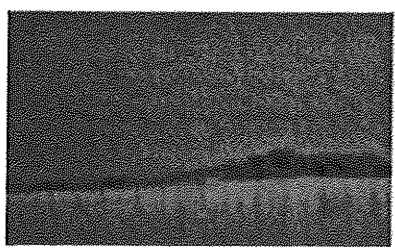
Light microscopy (top view)

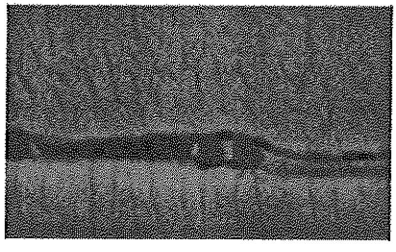


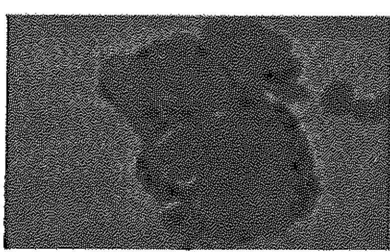




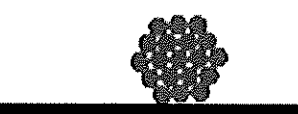
Histology (vertical cut; hematoxylin and eosin stain)



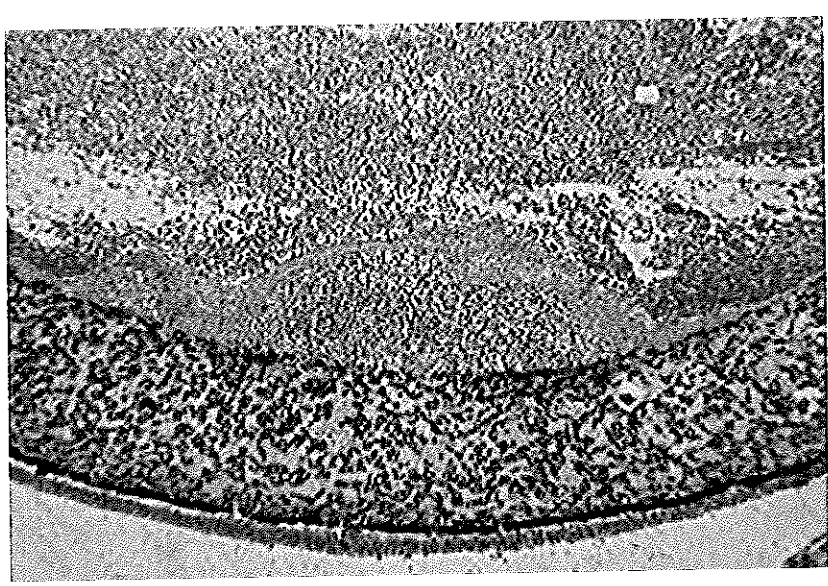




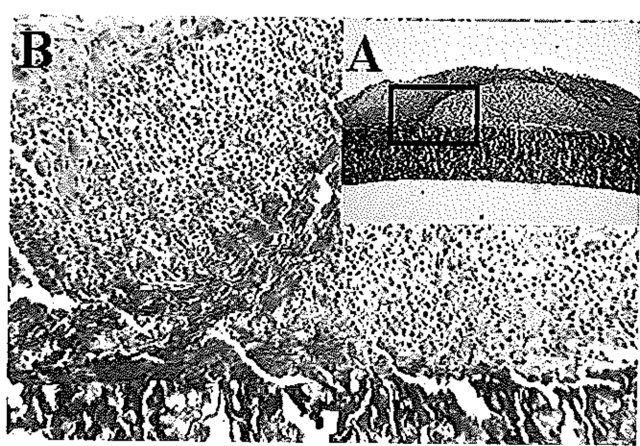
endothelial cells hepatocytes



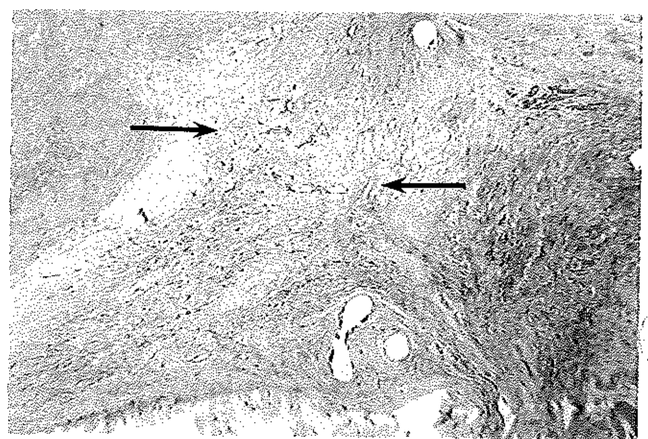
Chapter 3.4, Fig. 14 Different levels of type 1 collagen coating on a culture dish result in different organization of endothelial cells and hepatocytes. High collagen levels cause both cell types to spread across the substratum (left). On intermediate collagen levels, endothelial cells form a layer on the substratum whereas hepatocytes form a layer on top of the endothelial cells (center). Low levels of collagen result in an inner layer of hepatocyte aggregate surrounded by endothelial cells (right). (Reproduced by permission from Lauffenburger, D. A., *et al.*, 2001. Who's got pull around here? Cell organization in development and tissue engineering. *Proc. Natl. Acad. Sci. USA* 98: 4282.)



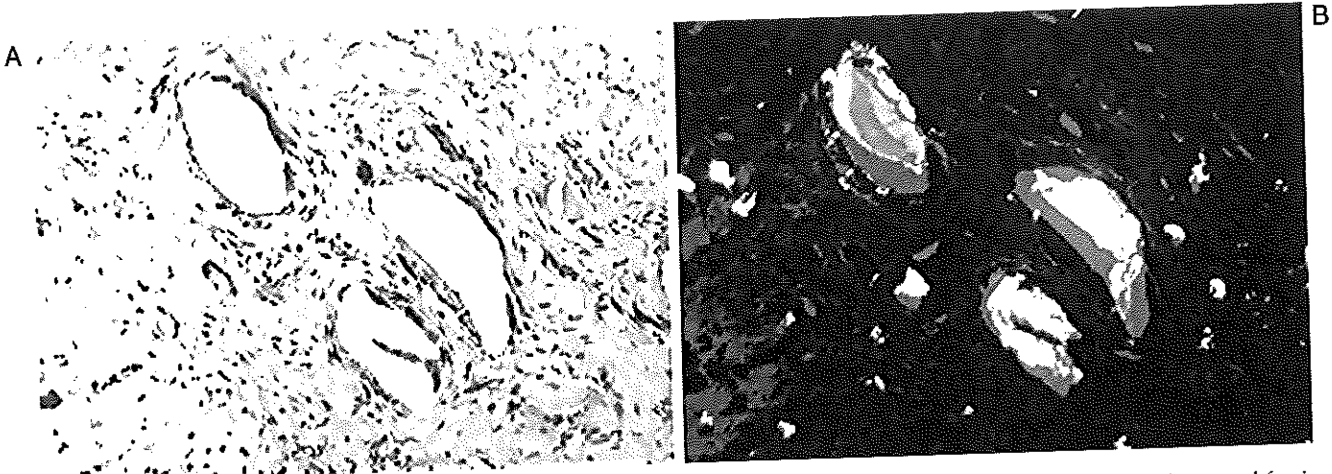
Chapter 4.2, Fig. 2 Acute inflammation, secondary to infection, of an ePTFE vascular graft. A focal zone of polymorphonuclear leukocytes is present at the lumenal surface of the vascular graft, surrounded by a fibrin cap, on the blood-contacting surface of the ePTFE vascular graft. Hematoxylin and eosin stain. Original magnification 4×.



Chapter 4.2, Fig. 3 Chronic inflammation, secondary to infection, of an ePTFE arteriovenous shunt for renal dialysis. (A) Low-magnification view of a focal zone of chronic inflammation. (B) High-magnification view of the outer surface with the presence of monocytes and lymphocytes at an area where the outer PTFE wrap had peeled away from the vascular graft. Hematoxylin and eosin stain. Original magnification (A) 4×, (B) 20×.

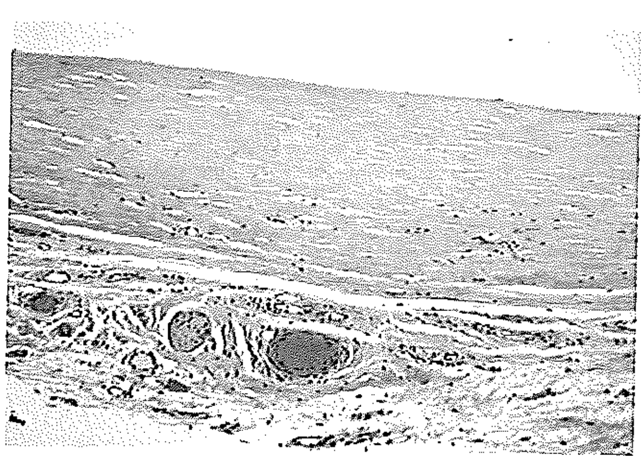


Chapter 4.2, Fig. 4 Granulation tissue in the anastomotic hyperplasia at the anastomosis of an ePTFE vascular graft. Capillary development (red slits) and fibroblast infiltration with collagen deposition (blue) from the artery form the granulation tissue (arrows). Masson's Trichrome stain. Original magnification 4×.

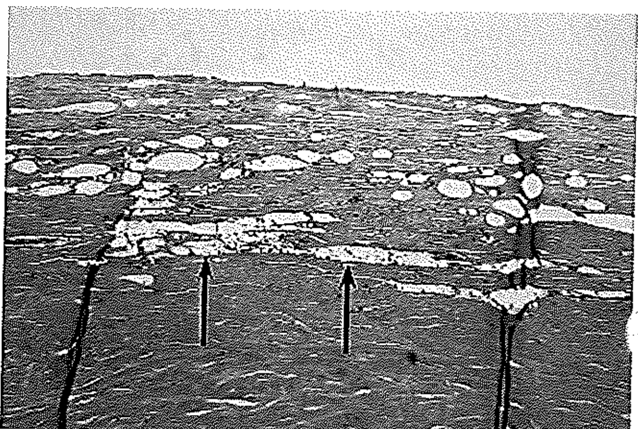


Chapter 4.2, Fig. 6 (A) Focal foreign-body reaction to polyethylene wear particulate from a total knee prosthesis. Macrophages and foreign-body giant cells are identified within the tissue and lining the apparent void spaces indicative of polyethylene particulate. Hematoxylin and eosin stain. Original magnification 20×. (B) Partial polarized light view. Polyethylene particulate is identified within the void spaces commonly seen under normal light microscopy. Hematoxylin and eosin stain. Original magnification 20×.

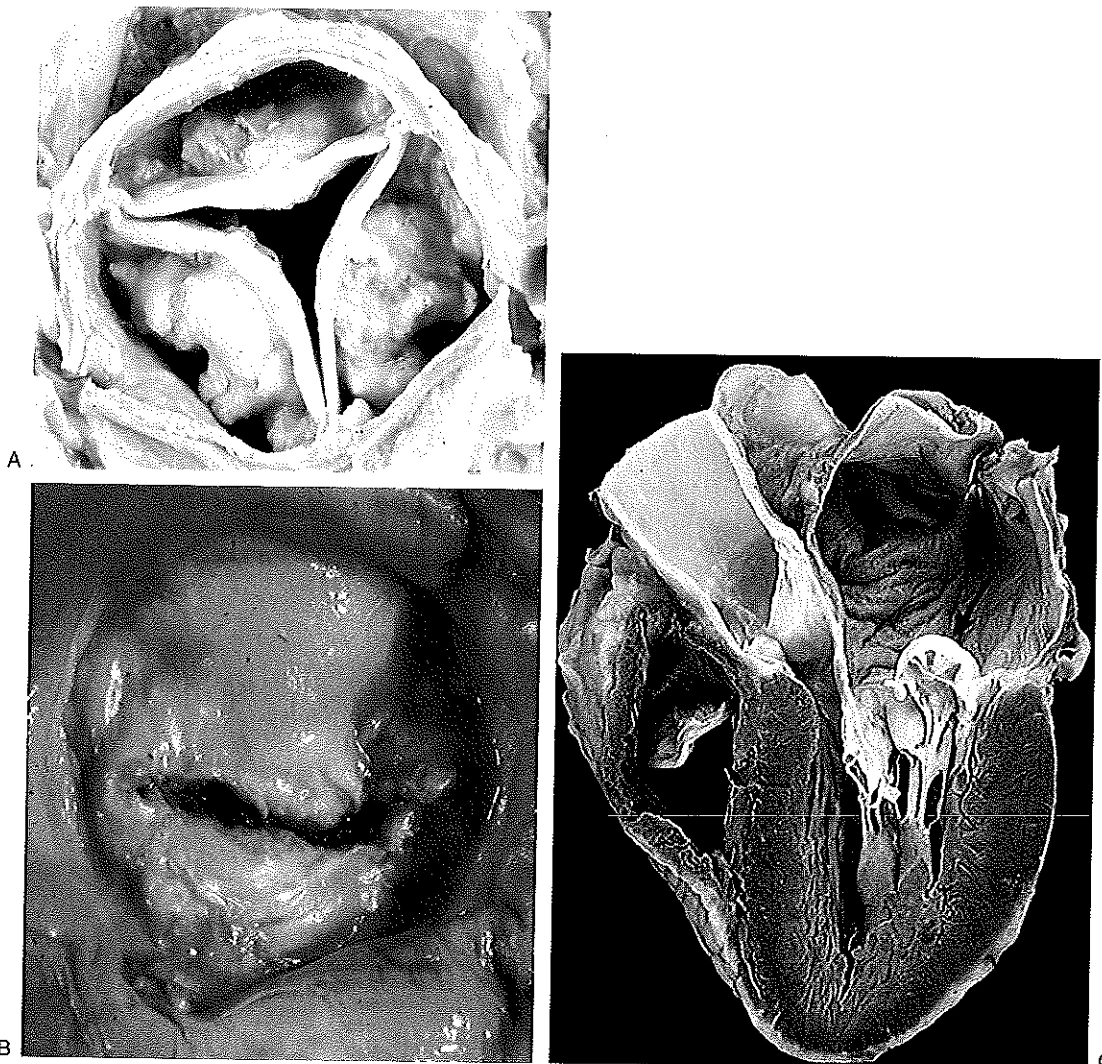
Chapter 4.2, Fig. 7 Foreign-body reaction with multinucleated foreign body giant cells and macrophages at the periadventitial (outer) surface of a Dacron vascular graft. Fibers from the Dacron vascular graft are identified as clear oval voids. Hematoxylin and eosin stain. Original magnification 20×.



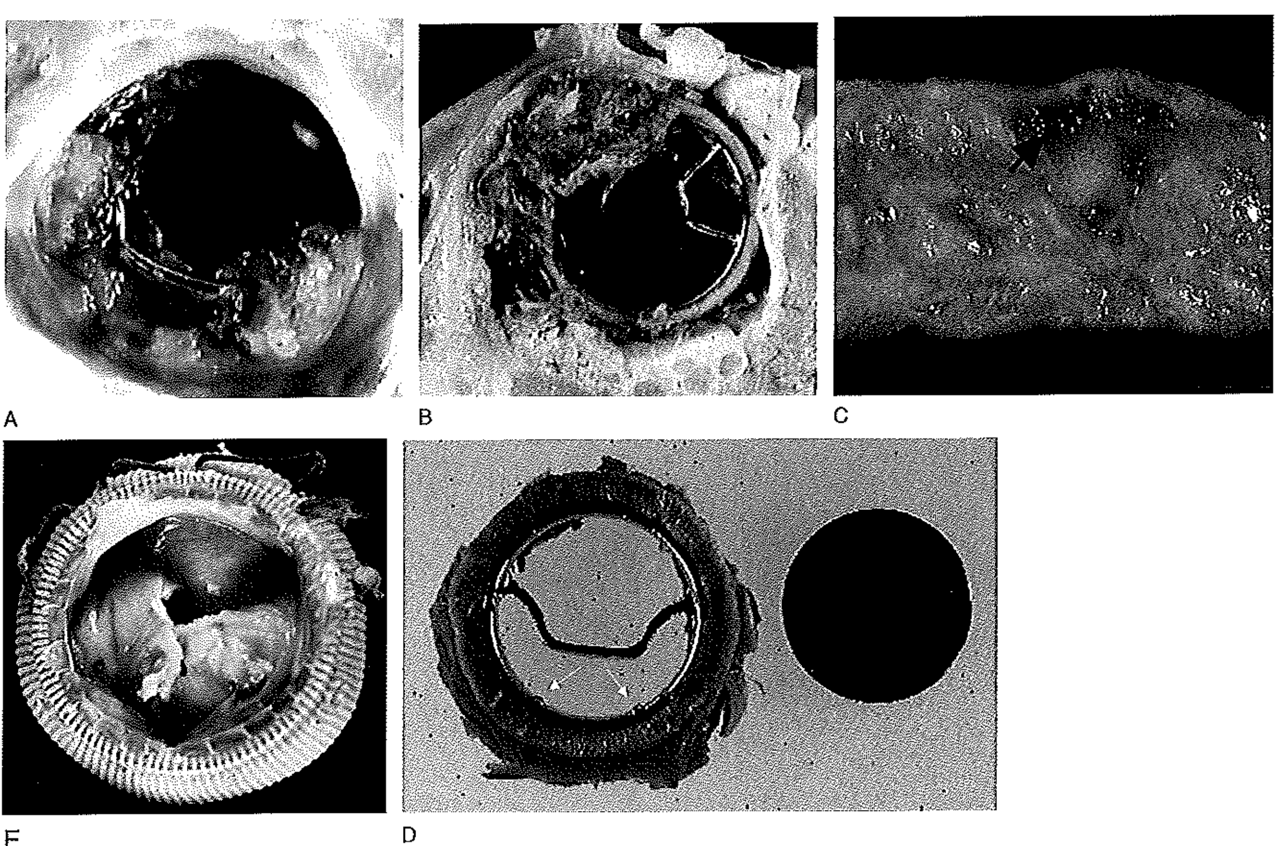
Chapter 4.2, Fig. 9 Fibrous capsule composed of dense, compacted collagen. This fibrous capsule had formed around a Mediport catheter reservoir. Loose connective tissue with small arteries, veins, and a nerve is identified below the acellular fibrous capsule.



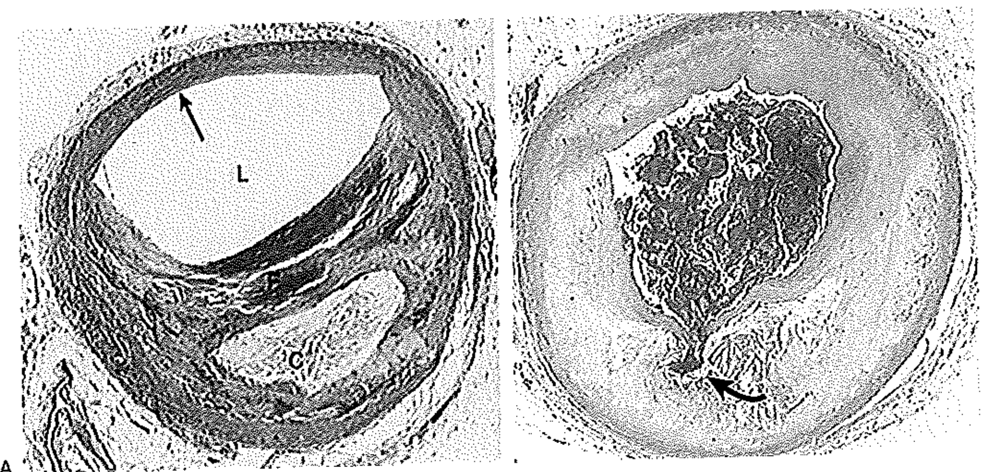
Chapter 4.2, Fig. 10 Fibrous capsule with a focal foreign-body reaction to silicone gel from a silicone gel–filled silicone-rubber breast prosthesis. The breast prosthesis–tissue interface is at the top of the photomicrograph. Oval void spaces lined by macrophages and a few giant cells are identified and a focal area of foamy macrophages (arrows) indicating macrophage phagocytosis of silicone gel is identified. Hematoxylin and eosin stain. Original magnification 10×.



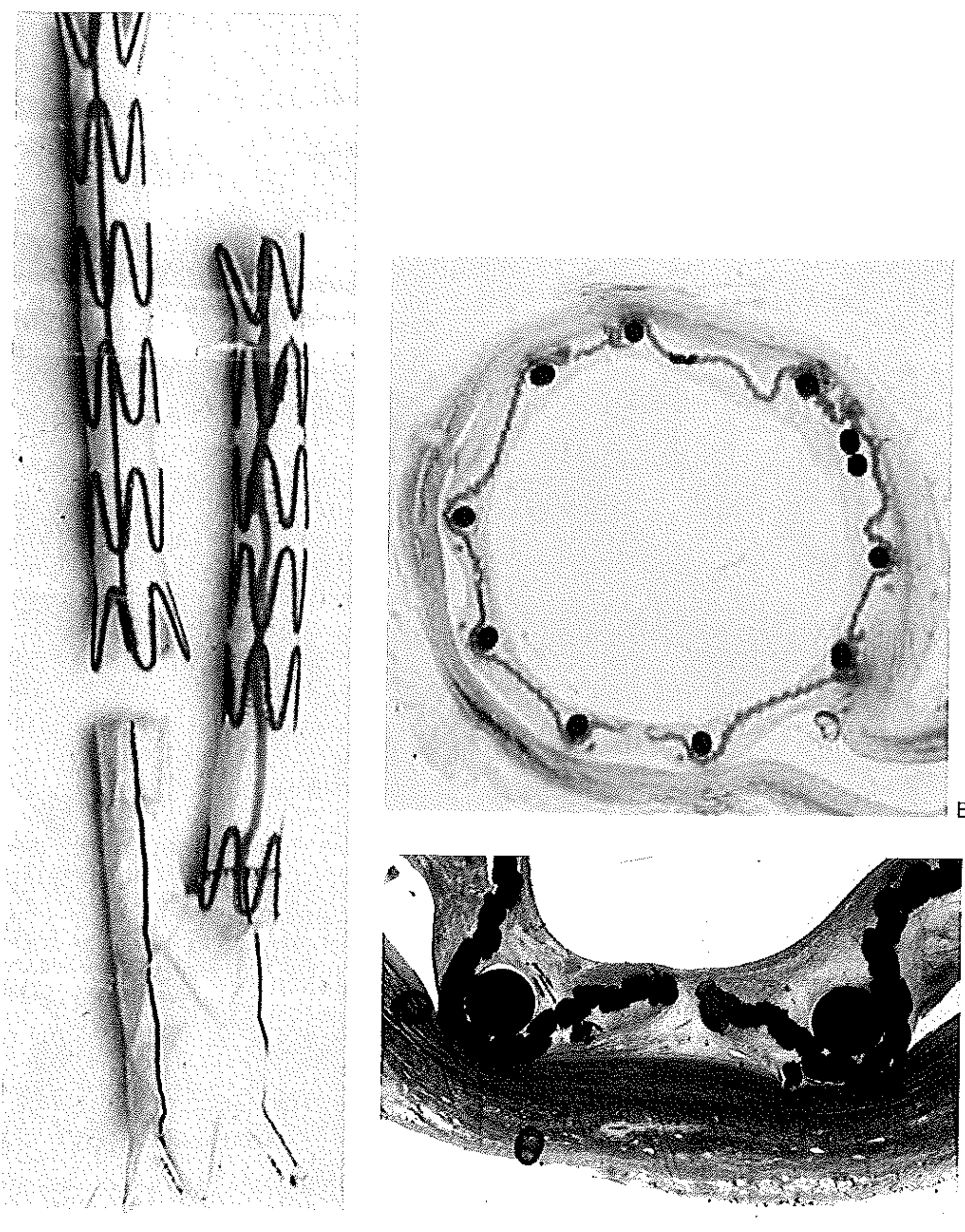
Chapter 7.3, Fig. 2 (A) Severe degenerative calcification of a previously anatomically normal tricuspid aortic valve, the predominant cause of aortic stenosis. (B) Chronic rheumatic heart disease, manifest as mitral stenosis, viewed from the left atrium. (C) Myxomatous degeneration of the mitral valve, demonstrating hooding with prolapse of the posterior mitral leaflet into the left atrium (*arrow*). A, B: Reproduced by permission from Schoen, F. J., and Edwards, W. D. (2001). Valvular heart disease: General priciples and stenosis. in *Cardiovascular Pathology*, 3rd ed. Silver, M. D., Gotlieb, A. I., and Schoen, F. J., eds. Churchill Livingstone, New York. C: Reproduced by permission from Schoen, F. J. (1999). The heart. in *Robbins Pathologic Basis of Disease*, 6th ed., R. S. Cotran, V. Kumar, T. Collins, eds. W.B. Saunders, Philadelphia.



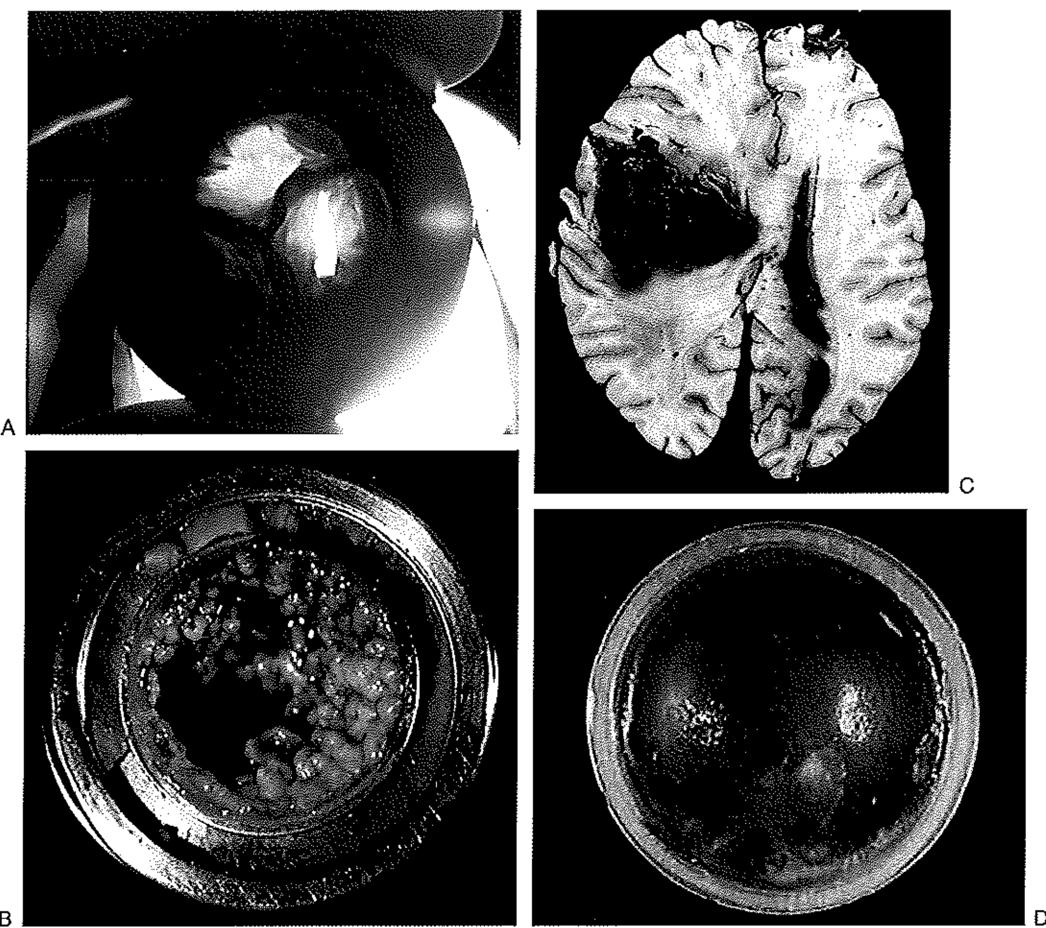
Chapter 7.3, Fig. 6 Prosthetic valve complications. (A) Thrombosis on a Bjork–Shiley tilting disk aortic valve prosthesis, localized to outflow strut near minor orifice, a point of flow stasis. (B) Thromboembolic infarct of the small bowel (arrow) secondary to embolus from valve prosthesis. (C) Prosthetic valve endocarditis with large ring abscess, viewed from the ventricular aspect of an aortic Bjork–Shiley tilting disk aortic valve. (D) Strut fracture of Bjork–Shiley valve, showing valve housing with single remaining strut and adjacent disk. (E) Structural valve dysfunction (manifest as calcific degeneration with tear) of porcine valve. B: Reproduced by permission from Schoen, F. J. (2001). Pathology of heart valve substitution with mechanical and tissue prostheses. in *Cardiovascular Pathology*, 3rd ed. M. D. Silver, A. I. Gotlieb, and F. J. Schoen, eds. Churchill Livingstone, New York. C: Reproduced by permission from Schoen, F. J. (1987). Cardiac valve prostheses: pathological and bioengineering considerations. *J. Card. Surg.* 2: 65. A and D: Reproduced by permission from Schoen, F. J., Levy, R. J., and Piehler, H. R. (1992). Pathological considerations in replacement cardiac valves. *Cardiovasc. Pathol.* 1: 29.



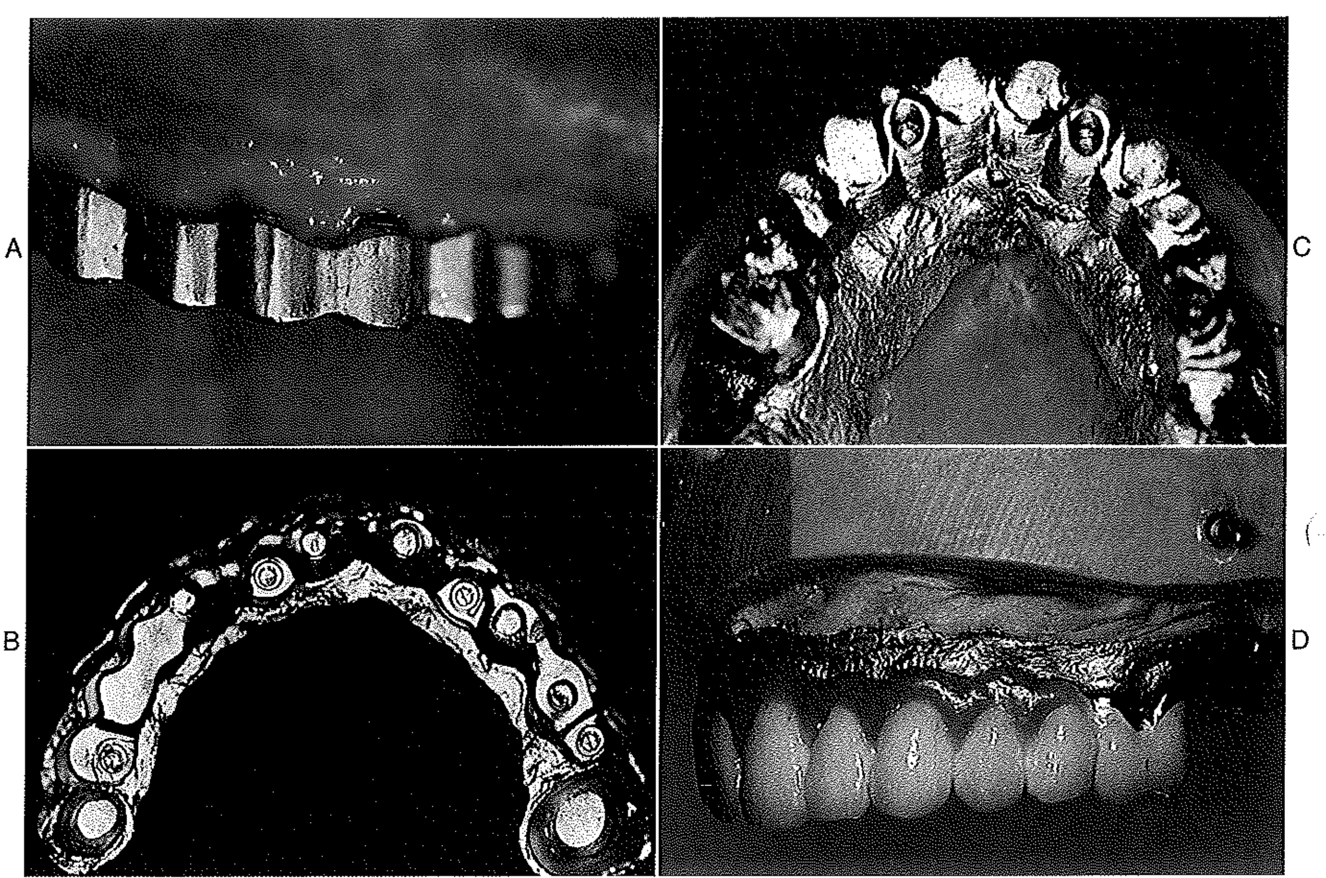
Chapter 7.3, Fig. 7 Atherosclerotic plaque in the coronary artery. (A) Overall architecture demonstrating a fibrous cap (F) and a central lipid core (C) with typical cholesterol clefts. The lumen (L) has been moderately narrowed. Note the plaque-free segment of the wall (*arrow*). (B) Coronary thrombosis superimposed on an atherosclerotic plaque with focal disruption of the fibrous cap (*arrow*), triggering fatal myocardial infarction. A: Reproduced by permission from Schoen, F. J., and Cotran, R. S. (1999). Blood vessels. in *Robbins Pathologic Basis of Disease*, 6th ed., R. S. Cotran, V. Kumar, and T. Collins, eds. W.B. Saunders, Philadelphia. B: Reproduced by permission from Schoen, F. J. (1989). *Interventional and Surgical Cardiovascular Pathology: Clinical Correlations and Basic Principles.* W.B. Saunders, Philadelphia.



Chapter 7.3, Fig. 10 Stent grafts. (A) Configuration of device showing composite metal and fabric portions. (B) Low-power photomicrograph of well-healed experimental device explanted from a dog aorta. The lumen is widely patent and the fabric and metal components are visible. (C) High-power photomicrograph of stent graft interaction with the vascular wall, demonstrating mild intimal thickening. B and C: courtesy Jagdish Butany, MD, University of Toronto.



Chapter 7.3, Fig. 17 Complications of cardiac assist devices. (A) Cuspal tear in inflow valve of LVAD. (B) Hemorrhage into the brain in a patient with an LVAD. (C) Fungal infection in LVAD outflow graft. (D) Thrombosis on pumping bladder. A: Reproduced by permission from Schoen, F. J., and Padera, R. F. (2003). Pathologic considerations in the surgery of adult heart disease. in *Cardiac Surgery in the Adult*, 2nd ed., L. H. Cohn, ed. McGraw-Hill, New York. C: Reproduced by permission from Schoen, F. J., and Edwards, W. D. (2001). Pathology of cardiovascular interventions. in *Cardiovascular Pathology*, 3rd ed., M. D. Silver, A. I. Gotlieb, and F. J. Schoen, eds. Churchill Livingstone, New York. D: Reproduced by permission from Fyfe, B., and Schoen, F. J. (1993). Pathologic analysis of 34 explanted Symbion ventricular assist devices and 10 explanted Jarvik-7 total artificial hearts. *Cardiovasc. Pathol.* 2: 187–197.



Chapter 7.8, Fig. 21 (A) Electron discharge machining or spark erosion techniques were used to mill this mesostructure bar shown attached to implants with fixation screws. (B) The armature or skeleton to which the porcelain is baked and the denture teeth are processed is milled intimately to fit by a precise frictional relationship to the mesostructure bar. (C) The external surface of this structure is prepared to receive the processed prosthesis. (D) The completed spark erosion fabricated mesostructure bar, with a totally porcelain baked superstructure, may be maintained by a frictional relationship or, if additional retention is desired, by the use of strategically related latches, such as Ceka-like attachments.

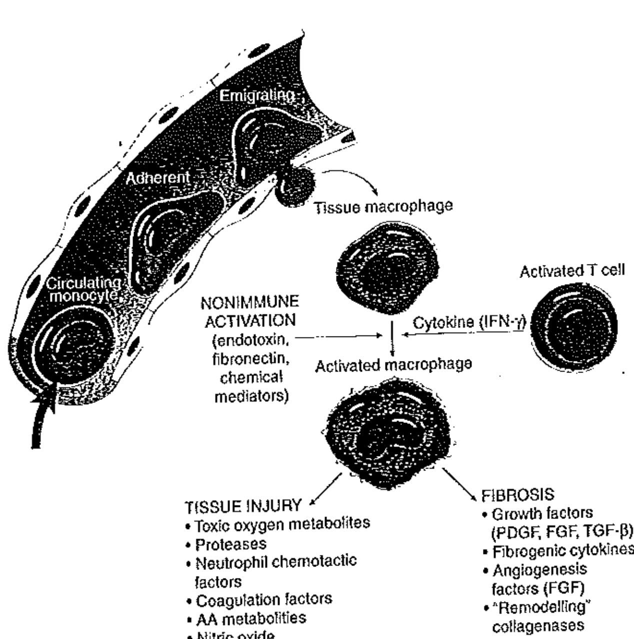


FIG. 6. Macrophage recruitment and local tissue effects after activation. Circulating monocytes are recruited to sites of tissue injury by changes in adhesion molecule expression on endothelial cells in the vicinity, and by chemotactic signals (chemokines) delivered by injured cells or neutrophils, by complement components (generated during complement activation), and by microorganisms themselves. Once these monocytes emigrate into the tissues, they become macrophages and may be activated by IFN-γ from various sources (including activated NK cells or T cells) or by nonimmunologic stimuli such as endotoxin. Activated macrophages will ingest microorganisms and necrotic debris, but will also make a number of eicosanoids (arachadonic acid or AA metabolites), reactive oxygen intermediates, and cytokine mediators that will affect the local tissue environment. Figure reprinted with permission from Kumar *et al.* (2003).

TYPES OF ADAPTIVE IMMUNITY

Adaptive immune responses adopt two basic (and interrelated) forms, humoral and cell-mediated immunity. These are accomplished by different components of the immune system and function to eliminate different types of microorganisms (Fig. 7).

- *Humoral immunity* is mediated by proteins called antibodies that are produced by B lymphocytes. Antibodies bind to unique microbial (or any molecular) antigens with exquisite specificity and target bound molecules or microbes for elimination by phagocytosis and digestion (e.g., via neutrophils and macrophages), direct killing (via NK cells), or lysis (via complement). Humoral immunity is the principal adaptive defense response against extracellular microorganisms (or their toxins), since antibodies can bind to them and assist in their clearance. Antibodies come in different types, e.g., IgA, IgG, IgM, IgE; IgG is the most common, although it is further subdivided into several different subtypes with different functionalities. The different antibody types are specialized to activate specific effector mechanisms (e.g., phagocytosis, complement activation, or release of mediators from mast cells); the details are beyond the scope of this discussion.
- *Cellular immunity* is mediated by T lymphocytes, this form of immune response can participate in the elimination of extracellular microbes (Fig. 7). However, it is also the main mechanism by which intracellular pathogens (e.g., viruses and certain bacteria) that are not accessible to circulating antibodies can be targeted (Fig. 8).

T cells have surface receptors that cannot "see" intact foreign antigen, but rather recognize digested antigen fragments ("processed antigen") presented on the surface of certain host cell types in association with major histocompatibility complex (MHC) molecules (see later discussion). For helper T cells, these accessory or antigen-presenting cells include macrophages, one of the major cell types of the innate immune response. Thus, the innate system directs the response of the adaptive immune system. In return, recognition of foreign peptides leads to T-cell activation. Helper T cells (identified by their expression of the CD4 surface marker) assist in B-cell activation, as well as in the recruitment and activation of macrophages and neutrophils of the innate immune system. Helper T cells can also participate in the activation of NK cells, as well as cytototoxic or killer T cells (identified by their expression of the CD8 surface marker); in this manner infected cells containing intracellular pathogens may be recognized and deleted (Fig. 8).

Not all the possible responses are elicited at the same time in response to a particular pathogen. In some cases, it may be more advantageous to induce primarily a B-cell antibody-mediated response; in other circumstances, a cytotoxic T-cell response may be most warranted. Moreover, the adaptive immune response needs to be tightly regulated to prevent ongoing tissue injury, and therefore a negative-regulatory feedback must exist. The central regulation of these potential outcomes derives from the helper T cells, and more specifically the nature of the cytokines that they produce. Two basic types of helper T cells are currently recognized, called Th1 and Th2, each secreting fairly distinct subsets of cytokines (other T cell subsets are increasingly being identified, but the basic Th1 vs Th2 paradigm is sufficient for this discussion) (Abbas *et al.*, 1996). Thus, whether helper T cells induce or inhibit macrophage activation (for example) is largely a function of their differentiation and their ultimate cytokine repertoires (Fig. 9). The regulatory pathways that determine helper T-cell differentiation are an extremely active area of investigation.

RECOGNITION AND EFFECTOR PATHWAYS IN ADAPTIVE IMMUNITY

Any given T or B cell can only recognize one antigen; we are therefore able to respond to the wide diversity of foreign molecules because of an enormous repertoire of cells arising as a consequence of somatic recombination (see earlier discussion), each with different antigen specificity. Although antibodies and B cells bind to intact foreign molecules, most B-cell responses also require interactions with helper T cells. It is important to reiterate that T cells cannot recognize

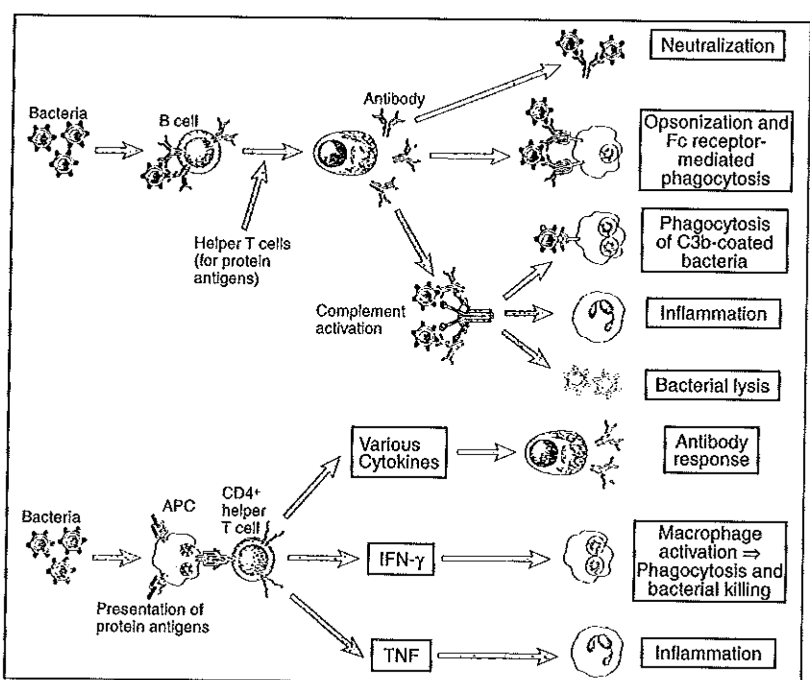


FIG. 7. T- and B-cell adaptive immune responses to extracellular microbes. Adaptive immune responses to extracellular microorganisms (and their toxins) include B-cell responses to generate antibody, and helper T-cell responses that can direct both B-cell antibody production and secondary cellular activation of macrophages and other inflammatory cells. Binding of antibodies can prevent microbes from entering host tissues (neutralization), can opsonize them for phagocytes, or can help activate complement more efficiently to increase inflammatory responses or induce microbial lysis. T-cell activation requires that antigen presenting cells (APC, such as macrophages) degrade the microbe first and present peptide fragments. After helper T-cell activation, and depending on the nature of the cytokines that are produced, B-cell responses can be augmented, or macrophages and other inflammatory cells may be activated. IFN-γ, interferon-γ; TNF, tumor necrosis factor. Figure reprinted with permission from Abbas and Lichtman (2003).

proteins until they have been degraded into smaller fragments and been bound to self histocompatibility molecules on antigen-presenting cells. Thus, most of the adaptive immune response, involving both B and T cells, is dependent on recognition of processed antigen fragments in the context of self histocompatibility proteins (Fig. 10).

In all mammals, histocompatibility molecules are grouped together on chromosomes into clusters generically called major histocompatibility complexes or MHCs. Proteins of this complex are denoted as "histocompatibility" molecules because they were first recognized as the major determining element in tissue ("histo") compatibility in organ transplantation. When inbred strains of animals shared the same MHC determinants, tissue grafts could be transplanted with relative impunity; if the donor and host were MHC-disparate, grafts were said to be histo-incompatible and the organs ultimately failed by a process called rejection (see discussion at the end of the chapter).

In humans, this MHC cluster occurs on chromosome 6, and the molecules are called human leukocyte antigens or HLA. There are two general categories of MHC molecules, called class I and class II. In humans, MHC class I (MHC I) molecules are called HLA-A, -B, and -C; MHC class II (MHC II) molecules are called HLA-DP, -DQ, and -DR. The MHC class I molecules present peptide fragments derived from the antigens of intracellular pathogens to $CD8^+$ cytotoxic T cells; MHC class II molecules present peptide fragments from the antigens of extracellular pathogens to $CD4^+$ helper T cells (Fig. 10) (Klein and Sato, 2000a, b).

There is a basic dichotomy of responses depending on the original source of a particular antigen. Thus, proteins that come from the inside of cell (for example, viruses) associate with MHC I molecules and are recognized selectively by cytotoxic T cells (also called $CD8^+$ T cells). Proteins that come from the outside of cells (for example, bacteria) associate with MHC II molecules and are selectively recognized by helper T cells (also called $CD4^+$ T cells) (Fig. 10) (Germain, 1994).

When cytotoxic T cells encounter their specific antigen, their response is to kill the target cell bearing that antigen. When helper T cells encounter their specific antigen, their response is to make stimulatory molecules (cytokines) that cause the proliferation and activation of other cells; the major cytokine resulting in lymphocyte proliferation is interleukin-2 (IL-2). Besides increasing the numbers of T and B cells in the area of the immune response, helper T cells can also (a) activate B cells to secrete antibody; (b) activate macrophages and neutrophils

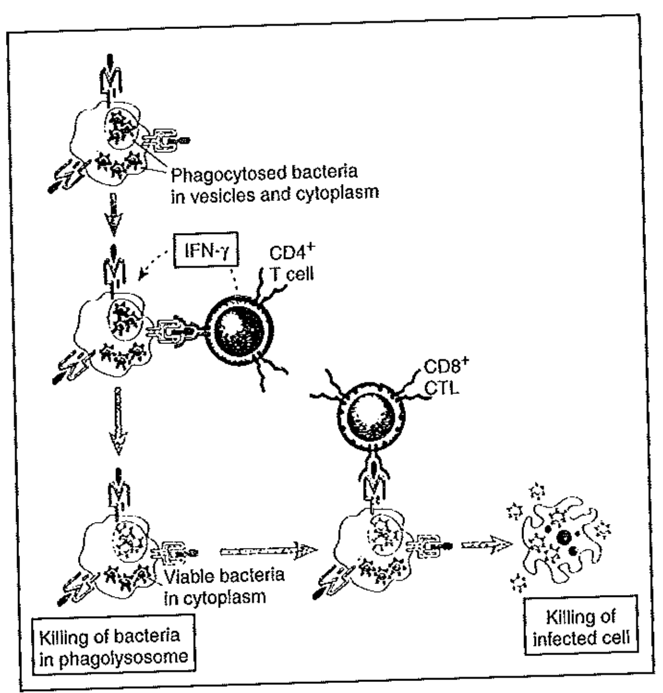


FIG. 8. Helper ($CD4^+$) and cytotoxic ($CD8^+$) T-cell collaboration in defense against intracellular microbes. Intracellular bacteria are partially degraded within the phagolysosomes of APC such as macrophages; the resulting peptide fragments are presented in the context of MHC molecules to activate helper and/or killer T cells. Cytokines elaborated by activated helper T cells can participate in turning on cytotoxic T cells, as well as in the activation of the original APC. In this manner, either cytotoxic T cells will directly kill the infected cell, or the additional booster activation of the APC by helper cytokines will enable them to completely destroy the microbe. Similar pathways exist to allow activated cytotoxic T cells or NK cells to kill cells infected with viruses. Figure reprinted with permission from Abbas and Lichtman (2003).

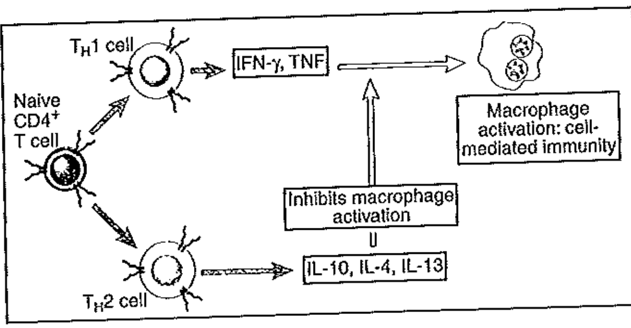


FIG. 9. Role of helper T-cell cytokines in determining immune responses. Naïve $CD4^+$ helper T cells can differentiate into either Th1 or Th2 type cells, each with distinct cytokine profiles and with distinct functions in immune regulation. In the example shown, interferon-γ (IFN-γ) and TNF secreted by Th1-type helper T cells drive macrophage activation, whereas interleukins-4, -10, and –13 (IL-4, IL-10, and IL-13) made by Th2 helper T cells inhibit macrophage activation. A similar dichotomy exists for the activation of the other elements of both the innate and adaptive immune response. Figure reprinted with permission from Abbas and Lichtman (2003).

to help clear infectious agents; (c) activate natural killer cells to be more cytotoxic; and (d) activate endothelium lining blood vessels to recruit even more inflammatory cells (Figs. 7 and 8).

T-cell recognition of antigen fragments bound to MHC molecules results in T-cell activation (Garcia *et al.*, 1999). This recognition step is accomplished by T cell receptors (TCR) on the surface of T lymphocytes; the TCR interact with a group of molecules (collectively called the CD3 complex) and send a signal to the nucleus resulting in cellular stimulation. Complete activation of T cells also requires additional interplay between other molecules (called costimulator molecules) on the surface of T cells and antigen-presenting cells of the innate immune system. Incomplete activation of T cells (i.e., without the costimulators) may result in anergy (no response) to the antigen (Fig. 11).

It bears repeating that although many aspects of immunity involve exquisitely sensitive responses to only selected foreign molecules (antigens), the immune response also involves cells (macrophages, neutrophils, and natural killer cells) and proteins (complement and cytokines) which are antigen nonspecific. Antigen-specific and nonspecific pathways interact with each other.

PATHOLOGY ASSOCIATED WITH IMMUNE RESPONSES

The innate and adaptive immune system exists primarily to defend us against infection (immune surveillance to neoplasm was a later evolutionary adaptation). Unfortunately, immune activation leads not only to the activation of host defenses and production of protective immunoglobulins and T-cells, but also occasionally to the development of responses that may potentially damage host tissues.

Both innate and adaptive immune responses may be implicated in causing disease states. As highlighted earlier, in the setting of prolonged activation, macrophages of the innate immune response will ultimately mediate tissue fibrosis and scarring. Indeed, the response to foreign materials—causing much of the local pathology associated with implants—is attributable to such persistent macrophage activation. Moreover, certain bacterial toxins (LPS) nonspecifically stimulate macrophages (as well as other cell types) and result in systemic pathology from excessive cytokine elaboration.

By having increased specificity, adaptive immunity might be expected to lead overall to less secondary damage. Normally, an exquisite system of checks and balances optimizes the antigen-specific eradication of infecting organisms with only trivial innocent by-stander injury. However, certain types of infection (e.g., virus) may require destroying host tissues to eliminate the disease (see Fig. 10B). Still other types of infections (e.g., tuberculosis) may only be controlled by a cellular response that walls off the offending agent with activated macrophages and scar, often at the expense of adjacent normal parenchyma (similar to foreign-body responses). Even when the host response to an infectious agent is specific antibody, the antibody occasionally cross-reacts with self-antigens (e.g., anti-cardiac antibodies following certain streptococcal infections, causing rheumatic heart disease). Immune complexes

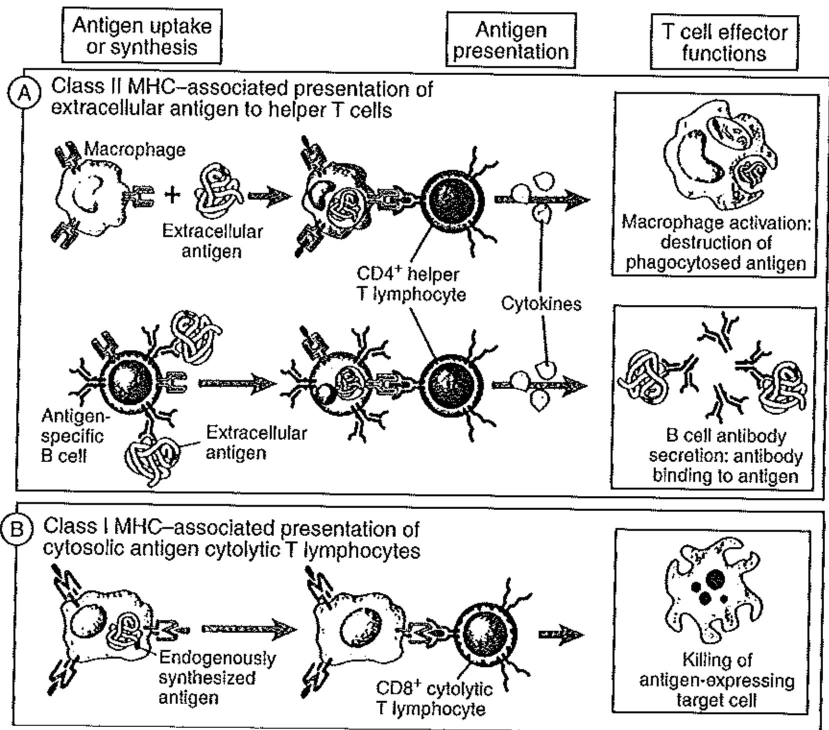


FIG. 10. Presentation of extracellular versus intracellular antigens to cytotoxic versus helper T cells. (A) Extracellular antigens (e.g., from extracellular bacteria) are ingested and degraded by macrophages or other APC (such as B cells), and are then presented in association with MHC II surface molecules to $CD4^+$ helper T cells. Helper T cells activated in this manner lead to macrophage and/or B-cell activation that will eliminate the extracellular microbe antigens. (B) Intracellular antigens (e.g., from intracellular viruses) are degraded and presented in association with MHC I surface molecules to $CD8^+$ cytotoxic T cells. Killer T cells activated in this manner then lyse (kill) the cell that originally harbored the intracellular pathogen. Figure reprinted with permission from Abbas and Lichtman (2003).

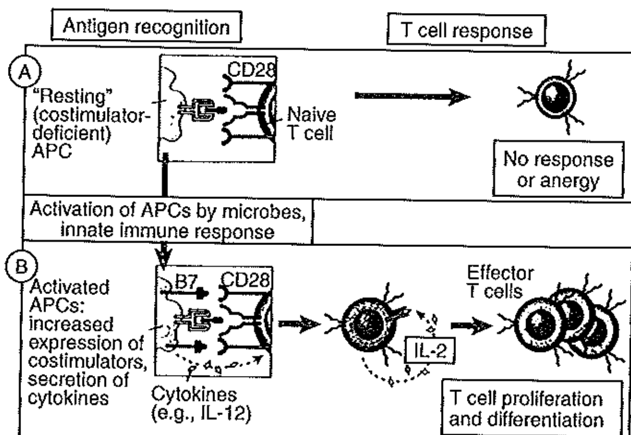


FIG. 11. Role of costimulation in T-cell activation. (A) Antigen-presenting cells (APC) that are not activated will express few or no costimulator molecules. In that setting, even though the APC display processed antigen in the appropriate MHC context, the T cells will fail to respond. Indeed, such costimulator-poor APC presentation may result in a long-term anergy (inability to respond) to particular antigens. (B) Microbes and cytokines produced during innate immune responses activate APC to make costimulator molecules (such as B7, shown here) that will result in "complete" activation of the T cells. Activated APC also produce additional cytokines such as interleukin-12 (IL-12) that also participate in stimulating T-cell activation and differentiation. Figure reprinted with permission from Abbas and Lichtman (2003).

composed of specific antibody and circulating antigens may precipitate at inappropriate sites (see later discussion) and cause injury by activation of the complement cascade, or by facilitating binding of neutrophils and macrophages (e.g., post-streptococcal glomerulonephritis). If the antibody made in response to a particular antigen is IgE, any subsequent response to that antigen will be immediate hypersensitivity (allergy), potentially culminating in anaphylaxis. Finally, not all antigens that attract the attention of lymphocytes are exogenous. The immune system occasionally (but fortunately, rarely) loses tolerance for endogenous antigens, which results in autoimmune disease.

All of these forms of immune-mediated injury are collectively denoted as hypersensitivity. As discussed below and in Chapter 4.5, they are traditionally subdivided into four types; three are variations on antibody (immunoglobulin or Ig)-mediated injury, while the fourth is cell-mediated:

- IgE-mediated "immediate hypersensitivity"; allergy and anaphylaxis
- Mediated by antibody against fixed or circulating tissue antigens
- Immune complex (antigen–antibody)-mediated
- Immune cell–mediated

Pathogenesis of Antibody-Mediated Disease

Antibodies involved in immune-mediated diseases may bind to antigenic determinants that are intrinsic to (synthesized by) a particular tissue or cell, or that are exogenous and have been passively adsorbed (e.g., certain antibiotics or foreign proteins). Regardless of what they recognize, or how they got there, antibodies bound to the surfaces of cells or to extracellular matrix cause injury by certain basic mechanisms.

IgE-Mediated (Immediate Hypersensitivity)

Mast cells and basophils express surface Fc-receptors that can bind the Fc constant region of immunoglobulin E (IgE), one of the five basic immunoglobulin isotypes (Kay, 2001a, b). When circulating IgE's bind to the Fc-receptors and are subsequently cross-linked by specific allergen (antigen), they induce mast cell or basophil degranulation with release of preformed mediators, as well as synthesis of other potent effectors (Fig. 12):

- Preformed mediators: amines such as histamine and serotonin (cause vasodilation and increased vascular permeability)
- Mediators synthesized de novo:

Prostaglandins (e.g., PGD_2) that can affect vessel and airway contraction and vascular permeability

Leukotrienes (e.g., LTC_4, LTD_4, and LTE_4) that are exceptional vasoconstrictors and bronchoconstrictors previously identified as "slow-reacting substance(s) of anaphylaxis" (SRS-A)

Platelet activating factor (PAF), a rapidly catabolized phospholipid derivative that increases vascular permeability and diminishes vascular smooth muscle tone; it also causes bronchoconstriction

Cytokines, in particular TNF (recruits sequential waves of neutrophils and monocytes), and IL-4 (interleukin 4, induces local epithelial and macrophage expression of chemokines such as eotaxin, and also induces endothelial adhesion molecule expression: the combined effect will be to recruit eosinophils).

In most vascular beds, the overall result is vasodilation and increased vascular permeability, with a variable infiltrate classically predominated by eosinophils. Eosinophils are an inflammatory cell type classically associated with parasitic infections, as well as with allergies; they contain specific granules with potent cytotoxic activity for a variety of cell types. In the respiratory tree, the net result of an allergic stimulus is increased mucus secretion and bronchoconstriction.

The nature of the symptoms in any particular instance will depend on the portal of antigen entry, e.g., cutaneous (hives and rash, although these can also occur with inhaled or ingested allergen), inhaled (wheezing, airway congestion), ingested (diarrhea, cramping), or systemic (hypotension). The associated diseases range from the merely annoying (seasonal rhinitis or "hay fever") to debilitating (asthma) to life-threatening (anaphylaxis).

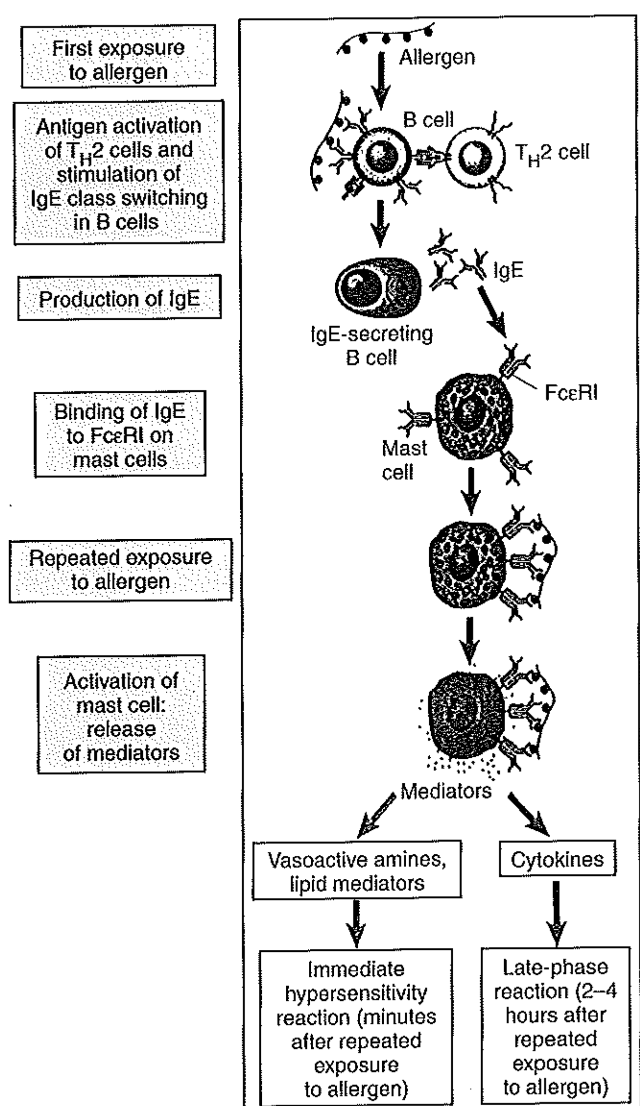


FIG. 12. Events in immediate-type hypersensitivity (allergy). Immediate hypersensitivity is initiated following contact with a specific allergen (an antigen that induces an IgE response). For unclear reasons, allergens induce in a susceptible host a predominant Th2 response that ultimately promotes an IgE antibody response. IgE then binds to mast cells in tissues (and basophils in the circulation, not shown) via specific IgE Fc receptors. Subsequent encounter with the relevant allergen results in IgE-Fc receptor cross-linking which activates the mast cells and basophils. Once activated, the cells secrete preformed mediators causing the characteristic immediate response (vasodilation and increased vascular permeability; may also cause bronchoconstriction). Over the next few hours (up to 24 hours), these activated cells will also synthesize and release additional mediators (prostaglandins, leukotrienes, PAF, and cytokines; see text). Figure reprinted with permission from Abbas and Lichtman (2003).

Antibody Bound to Cell Surfaces or Fixed Tissue Antigens

Antibodies bound to either intrinsic or extrinsic tissue antigens can induce tissue injury by promoting complement activation, inducing opsonization, or by interacting with important cell-surface molecules (Fig. 13).

Recall that complement may induce injury either by direct cytolysis via the C5b-9 membrane-attack complex (MAC)

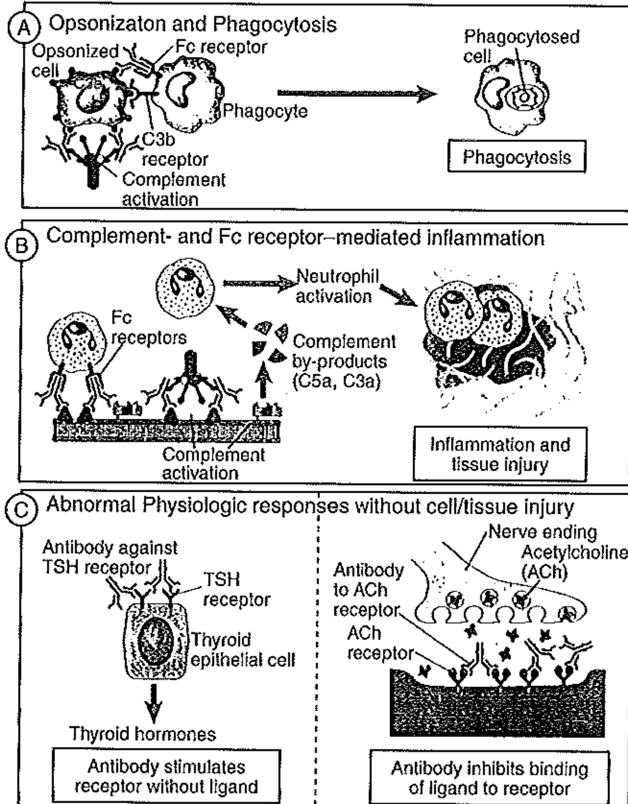


FIG. 13. Effector mechanisms in antibody-mediated disease. (A) Antibodies, with or without complement activation, will opsonize cells leading to phagocytosis and destruction. (B) Antibodies and secondarily generated complement fragments bound to large non-phagocytosable cells or tissues will recruit inflammatory cells such as neutrophils and macrophages. If these inflammatory cells cannot complete ingest the target, frustrated phagocytosis will result in the release of lysosomal contents and reactive oxygen intermediates into the tissues with subsequent extracellular damage. (C) Antibodies can also elicit pathology without causing tissue damage. In the panel on the left, antibodies to the thyroid stimulating hormone (TSH) receptor will mimic authentic TSH and will cause hyperstimulation of the thyroid (Graves' disease). In the panel on the right, antibodies to the acetylcholine (ACh) receptor at the neuromuscular junction will block normal ACh stimulation of muscle contraction leading to weakness (myasthenia gravis). Figure reprinted with permission from Abbas and Lichtman (2003).

punching holes in a cell's plasma membrane, or by opsonization (via the C3b fragment), enhancing phagocytosis by macrophages and neutrophils. In addition to direct cell killing, local activation of the complement cascade will result in the generation of complement fragments such as C3a and C5a (Fig. 5 and Chapter 4.3) (Barrington *et al.*, 2001; Walport, 2001a, b).

- C3a and C5a (so-called anaphylotoxins) mediate increased vascular permeability and smooth muscle relaxation (vasodilation), mainly via releasing histamine from mast cells
- C5a also activates the lipoxygenase pathway in arachidonic acid catabolism, resulting in increased leukotriene synthesis
- C5a-mediated chemotaxis of PMN and monocytes
- On circulating blood cells, bound complement may directly mediate cell lysis; in addition, bound antibody and opsonizing complement fragments induce efficient uptake and destruction by cells of the splenic and hepatic mononuclear phagocyte system

Antibody binding in conjunction with C3b opsonization may also lead indirectly to tissue injury. Large, nonphagocytosable cells or tissue may promote "frustrated phagocytosis" by neutrophils or macrophages; the attempted intracellular lysis results instead in the extracellular release of proteases and toxic oxygen metabolites (Fig. 13B).

Instead of fixing complement, target cells coated with low concentrations of antibody can also attract a variety of nonsensitized cells of innate immunity with Fc-receptors, most importantly the natural killer (NK) cells. These bind to the exposed Fc portion of the bound immunoglobulin and induce cell lysis without phagocytosis.

Binding of antibodies to certain receptors can induce pathology even without causing tissue injury. For example, in the case of Graves' disease, antibodies bind to the thyroid stimulating hormone (TSH) receptor on thyroid epithelial cells and mimic authentic TSH ligand interaction; the result is autonomous stimulation of the gland with hyperthyroidism. Alternatively, antibodies that cross-react with the acetylcholine receptor at the nerve–muscle synapse can block binding of acetylcholine and result in the weakness seen in the disease myasthenia gravis (Fig. 13).

Immune Complex (IC)-Mediated Injury

In many circumstances, circulating antigen and antibody combine to form insoluble aggregates called immune complexes (IC). These are usually efficiently cleared by macrophages in the spleen and liver, but occasionally deposit in certain vascular beds. Once ICs are deposited, the mechanism of injury is basically the same regardless of where or for what reason ICs have accumulated; the major sources of pathology are complement activation (see above) and neutrophil and/or macrophage injury (Fig. 14).

Pathogenesis of Cell-Mediated Disease

T-cell-mediated responses are of two general types (Fig. 15):

- *T cell-mediated cytolysis (caused by antigen-specific $CD8^+$ cytotoxic T lymphocytes or CTL).* In CTL-mediated reactions, cytotoxic lymphocytes recognize specific antigen in association with class I MHC and induce direct cytolysis. It is important to emphasize that CTL-mediated cytolysis is highly specific, without significant "innocent bystander" injury.
- *Delayed-type hypersensitivity (mediated by cytokines and antigen nonspecific effector cells).* In the case of cell-mediated immunity, $CD4^+$ helper T-cells recognize specific antigen in the context of class II MHC, and respond by producing a host of soluble antigen-nonspecific cytokines. These soluble mediators induce further T-lymphocyte recruitment and proliferation, and attract

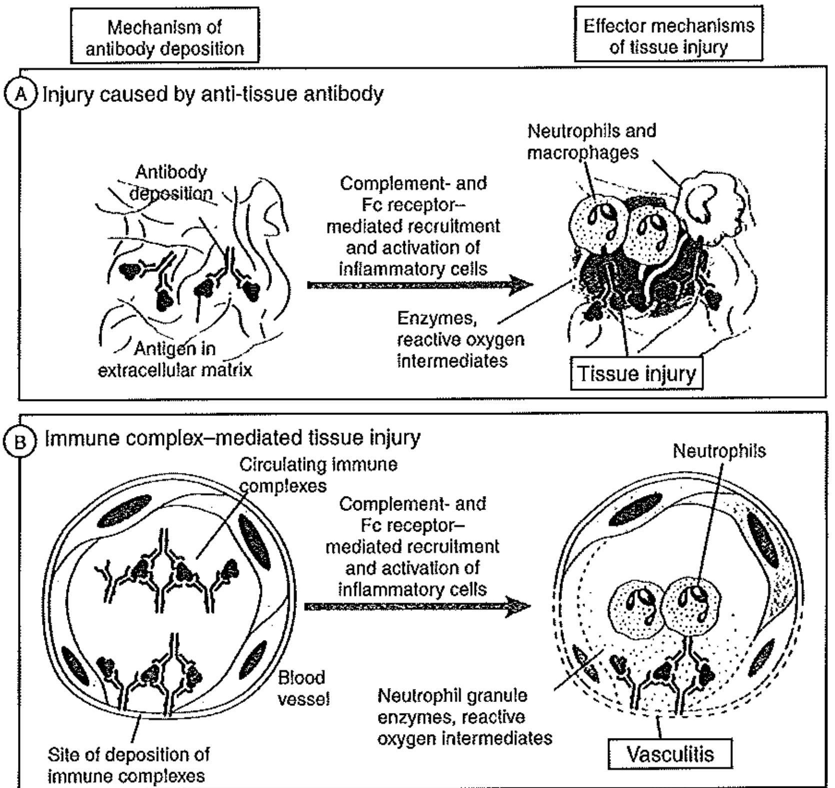


FIG. 14. Antibody-mediated pathology. (A) Direct binding of antibodies to tissue antigens will cause tissue injury by recruiting inflammatory cells and activating complement. (B) Circulating antigen–antibody complexes (also called immune complexes) can deposit in vessels and tissues also leading to inflammatory cell recruitment and complement activation. Figure reprinted with permission from Abbas and Lichtman (2003).

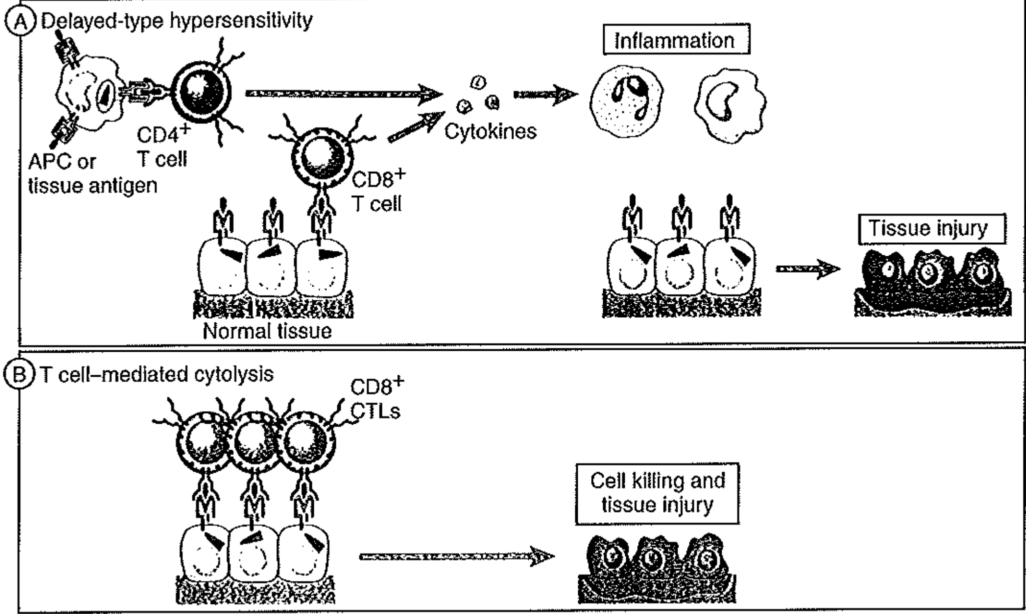


FIG. 15. Mechanisms of T-cell-mediated disease. (A) In delayed-type hypersensitivity responses, T cells (typically $CD4^+$ helper T cells) respond to tissue or cellular antigens by secreting cytokines that stimulate inflammation, and ultimately promote tissue injury (APC, antigen-presenting cell). (B) In some diseases, $CD8^+$cytotoxic T cells directly kill tissue cells. Figure reprinted with permission from Abbas and Lichtman (2003).

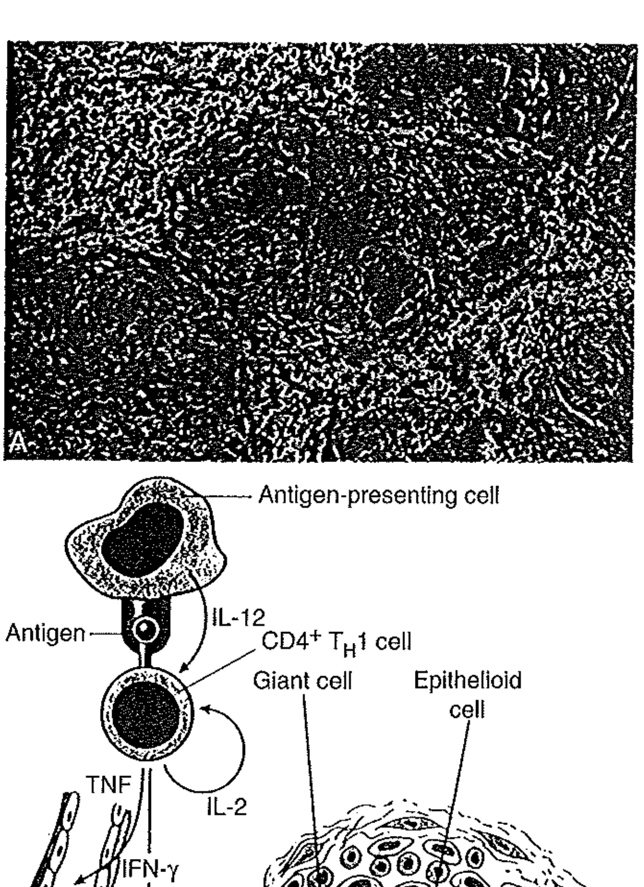


FIG. 16. Granulomatous inflammation. (A) A histologic section of a lymph node showing numerous granulomas, in this case, in response to tuberculosis. Granulomas are aggregates of activated macrophages, surrounded by activated lymphocytes. Note the presence of numerous multinucleated forms of the macrophages, so-called giant cells, which result from cell–cell fusion of macrophages under the influence of certain T-cell cytokines. (B) Schematic illustration of the events that lead to granuloma formation in response to persistent antigens. Antigen-presenting cells (APC) of the innate immune system process antigen and subsequently present it to $CD4^+$ helper T cells; the APC also provide interleukin-12 (IL-12) and other cytokines to drive T-cell activation. Activated T cells, in turn, elaborate cytokines such as tumor necrosis factor (TNF) that will recruit inflammatory cells, and interferon-γ (IFN-γ) that will induce the activation of the recruited cells, in particular macrophages. These cytokines can also induce macrophage fusion to generate giant cells. If the antigen is not effectively eliminated, the constant cycle of T-cell and macrophage activation leads to the accumulation of an aggregate of activated cells. Activated macrophages will also elaborate mediators that result in tissue injury, as well as cytokines resulting in tissue fibrosis (see also Fig. 6). The end result is loss of tissue function and scar formation. In the case of "inert" foreign bodies, adsorption of host proteins onto the foreign-body surface with subsequent denaturation and modification can lead to direct macrophage activation via the receptors involved in innate immunity. Figure reprinted with permission from Kumar *et al.* (2003).

and activate antigen nonspecific macrophages; at the site of a $CD4^+$ T-cell mediated response, the vast majority (greater than 90%), of newly recruited cells are not specific for the original inciting antigen. Cytokine-mediated CMI is critical in clearing intracellular infections not accessible to antibodies or CTL (e.g., tuberculosis, leishmania, histoplasmosis), as well as a variety of large infectious agents not well controlled by antibodies alone (e.g., fungi, protozoans, parasites). Although tightly regulated, the relatively nonspecific effector components of cell-mediated immunity (cytokines and activated macrophages) are largely responsible for the injury seen in delayed-type hypersensitivity (DTH).

In comparison to CTL, cytokine-mediated immunity may ultimately develop an antigen-nonspecific component; that is, after the initial antigen-specific T-cell response, the recruited antigen-nonspecific T-cells and macrophages can cause significant bystander injury. Macrophages in particular are an important component of the recruited inflammatory cells in DTH and mediate much of the subsequent immune effector responses. By virtue of the release of reactive oxygen intermediates, prostaglandins, lysosomal enzymes, and cytokines such as TNF (which, in turn, have potent effects, e.g., on the synthetic function of fibroblasts, lymphocytes, and endothelium), activated macrophages can potentially wreak significant havoc.

An important variant of DTH with a prominent localized component of activated macrophages is called granulomatous inflammation (Fig. 16). Granulomas (the designation of a nodule of granulomatous inflammation is a granuloma) are the characteristic response of the immune system to foreign objects (such as implanted devices), and are thus important elements in most tissue–materials interactions. Granulomas can be mediated by the same basic DTH pathways (antigen-specific T-cells and recruited nonspecific macrophages) in the setting of persistent antigenic stimuli (such as tuberculosis bacteria that may be difficult to eradicate). With persistent antigen, chronic macrophage activation results in cytokine elaboration culminating in a surrounding fibrosis. Presumably by organizing a local accumulation of activated macrophages, granulomas serve to eradicate, or at least wall off, infectious organisms that would otherwise be difficult to contain.

Granulomas also occur in the setting of large, inert, or indigestible substances (see list below); in that case, direct macrophage activation occurs by binding to denaturated or modified host proteins that have adsorbed on the surfaces of the foreign materials via the receptors used for innate immunity (Tang and Eaton, 1993, 1999; Tang *et al.*, 1996). A diagnosis of granuloma suggests only a limited number of disease entities; clinically, the most common are foreign body, tuberculosis, and sarcoidosis (see also Table 2). Final confirmation of the

TABLE 2 Examples of Granulomatous Inflammation

Direct macrophage activation
- Dusts, e.g., beryllium, silica
- Foreign body, e.g., surgical suture, breast implant
- Gout (urate crystals)

T-cell-mediated macrophage activation
- Infections (TB, leprosy, syphilis, cat-scratch disease, schistosomiasis, fungus)
- Necrotizing vasculitis with granulomas (Wegener's granulomatosis, temporal arteritis)
- "Autoimmune" disorders with granulomas (Crohn's disease, de Quervain's thyroiditis)
- Sarcoidosis (inciting agent unknown)

particular inciting agent requires cultures, serologies, or special stains, or may be a diagnosis of exclusion (e.g., sarcoidosis).

Injury associated with granulomas may be due to displacement, compression, and necrosis of adjacent healthy tissue, or may be a consequence of the persistent chronic inflammation that led to the granuloma in the first place (e.g., berylliosis). Granulomas associated with a variety of "autoimmune disorders," such as temporal arteritis, Crohn's disease, and Wegener's granulomatosis, presumably reflect diseases with persistent antigen stimulation, or a heightened DTH response to specific self antigens.

SIMILARITIES AND DIFFERENCES BETWEEN ORGAN REJECTION AND THE RESPONSE TO SYNTHETIC MATERIALS OR TISSUE-DERIVED BIOMATERIALS

When foreign cells or organs are transplanted into a new host, the histocompatibility proteins on the cell surfaces of the graft are recognized by the components of adaptive immunity as being non-self. Note that except for minor genetic polymorphisms, most of the structural proteins and other molecular components in a graft are nearly identical to those that the host will also express (e.g., the contractile proteins in heart muscle, the collagenous extracellular matrix, the usual housekeeping proteins). The MHC molecules, however, are distinctly different between most humans (except identical twins!) and will elicit helper and cytotoxic T-cell activation, as well as B-cell antibody production. Clearly, once these pathways have been activated, the usual physiologic effector mechanisms (direct cell killing, complement activation, phagocytosis, cytokine elaboration, etc.) will be brought to bear on the graft and will in most cases effect its destruction. Again, although components of innate immunity are recruited and activated in the process of graft damage, the initial recognition step and the driving force for transplant rejection is via the cells of adaptive immunity (you are also referred to the basic immunology texts for excellent overviews of the rejection phenomenon; see Abbas and Lichtman, 2003; Benjamini *et al.*, 2000; Janeway, 2001). To prevent or reverse such rejection requires a whole armamentarium of immunosuppressive agents (e.g., cytotoxic drugs or agents such as cyclosporine, which put the recipient at risk of serious infections and certain tumors).

The point is emphasized here because in the immunologic sense, the synthetic materials that make up implanted devices are not rejected. In addition, tissue- or collagen-based biomaterials (e.g., a biological heart valve substitute or a processed collagen) derived from the same species (or sufficiently related species so that there are not major antigenic differences in, e.g., collagen proteins) are also not rejected. Such materials/devices do not elicit specific (adaptive) immune responses, and therefore will not have antibodies or lymphocytes that recognize the materials and cannot therefore drive the overall response. Moreover, although tissue-derived biomaterials derived from non-self [e.g., heart valve from another person (homograft) or an animal (porcine aortic valve or bovine pericardial bioprostheses)] may express foreign histocompatibility antigens, be antigenic, and be capable of eliciting adaptive immune responses (including antibodies and antigen-specific T cells), any failure of the device does not necessarily equate to immune-mediated device dysfunction. Stated another way, even immunogenetic tissue does not necessarily progress to device failure. Moreover, specific immunological responses can even be secondarily induced by device failure, but have nothing to do causally with the actual failure of the device. As a corollary statement, simply finding inflammatory cells (and even T cells and antibodies) does not in any way prove that the response is "rejection"; such elements will accrue at any site of injury in a nonspecific way (recall that some 90% of T cells in a DTH response are not antigen-specific, but are nonspecifically recruited to the site of injury). This is much more than a semantic point, in that synthetic or natural biomaterial device functions or longevity are not likely to benefit from specific immunosuppression. Of course, if a device incorporates viable cells in its manufacture (e.g., endothelial cells lining a vascular conduit), those cells will express MHC proteins and will elicit adaptive immune responses that materially contribute to device failure. In that instance, it will be necessary in the long term either to engineer such devices using cells derived from the individual who will eventually receive the implant, or to rely on long-term immunosuppression much as is done for organ transplants.

It should also be emphasized that although synthetic and biomaterials are not rejected in the immunologic sense, components of the immune system (particularly innate immunity) can contribute to device dysfunction and failure. In particular, and as described above, nonspecific activation of macrophages and complement will lead to local tissue damage via proteolysis, accumulation of other inflammatory cells, and/or cytokine elaboration; in most cases, with an ongoing, persistent innate response to a device that cannot be eliminated, fibrous scar tissue will also develop.

Thus, under certain circumstances, synthetic materials and biomaterials can have failure modes that are attributable to activation of the immune system (particularly innate immunity). An "inert" Silastic-clad breast prosthesis, for example, can accumulate dense scar tissue around it (secondary to persistent macrophage activation) that is not aesthetically ideal. Similarly, a metal hip prosthesis can induce ongoing macrophage activation that in the bone will lead to cytokine production that ultimately drives bone resorption and prosthesis loosening. Although administration of steroids

in these settings may have some beneficial effect by limiting macrophage activation, it may also induce complications since steroids (among other side effects) also inhibit healing and increase susceptibility to infections.

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4.4 The Complement System

Richard J. Johnson

As discussed in the previous chapter, the immune system acts to protect each of us from the constant exposure to pathogenic agents such as bacteria, fungi, viruses, and cancerous cells that pose a threat to our lives. The shear multitude of structures that the immune system must recognize, differentiate from "self," and mount an effective response against has driven the evolution of this system into a complex network of proteins, cells, and distinct organs. An immune response to any foreign element involves all of these components, acting in concert, to defend the host from intrusion. Historically, the immune system has been viewed from two perspectives: cellular or humoral. This is a somewhat subjective distinction, since most humoral components (such as antibodies, complement components, and cytokines) are made by cells of the immune system and, in turn, often function to regulate the activity of these same cells. The focus of this chapter will be on the basic biochemistry and pathobiology of the complement system, a critical element of the innate immune response, and its relevance to biomaterials research and development.

INTRODUCTION

Complement is a term devised by Paul Ehrlich to refer to plasma components that were known to be necessary for antibody-mediated bactericidal activity. We now know that complement is composed of more than 30 distinct plasma and membrane bound proteins involving three separate pathways: classical, alternative, and the more recently described lectin pathway. The complement system directly and indirectly contributes both to innate inflammatory reactions and to cellular (i.e., adaptive) immune responses. This array of effector functions is due to the activity of a number of complement components and their receptors on various cells. These activities are summarized in Table 1, along with the responsible complement protein(s). One of the principal functions of complement is to serve as a primitive self–nonself discriminatory defense system. This is accomplished by coating a foreign material with complement fragments and recruiting phagocytic cells that attempt to destroy and digest the "intruder." Although the system evolved to protect the host from the invasion of adventitious pathogens, the nonspecific and spontaneous nature of the alternative pathway permits activation by various biomaterial surfaces. Because complement activation can follow three distinct but interacting pathways, the various ways of activating the cascade will be outlined separately below.

TABLE 1 Complement Activities

Activity	Complement protein
Identification/opsonization of pathogens	C3, C4
Recruitment/activation of inflammatory cells	C3a, C5a
Lysis of pathogens/cytotoxicity	C5b-9 (MAC)
Clearing immune complexes and apoptotic cells	C1q, C3b, C4b
Augment cellular immune responses (T and B cells)	C3, C4, C3a, C5a

CLASSICAL PATHWAY

The classical pathway (CP) is activated primarily by immune complexes (ICs) composed of antigen and specific antibody, although other proteins such as C-reactive protein, serum amyloid protein, and amyloid fibrils as well as apoptotic bodies can also activate the CP (Cooper, 1985). The proteins of this pathway are C1, C2, C4, C1 inhibitor (C1-Inh), and C4 binding protein (C4bp). Some of their basic biochemical characteristics are summarized in Table 2.

Complement activation by the CP is illustrated in Fig. 1. This system is an example of an enzyme cascade in which each step in the series, from initiation to the final product, involves enzymatic reactions (in this case, proteolytic cleavage reactions) that result in some degree of amplification. Recent work with knockout mice (mice deficient in C1q, C2, C4, or IgG) has shown that the CP is in a state of continuous low-level activation, essentially primed to react vigorously in the presence of an IC. When an IC forms, the cascade is initiated when C1 binds to the Fc portion of an antigen–antibody complex. The C1 protein is composed of three different types of subunits called C1q, C1r, and C1s (Fig. 2). One end of C1q binds to an IC formed between an antigen and one molecule of (pentameric) immunoglobulin (Ig) M or several closely spaced IgG molecules. This interaction is believed to produce a conformational change in the C1q that results in activation (i.e., autocatalytic proteolysis) of the two C1r and then the two C1s subunits, bound to the other end of the C1q protein. Both C1r and C1s are zymogen serine proteases that are bound to the C1q in a calcium-dependent manner that is inhibited by calcium chelators such as citrate or EDTA. The proteolysis of C1s completes the activation of C1, which then proceeds to act on the next proteins in the cascade, C4 and C2.

TABLE 2 Proteins of the Classical Pathway of Complement

Protein	Molecular weight	Subunits	Plasma concentration (μg/ml)
C1q	410,000	6A, 6B, 6C	70
C1r	85,000	1	35
C1s	85,000	1	35
C2	102,000	1	25
C4	200,000	$\alpha\beta\gamma$	600
C1-Inh	104,000	1	200
C4bp	570,000	8	230

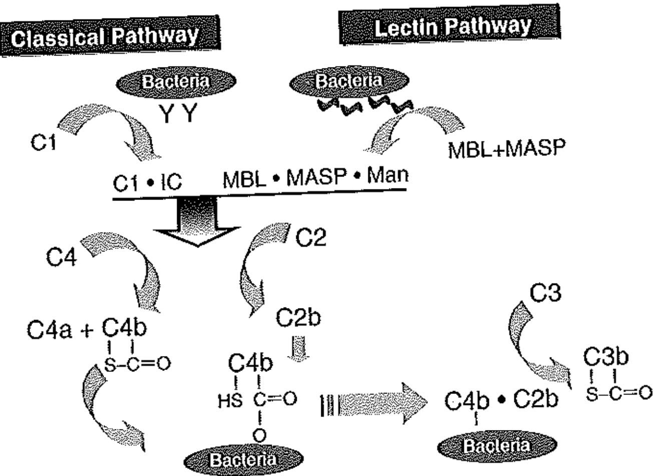

FIG. 1. Complement activation by the classical pathway (CP). Upon binding to the Fc region of an immune complex, C1 is activated and cleaves C4, exposing its thioester, which permits covalent attachment of C4b to the activating surface. C2 is cleaved, producing C2b, which binds to C4b to form the CP C3 convertase. C2b is a serine protease that specifically acts on C3 to generate C3b and C3a. The lectin pathway is also illustrated. MBL recognizes certain sugar residues (mannose, N-acetylglucosamine) on the surface of an activator (bacteria). MASP-1 appears to activate MASP-2, which then cleaves both C4 and C2 of the CP, generating the CP C3 convertase.

C4 is composed of three separate chains, α, β and γ (Fig. 2), bound together by disulfide bonds. Activated C1s cleaves C4 near the amino-terminus of the α chain, yielding a 77-amino acid polypeptide called C4a and a much larger (190,000 Da) C4b fragment. The C4 protein contains a unique structural element called a thioester (Fig. 2). Thioesters have only been detected in two other plasma proteins, α2-macroglobulin and C3. Upon cleavage of C4, the buried thioester becomes exposed and available to react with a surface containing amino or hydroxyl moieties. About 5% of the C4b molecules produced react through the thioester and become covalently attached to the surface. This represents the first amplification step in the pathway since each molecule of C1 produces a number of surface-bound C4b sites.

The C4b protein, attached to the surface, acts as a receptor for C2. After binding to C4b, C2 becomes a substrate for C1s. Cleavage of C2 yields two fragments: A smaller C2a portion diffuses into the plasma, while the larger C2b remains bound to the C4b. The C2b protein is another serine protease that, in association with C4b, represents the classical pathway C3/C5 convertase.

As the name implies, the function of the C4b·C2b complex is to bind and cleave C3. The C3 protein sits at the juncture of the classical and alternative pathways and represents one of the critical control points. Cleavage of C3 by C2b yields a 9000-Da C3a fragment and a 175,000-Da C3b fragment that is very similar to C4b in both structure and function. Cleavage of C3 produces a conformational change in the C3b protein that results in exposure of its thioester group (Fig. 2). Condensation with water or surface carbohydrates results in covalent attachment of 10–15% of the C3b to the surface of the activator. This is the second amplification step in the sequence since as many as 200 molecules of C3b can become attached to the surface surrounding every C4b·C2b complex. Eventually one of the C3b molecules reacts with a site on the C4b protein, creating a C3b–C4b·C2b complex that acts as a C5 convertase (Fig. 4).

In contrast to C3, which can be cleaved in the fluid phase (see later discussion), proteolytic activation of C5 occurs only after it is bound to the C3b portion of the C5 convertase on the

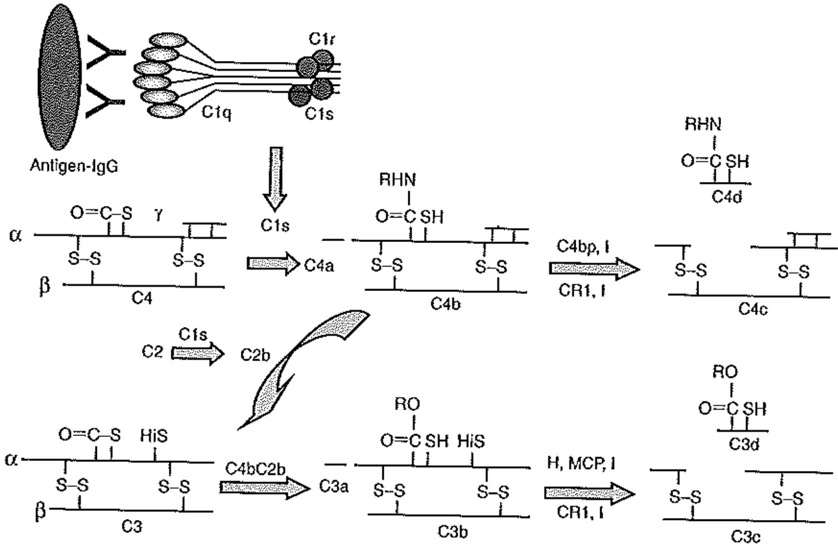

FIG. 2. Schematic illustration of C4 and C3 protein structures. O=C–S represents the reactive thioester bond that permits covalent attachment to surface nucleophiles (hydroxyl or amino groups). The pattern of proteolytic degradation and the resulting fragments are also shown. Although factor I is the relevant *in vivo* protease, some of these same fragments can be generated with trypsin, plasmin, and thrombin.

FIG. 3. Complement activation by the alternative pathway (AP). The spontaneous conversion of C3 to C3(H_2O) permits the continuous production of C3b from C3, a process called C3 tickover. In the presence of an activating surface, the C3b is covalently bound and becomes the focal point for subsequent interactions. The bound C3b is recognized by factor B, which is then cleaved by factor D to produce a surface-bound C3 convertase (C3b·Bb). This results in amplification of the original signal to produce more convertase.

surface of an activator (e.g., the immune complex). Like C3, C5 is also cleaved by C2b to produce fragments designated C5a (16,000 Da) and C5b (170,000 Da). The C5b molecule combines with the proteins of the terminal components to form the membrane attack complex described later. C5a is a potent inflammatory mediator and is responsible for many of the adverse reactions normally attributed to complement activation in various clinical settings.

LECTIN PATHWAY

In the 1990s, investigators working with a protein called mannose binding protein (or mannan binding lectin, MBL) discovered of a third pathway that leads to complement activation (Matsushita, 1996). This scheme is called the lectin pathway and is composed of lectins such as MBL and two MBL-associated serine proteases or MASPs (Table 3). MBL is an acute phase protein, so its concentration in plasma increases substantially during an infection. MBL binds to terminal mannose, *N*-acetylglucosamine, and *N*-acetylmannosamine residues in complex carbohydrate structures. MBL has long been recognized as an opsonin, i.e., a protein that facilitates phagocytosis of bacteria. Low concentrations of MBL in children are associated with recurrent bacterial infections. MBL is similar in structure to C1q, having an amino-terminal domain with a collagen-like structure that binds the MASP proteins, followed by a globular carbohydrate recognition domain (CDR) that binds to sugar residues. There are two MASP proteins, called MASP-1 and MASP-2, that are very similar in structure to C1r and C1s (Wong *et al.*, 1999). Upon activation of MBL·MASP-1·MASP-2, the MASP protease components cleave C4 and C2, forming a CP C3 convertase (Fig. 1).

ALTERNATIVE PATHWAY

The alternative pathway (AP) was originally discovered in the early 1950s by Pillemer *et al.* (1954). Pillemer's group studied the ability of a yeast cell wall preparation, called zymosan, to consume C3 without affecting the amount of C1, C2, or C4. A new protein, called properdin, was isolated and implicated

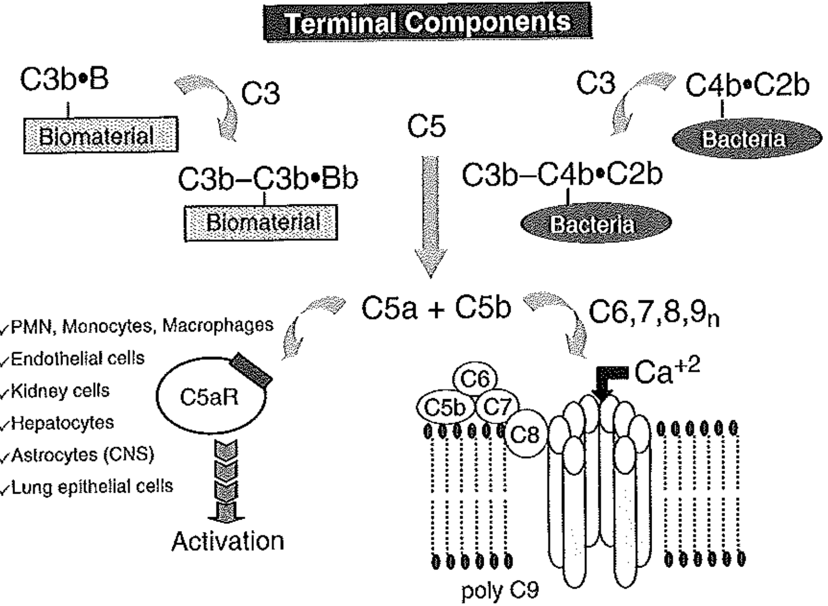

FIG. 4. Conversion of C5 produces C5a and leads to formation of the membrane attack complex (MAC). C5a binds to receptors on a variety of cells and results in numerous activities. C5b, formed by the CP, lectin, or the AP, binds C6 and C7 to form a complex that associates with the plasma membrane. This C5b67 multimer then binds C8, which results in the formation of a small hole in the lipid bilayer that allows small molecules to pass through. Association of multiple C9 proteins enlarges the pore, leading to loss of membrane integrity and cell death.

TABLE 3 Proteins of the Lectin Pathway of Complement

Protein	Molecular weight	Subunits	Plasma concentration (μg/ml)
MBL	270–650,000	18	1–3
MASP-1	93,000	2 (H,L)	6
MASP-2	76,000	2 (H,L)	

in initiating C3 activation independent of the CP. This new scheme was called the properdin pathway. However, this work fell into disrepute when it was realized that plasma contains natural antibodies against zymosan, which implied CP involvement in Pillemer's experiments. The pathway was rediscovered in the late 1960s with the study of complement activation by bacterial lipopolysaccharide and with the discovery of a C4-deficient guinea pig. The 1970s witnessed the isolation and characterization of each of the proteins of this pathway until it was possible to completely reconstruct the entire AP by recombining each of the purified proteins (Schreiber *et al.*, 1978). Most biomaterials activate complement through the AP, although there is evidence that the CP can also contribute (presumably subsequent to IgG binding).

The proteins of this pathway are described in Table 4. Their actions can be conceptually divided into three phases: initiation, amplification, and regulation (Figs. 3 and 5). Initiation is a spontaneous process that is responsible for the nonselective nature of complement. During this stage, a small portion of the C3 molecules in plasma undergo a conformational change that results in hydrolysis of the thioester group, producing an activated form of C3 called C3(H_2O) ("C3-water"), that will bind to factor B. The C3(H_2O)·B complex is a substrate for factor D (another serine protease), which cleaves the B protein to form a solution-phase alternative pathway C3 convertase: C3(H_2O)·Bb. Analogous to C2b in the CP, Bb is a serine protease that (in association with C3(H_2O)) will cleave more C3 to form C3b. Under normal physiological conditions, most of the C3b produced is hydrolyzed and inactivated, a process that has been termed "C3 tickover." C3 tickover is a continuous process that ensures a constant supply of reactive C3b molecules to deposit on foreign surfaces, such as cellulosic- or nylon-based biomaterials. Recognition of the C3b by factor B, cleavage by factor D, and generation of more C3 convertase leads to the amplification phase (Fig. 3). During this stage, many more C3b molecules are produced, bind to the surface, and in turn lead to additional C3b·Bb sites. Eventually, a C3b molecule attaches to one of the C3 convertase sites by direct attachment to the C3b protein component of the enzyme. This C3b–C3b·Bb complex is the alternative pathway C5 convertase and, in a manner reminiscent of the CP C5 convertase, converts C5 to C5b and C5a (Fig. 4).

Recent work with purified proteins and techniques to measure direct interactions with polymer surfaces has revealed an additional potential mechanism for alternative pathway activation (Andersson *et al.*, 2002). Both C3b and C3 will adsorb

TABLE 4 Proteins of the Alternative Pathway of Complement

Protein	Molecular weight	Subunits	Plasma concentration (μg/ml)
C3	185,000	$\alpha\ \beta$	1300
B	93,000	1	210
D	24,000	1	1
H	150,000	1	500
I	88,000	$\alpha\ \beta$	34
P	106–212,000	2–4	20

TABLE 5 Proteins of the Membrane Attack Complex

Protein	Molecular weight	Subunits	Plasma concentration (μg/ml)
C5	190,000	$\alpha\ \beta$	70
C6	120,000	1	60
C7	105,000	1	60
C8	150,000	$\alpha\ \beta$	55
C9	75,000	1	55
S-protein	80,000	1	500

FIG. 5. Control of complement activation by factors H, I, and C4 binding protein. The extent to which complement activation occurs on different surfaces is dependent on the ability of fH or C4BP to recognize C3b or C4b on the surface. Degradation by factor I results in irreversible inactivation and the production of C3 and C4 fragments recognized by various complement receptors on WBC.

to (not react with) polystyrene. A portion (about 10%) of the bound C3 or C3b binds factor B. This complex is recognized by factor D, which then catalyzes the formation of an AP C3 convertase. This process is facilitated by properdin, which increases the amount of convertase formed under these conditions. Interestingly, while the C3b·Bb convertase is controlled by factors H and I (see later discussion), the surface-bound C3·Bb convertase is not. The adsorption of C3 does not occur if the polystyrene surface is precoated with fibrinogen, so the extent to which this occurs in whole blood, where many other proteins can compete with C3 for binding to a biomaterial surface, has not been demonstrated.

MEMBRANE ATTACK COMPLEX

All three pathways lead to a common point: cleavage of C5 to produce C5b and C5a. C5a is a potent inflammatory mediator and is discussed later in the context of receptor-mediated white-blood-cell activation. The production of C5b initiates the formation of a macromolecular complex of proteins called the membrane attack complex (MAC) that disrupts the cellular lipid bilayer, leading to cell death (Table 5). The sequence of events in MAC formation is outlined in Fig. 4.

Following cleavage of C5 by C5 convertase, the C5b remains weakly bound to C3b in an activated state in which it can bind C6 to form a stable complex called C5b6. This complex binds to C7 to form C5b67, which has ampiphilic properties that allow it to bind to, and partially insert into, lipid bilayers. The C5b67 complex then binds C8 and inserts itself into the lipid bilayer. The C5b678 complex disrupts the plasma membrane and produces small pores ($r \sim 1$ nm) that permit leakage of small molecules. The final step occurs when multiple copies of C9 bind to the C5b678 complex and insert into the membrane. This enlarges the pore to about 10 nm and can lead to lysis and cell death. Even at sublytic levels, formation of MAC on host cells results in a number of activation responses (elevated Ca^{2+}, arachidonic acid metabolism, cytokine production).

In addition to the usual means of generating C5b (i.e. through C5 convertase activity), several groups have shown that C5 can be modified by a variety of oxidizing agents (H_2O_2, superoxide anion, and others) to convert C5 into a C5b-like structure that will bind C6. The oxidized C5·C6 complex can bind C7, C8, and C9 to form lytic MAC. This mechanism of MAC formation may be relevant at sites where neutrophils and macrophages attempt to phagocytize a biomaterial, producing a variety of reactive oxygen species, or in hypoxia/reperfusion settings (angioplasty, cardiopulmonary bypass [CPB]).

CONTROL MECHANISMS

Various types of control mechanisms have evolved to regulate the activity of the complement system at numerous points in the cascade (Liszewski *et al.*, 1996). These mechanisms are shown in Fig. 6 and include (1) decay (dissociation) of convertase complexes, (2) proteolytic degradation of active components that is facilitated by several cofactors, (3) protease inhibitors, and (4) association of control proteins with terminal components that interfere with MAC formation. Without these important control elements, unregulated activation of the cascade results in overt inflammatory damage to various tissues and has been demonstrated to contribute to the pathology of many diseases (discussed later).

Control of Complement Activation

Decay Acceleration	Cofactor Activity	Protease/ Inhibitor	MAC Inhibitor
CR1	CR1	Factor I	CD59
Factor H	Factor H	C1Inh	S Protein
DAF/CD55	MCP/CD46	sCPN	Clusterin
C4BP	C4BP		

H Bb I

C3b•Bb → C3b•H → iC3b C3dg

FIG. 6. Control of complement activation occurs by various mechanisms and is facilitated by a number of different proteins in the plasma (fH, fI, C1 Inh, C4BP, sCPN, S-protein, and clusterin) or on cell surfaces (CR1, DAF, MCP and CD59). Decay acceleration refers to the increased rate of displacement of either C2b or fBb from CP or AP convertases. Cofactor activity refers to the increase rate of factor I–mediated proteolysis facilitated by some proteins.

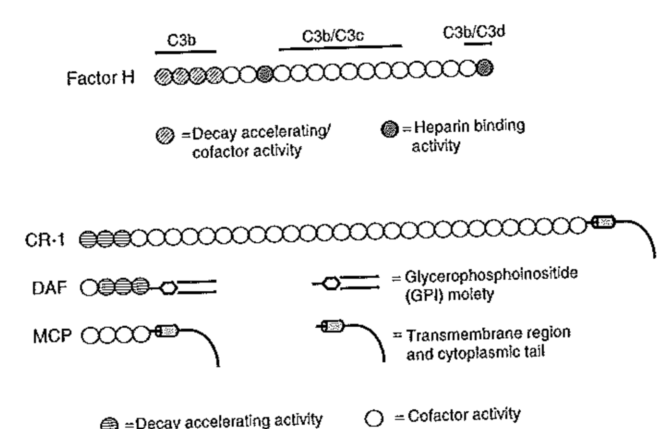

FIG. 7. Structure–activity relationships for complement control proteins. Each circle represents one short consensus repeat (SCR) domain, made up of about 60 amino acids. These SCR domains are strung together to create the different structures shown.

Starting at the top of the cascade, control of the classical and lectin pathway activation is mediated by a protein called C1 esterase inhibitor (C1-Inh). C1-Inh acts by binding to activated C1r and C1s subunits in C1 as well as MASP proteases bound to MBL. C1-Inh actually forms a covalent bond with these proteases, thus irreversibly inactivating these proteins. The effectiveness of this interaction is illustrated by the short half-life of C1s under physiological conditions (13 sec). The classical/lectin pathway C3/C5 convertase (C4b·C2b complex) spontaneously "decays" by dissociation of the C2b catalytic subunit. The rate of dissociation is increased by C4 binding protein (C4bp), which competes with C2 for a binding site on C4b. C4bp also acts as a cofactor for another control protein called factor I, which destroys the C4b by proteolytic degradation (Figs. 2 and 6).

The alternative pathway is also highly regulated by mechanisms that are very similar to the CP. The intrinsic instability of the C3b thioester bond (half-life = 60 μsec) ensures that most of the C3b (80–90%) is inactivated in the fluid phase. Once formed, the C3 convertase (C3b·Bb complex) also spontaneously dissociates and the rate of "decay" is increased by factor H. Aside from accelerating the decay of C3 convertase activity, factor H also promotes the proteolytic degradation of C3b by factor I (Figs. 2 and 6). Factors H and I also combine to limit the amount of active C3(H_2O) produced in the fluid phase.

In addition to factor H, there are several cell-membrane-bound proteins that have similar activities and structures (Fig. 7). These proteins act to limit complement-mediated damage to autologous, bystander cells. Decay-accelerating factor, or DAF, displaces Bb from the C3 convertase and thus destroys the enzyme activity. DAF is found on all cells in the blood (bound to the plasma membrane through a unique lipid group) but is absent in a disease called proximal nocturnal hemoglobinuria (PNH), which manifests a high spontaneous rate of red blood cell lysis. In addition to DAF, there are two other cell-bound control proteins: membrane cofactor protein (MCP) and CR1 (complement receptor 1, see later discussion). MCP is found on all blood cells except erythrocytes, while CR1 is expressed on most blood cells as well as cells in tissues such as the kidney. Both of these proteins display cofactor activity for the factor I–mediated cleavage of C3b. CR1 also acts like factor H and DAF to displace Bb from the C3 convertase. A soluble recombinant form of CR1 (sCR1) was originally described by Weisman *et al.* (1990) and later produced commercially (Avant Immunotherapeutics). A number of investigations have used sCR1 to limit complement activation in various disease models (Larsson *et al.*, 1997; Couser *et al.*, 1995).

In contrast to the inhibitory proteins discussed above, properdin, the protein originally discovered by Pillemer *et al.*, functions by binding to surface-bound C3b and stabilizing the C3 and C5 convertase enzymes. Although properdin is not necessary for activation of the alternative pathway, a genetic deficiency of this protein has been associated with an increased susceptibility to meningococcal infections.

As with the other stages of the cascade, there are several control mechanisms that operate to limit MAC formation and the potential for random lysis of "bystander" cells. The short half-life of the activated C5b (2 min) and the propensity of the C5b67 complex to self-aggregate into a nonlytic form help limit MAC formation. In addition, a MAC inhibitor, originally called S protein and recently shown to be identical to vitronectin, binds to C5b67 (also C5b678 and C5b6789) and prevents cell lysis. Recently another group of control proteins called homologous restriction factors (HRFs) have been discovered. They are called HRFs because they control assembly of the MAC on autologous cells (i.e., human MAC on human cells) but do not stop heterologous interactions (e.g., guinea-pig MAC on sheep red blood cells). One well-characterized member of this group is called CD59. It is widely distributed, found on erythrocytes, white blood cells, endothelial cells, epithelial cells, and hepatocytes. CD59 functions by interacting with C8 and C9, preventing functional expression of MAC complexes on autologous cells.

TABLE 6 Receptors for Complement Proteins

Receptors name/ligand	Structure	Cellular distribution/response
CR1/C3b, C4b	200,000-Da single chain	RBC, PMN, monocytes, B and T cells/clearance of immune complexes, phagocytosis, facilitates cleavage of C3b to C3dg by Factor I
CR2/C3dg	140,000-Da single chain	B cells/regulate B-cell proliferation
CR4	150,000-Da α chain 95,000-Da β chain	PMN, platelets, B cells/leukocyte–endothelial cell interaction
CR3/iC3b, ICAM-1, β glucan fibrinogen, factor Xa	185,000-Da α chain 95,000-Da β chain	PMN, monocyte/phagocytosis of microorganisms; respiratory burst activity
C5a/C5a		PMN, monocytes, T cells, epithelial cells, endothelial cells, hepatocytes, CNS, fibroblasts/chemotaxis, degranulation, hyperadherence, respiratory burst, cytokine production (IL-6, IL-8)
C3a/C3a	65,000 Da	Mast cells, eosinophils (various tissues)/histamine release, IL-6 production
C1q/C1q	70,000 Da	PMN, monocytes, B cells/respiratory burst activity
H/H	50,000 Da (three chains)	B cells, monocytes/secretion of factor I, respiratory burst activity

COMPLEMENT RECEPTORS

Except for the cytotoxic action of the MAC, most of the biological responses elicited by complement proteins result from ligand-receptor-mediated cellular activation (Sengelov, 1995). These ligands are listed in Table 6 and are discussed briefly here.

The ability of complement to function in the opsonization of foreign elements is accomplished in large part by a set of receptors that recognize various C3 and C4 fragments bound to these foreign surfaces. These receptors are called complement receptor 1, 2, 3, or 4 (CR1, CR2, etc). CR1 is found on a variety of cells including RBCs, neutrophils, monocytes, B cells, and some T cells and recognizes a site within the C3c region of C3b (Fig. 2). On neutrophils and monocytes, activated CR1 will facilitate the phagocytosis of C3b- and C4b-coated particles. On RBCs, CR1 acts to transport C3b–immune complexes to the liver for metabolism. As discussed above, CR1 is also a complement regulatory protein. CR2 is structurally similar to CR1 (with 16 SCR domains; see Fig. 7), but recognizes the C3d fragment of C3b that is bound to antigen. CR2 is expressed on antigen-presenting cells such as follicular dendritic cells and B cells where it facilitates the process of antigen–immune complex-driven B-cell proliferation, providing a link between innate and adaptive immunity. CR3 represents another complement receptor that binds to iC3b, and β-glucan structures found on zymosan (yeast cell wall). Also, on activated monocytes, CR3 has been shown to bind fibrinogen and factor Xa (of the coagulation cascade). CR3 is a member of the β2-integrin family of cell adhesion proteins that includes leukocyte functional antigen-1 (LFA-1) and CR4. LFA-1, CR3, and CR4 are routinely referred to as CD11a, CD11b, and CD11c, respectively. Each of these proteins associates with a molecule of CD18 to form a α–β heterodimer that is then transported and expressed on the cell surface. These proteins help mediate the cell–cell interactions necessary for such activities as chemotaxis and cytotoxic killing. A genetic deficiency in CR3/LFA proteins leads to recurrent life-threatening infections. CR4 is found on neutrophils and platelets and binds C3d and iC3b. CR4 may facilitate the accumulation of neutrophils and platelets at sites of immune complex deposition.

In contrast to the ligands discussed earlier, which remain attached to activating surfaces, C3a, C4a, and C5a are small cationic polypeptides that diffuse into the surrounding medium to activate specific cells. These peptides are called anaphylatoxins because they stimulate histamine release from mast cells and cause smooth muscle contraction, which can produce increased vascular permeability and lead to a fatal form of shock called anaphylactic shock. These activities are lost when the peptides are converted to their des Arg analogs (i.e., with the loss of their carboxyl terminal arginine residue, referred to as C3a $_{\text{des Arg}}$, C5a $_{\text{des Arg}}$, etc.). This occurs rapidly *in vivo* and is catalyzed by serum carboxypeptidase N.

In addition to its anaphylatoxic properties, C5a and C5a $_{\text{des Arg}}$ bind to specific receptors originally found on neutrophils and monocytes. Recently the receptors for both C5a and C3a have been cloned and sequenced. The C5aR (CD88) has been shown to be expressed on endothelial cells (EC), hepatocytes, epithelial cells (lung and kidney tubules), T cells, cells in the CNS as well as on the myeloid cell lines. In addition, expression levels of C5aRs are increased on EC and hepatocytes by exposure to LPS and IL-6. In myeloid cells (neutrophils and monocytes), the C5a-receptor interaction leads to a variety of responses, including chemotaxis of these cells into an inflammatory locus; activation of the cells to release the contents of several types of secretory vesicles and produce reactive oxygen species that mediate cell killing; increased expression of CR1, CR3, and LFA-1, resulting in cellular hyperadherence; and the production of other mediators such as various arachidonic acid metabolites and cytokines, e.g., IL-1, -6, and -8. Many of the adverse reactions seen during extracorporeal therapies, such as hemodialysis, are directly attributable to C5a production. C3aRs are expressed on a variety of cell types

TABLE 7 Clinical Settings Involving Complement

Hemodialysis and cardiopulmonary bypass
Kidney disease (especially glomerulonephritis)
Ischemia/reperfusion injury (e.g., angioplasty following heart attack)
Sepsis and adult respiratory distress syndrome
Recurrent infections
Transplantation
Rheumatoid arthritis
Systemic lupus erythematosus
Asthma
Alzheimer's disease
Hereditary angioedema

including eosinophils, neutrophils, monocytes, mast cells, and astrocytes (in the CNS), as well as γ-IFN-activated T cells. In eosinophils, C3a elicits responses similar to C5a, including intracellular calcium elevation, increases endothelial cell adhesion, and the generation of reactive oxygen intermediates.

CLINICAL CORRELATES

The normal function of complement is to mediate a localized inflammatory response to a foreign material. The complement system can become clinically relevant in situations where it fails to function or where it is activated inappropriately; some of these settings are shown in Table 7 (Lambris and Holers, 2000). In the first instance, a lack of activity due to a genetic deficiency in one or more complement proteins has been associated with increased incidence of recurrent infections (MBL deficiency in children), autoimmune disease (over 90% of C1-deficient patients develop SLE), and other pathologies (for example, a deficiency of C1 inhibitor is known to result in hereditary angioedema, where various soft tissues become extremely swollen because of overproduction of various vasoactive mediators). The second instance, inappropriate activation, also occurs in a variety of circumstances. It is now recognized that endothelial cells exposed to hypoxic conditions (ischemia due to angioplasty or a blocked artery due to atherosclerosis) activate complement following reperfusion of the blocked vessel. This results in further damage to the vessel wall and eventually to the surrounding tissue. Activation of the classical pathway by immune complexes occurs in various autoimmune diseases such as systemic lupus erythematosus and rheumatoid arthritis. Glomerular deposition of immune complexes results in local inflammation that can contribute to a type of kidney damage called glomerulonephritis (GN). Quite a number of experimental and clinical data have been accumulated demonstrating that complement directly contributes to the initiation and/or progression of GN (Table 8), resulting in the development of end–stage renal disease and the necessity of hemodialysis therapy.

TABLE 8 Types of Studies Demonstrating a Role for Complement in Kidney Disease

Deficiency or loss of complement regulatory activity results in tissue damage
Mechanistic and knockout studies implicate complement and C5 in particular
Ongoing glomerular disease is associated with various indices of complement activation
Inhibition of complement activation attenuates tissue damage in model systems

TABLE 9 Clinical Symptoms Associated with Cuprophan-Induced Biocompatibility Reactions

Cardiopulmonary:	Pulmonary hypertension
	Hypoxemia
	Respiratory distress (dyspnea)
	Neutropenia (pulmonary leukosequestration)
	Tachycardia
	Angina pectoris
	Cardiac arrest
Other:	Nausea, vomiting, diarrhea
	Fever, chills, malaise
	Urticaria, pruritus
	Headache

One of the major settings where complement has been implicated in adverse clinical reactions is during extracorporeal therapies such as hemodialysis, cardiopulmonary bypass, and apheresis therapy. The same nonspecific mechanism that permits the alternative pathway to recognize microbes results in complement activation by the various biomaterials found in different medical devices. The following discussion summarizes the clinical experience with hemodialysis and cardiopulmonary bypass, but many of the observations concerning complement activation and WBC activation are relevant to other medical biomaterial applications.

One of the most investigated materials (from the perspective of complement activation) is the cellulosic Cuprophan membrane used extensively for hemodialysis. Some of the adverse reactions that occur during clinical use of a Cuprophan dialyzer are listed in Table 9. In 1977, Craddock *et al.* showed that some of these same manifestations (neutropenia, leukosequestration, and pulmonary hypertension) could be reproduced in rabbits and sheep when the animals were infused with autologous plasma that had been incubated *in vitro* with either Cuprophan or zymosan. This effect could be abrogated by treatment of the plasma to inhibit complement activation (heating to 56°C or addition of EDTA), thus linking these effects with complement. The development and use of specific radioimmunoassays (RIAs) to measure C3a and C5a by Dennis Chenoweth (1984) led to the identification of these complement components in the plasma of patients during dialysis therapy. A typical patient response to a Cuprophan

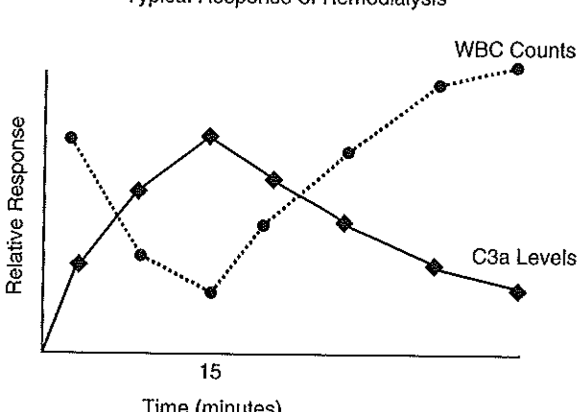

FIG. 8. A typical response pattern to dialysis with a complement-activating hemodialysis membrane. Many investigators have noted that the extent of C3a production is directly proportional to the degree of neutropenia at the same time points.

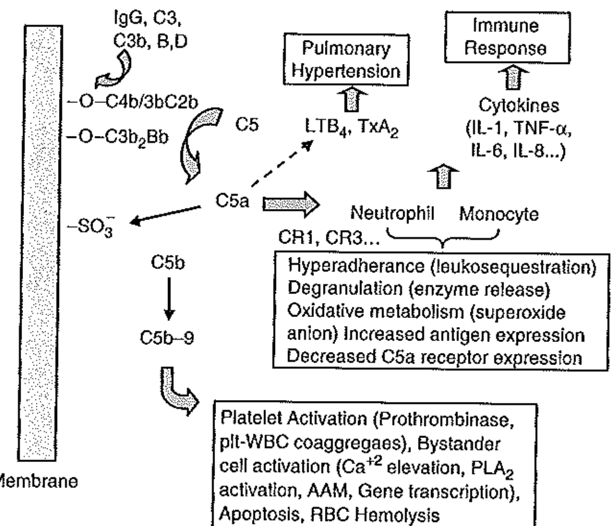

FIG. 9. The biochemical basis for complement-mediated adverse reactions during extracorporeal therapy. Production of C5a leads to receptor-dependent white blood cell activation. This results in profound neutropenia, increased concentrations of degradative enzymes, and reactive oxygen species that ultimately may lead to tissue damage and dysfunction of these important immune cells. Generation of secondary mediators, such as arachidonic acid metabolites (TxA2, LTB4) and cytokines, can have profound consequences on whole organ systems. Finally, formation of the MAC (C5b-9) has been linked with increased hemolysis during cardiopulmonary bypass and formation of microparticles and shown to increase platelet prothrombinase activity *in vitro*. This last observation suggests that surfaces that activate complement aggressively may be more thrombogenic.

membrane is shown schematically in Fig. 8. The C3a (and C5a) levels rise during the first 5–15 min, peaking between 10 and 20 min. For a Cuprophan membrane, typical peak C3a levels range from 4000 to 6000 ng/ml. During this period the white blood cells become hyperadherent and are trapped in the lung, resulting in a peripheral loss of these cells (neutropenia). As complement activation is controlled (e.g., by factor H), the C3a and C5a levels decrease to baseline levels and the WBCs return to the peripheral circulation, now in a more activated (primed) state. This is a very consistent response and many authors have noted a direct correlation between the extent of complement activation and the degree of neutropenia (as well as other responses such as CR3 expression) seen with various dialysis membranes.

Based on our understanding of the biochemistry of complement and its biological actions, the following scenario can be drawn (Fig. 9). Blood contact with the membrane results in initial protein deposition, including IgG, C3, and especially C3b, eventually leading to the formation of C3 and C5 convertase enzymes. Conversion of C5 results in C5a production, which leads to receptor-mediated neutrophil and monocyte activation. Production of C5b leads to MAC formation, which binds to bystander cells and results in subsequent activation of these cells through calcium-dependent mechanisms. Recognition of biomaterial-bound C3 and C4 fragments by WBC results in cell adherence and further activation of these cells. These various responses accounts for much of the pathophysiology seen clinically. The critical role of C5a in mediating many of these adverse reactions has been confirmed in experiments employing purified sheep C5a. Infusion of this isolated peptide into sheep, in a manner that would simulate exposure to this molecule during hemodialysis, produced dose-dependent responses identical to that seen when the sheep are subjected to dialysis (Johnson *et al.*, 1996). In addition, numerous *in vivo* and *in vitro* studies have documented the relationship between complement activation by biomaterials, the extent of WBC activation and the resulting inflammatory response illustrated in Fig. 9 (Tang *et al.*, 1998; Gemmell *et al.*, 1996; Larsson *et al.*, 1997; Lewis and Van Epps, 1987).

In the same time frame in which clinicians were linking complement with leukopenia in the hemodialysis setting, a number of cardiovascular scientists were demonstrating complement activation by the materials used to make cardiopulmonary bypass circuits. Typical levels of C3a produced in these procedures ranged from 300 to 2400 ng/ml. These investigations soon associated C3a and C5a production with a group of symptoms known as "postperfusion" or "postpump" syndrome (Table 10). Further analysis showed that complement was activated by the materials in the circuit (such as the polypropylene membranes and the nylon filters) but was also activated by during neutralization of the heparin anticoagulant with the protamine sulfate that was given to each patient at the end of the operation. This was further exacerbated by complement activation that occurred in the ischemic vascular bed upon reperfusion of the tissue that also occurred at the end of the procedure. The importance of complement activation, and C5 conversion in particular, to the clinical outcome of CPB patients was clearly demonstrated in a study by Fitch *et al.* (1999). Using a single chain anti-C5 antibody fragment that inhibited C5a and MAC generation during the procedure, these investigators showed that this antibody fragment lowered WBC activation, blood loss, cognitive deficits and myocardial injury. These results are consistent with other studies (Velthuis *et al.*, 1996; Hsu, 2001) using heparin-coated CPB circuits

TABLE 10 Postperfusion or Postpump Syndrome

Increased capillary permeability with accumulation of interstitial fluid
Blood loss requiring transfusions
Fever
Leukocytosis (increased WBC counts)
Organ dysfunction: heart, liver, kidney, brain and GI tract

that demonstrate lower inflammatory indices (complement, cytokine, and elastase levels) that are associated with improved clinical outcomes (decreased blood loss, length of ICU stays, and morbidity).

The CPB experience with heparin-coated devices demonstrates that modification of device materials (or the blood-contacting surfaces of those materials) can dramatically limit complement activation and the subsequent inflammatory response. Based in part on this and similar observations, hemodialyzer/membrane manufacturers began developing new membranes to produce more biocompatible (i.e., less complement-activating) devices. These new membranes tend to fall into two groups: moderately activating modified cellulosics [such as cellulose acetate (CA), hemophane, and cellulose triacetate (CT)] and low activating synthetics [such as polyacrylonitrile (AN69), poly(methyl methacrylate) (PMMA), and polysulfone (PS)]. Moderately activating modified cellulosics produce C3a levels and neutropenic responses that are about 50% of Cuprophan levels, while the synthetic materials display 0–20% activation compared to Cuprophan. Based on the known properties of complement and the structures of these membranes, the reasons for the improved biocompatibility can be rationalized as follows. Most of these materials have a diminished level of surface nucleophiles. In theory, this should result in lower deposition of C3b, and in fact this has been verified experimentally. The diminished capacity to bind C3b results in lower levels of C3 and C5 convertase activity and consequently an abated production of C3a and C5a. Patient exposure to C5a is reduced even further by materials that allow for transport through the membrane to the dialysate (for example, high-flux membranes such as polysulfone will do this) or by absorbing the peptide back onto the surface (the negatively charged AN69 has been shown to have a high capacity for binding cationic C5a). Thus, limiting C3b deposition and C5a exposure are two proven mechanisms of avoiding the clinical consequences of complement activation.

The same result can be also accomplished by facilitating the normal control of C3 convertase by factor H. Kazatchkine *et al.* (1979) have shown that heparin coupled to either zymosan or Sepharose limits the normal complement activation that occurs on these surfaces by augmenting C3b inactivation through factors H and I. Presumably, this accounts for the improved biocompatibility of heparin-coated circuits used in CPB described above. Mauzac *et al.* (1985) have prepared heparin-like dextran derivatives that are extensively modified with carboxymethyl and benzylamine sulfonate groups. These researchers have shown that these modifications diminish complement activation by the dextran substrate. A simple modification of cellulose membranes (Cuprophan) with maleic anhydride has been shown to limit the complement-activating potential of these materials by over 90% (Johnson *et al.*, 1990). Again, increased binding of factor H to surface-bound C3b appears to account for the improved biocompatibility of maleated cellulose. Thus materials that limit complement activation through normal regulatory mechanisms are on hand and may prove to be the next generation of complement-compatible materials. In addition, as the studies of Fitch *et al.* have shown, pharmaceutical control of complement is possible with agents that are now in clinical development.

SUMMARY AND FUTURE DIRECTIONS

The immune response to a biomaterial involves both humoral and cellular components. Activation of the complement cascade by classical, lectin, or alternative pathways leads to the deposition of C4b and C3b proteins. Recognition of these molecules by receptors on granulocytes can cause activation of these cells, leading to the production of degradative enzymes and destructive oxygen metabolites. Recognition of C4b or C3b by other proteins in the cascade leads to enzyme formation (C3 and C5 convertases), which amplifies the response and can lead to the production of a potent inflammatory mediator, C5a. C5a binds to specific receptors found on PMNs and monocytes. The interaction of C5a with these cells elicits a variety of responses including hyperadherence, degranulation, superoxide production, chemotaxis, and cytokine production. Systemic exposure to C5a during extracorporeal therapies has been associated with neutropenia and cardiopulmonary manifestations (Tables 9 and 10) that can have pathologic consequences. The other portion of the C5 protein, C5b, leads to formation of a membrane attack complex that activates cells at sublytic levels and has cytotoxic potential if produced in large amounts. The control of these processes is understood well enough to begin designing materials that are more biocompatible. Limiting C3b deposition (nucleophilicity), adsorbing C5a to negatively charged surface groups, and facilitating the role of factors H and I are three approaches that have been shown to be effective. Translating the last mechanism into commercial materials is one of the major challenges facing the development of truly complement-compatible membranes.

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4.5 Systemic Toxicity and Hypersensitivity

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Artificial implant devices comprise a variety of metallic alloys, polymers, ceramics, hydrogels, or composites for a large number of purposes and with widely different properties. With the exception of drug delivery systems, sutures, and other degradable biomaterial systems (Chapter 2.7), the implant devices are intended to resist chemical and biochemical degradation and to have minimal leaching of structural components or additives. However, synthetic devices are influenced by chemical and in some cases enzymatic processes resulting in the release of biomaterials-associated components. Since there is no natural repair mechanisms parallel to natural tissues, degradation (biodegradation) is a "one-way" process that brings about microscopic and macroscopic surface and bulk changes of the devices, sometimes enhanced by the biomechanical and bioelectrical conditions that the devices are intended to resist. With the exception of pathologic calcification of certain polymer implants, the surface changes may not be significant for the mechanical strength of the implant, whereas in contrast the released substances very often have biological effects on the surrounding tissues or, possibly, at other remote locations. Inflammatory, foreign body, or other local host reactions and tumorigenesis are discussed in Chapters 4.2, 4.3, and 4.7. The following discussion is concerned with the possibility of systemic toxic reactions and/or hypersensitive reactions caused by biomaterials-derived xenobiotics.

KINETICS AND NATURE OF BIOMATERIALS COMPONENTS

Xenobiotic components derived from *in vivo* medical devices have parenteral contact with connective tissue or other specialized tissues such as bone, dentin, and vascular or ocular tissue, whereas leachables from skin- and mucosa-contacting devices have to pass the epithelial lining of the oral mucosa, the skin, the gastrointestinal tract, or—for volatiles—the lung alveoli to get "inside" the body. In either case, further distribution of foreign substances to other tissues and organs is dependent on membrane diffusion into blood capillaries and lymph vessels. The transport may be facilitated by reversible binding to plasma proteins, globulins (metal, metal compounds), and chylomicrons (lipophilic substances). Storage—and later release—may take place for certain components in tissues such as fat and bone.

In addition to particulate matter the released components consist of chemical substances of different atomic and molecular size, solubility, and other chemical characteristics depending on the mother material. Examples are metal ions such as cobalt, chromium, nickel, molybdenum, and titanium from metallic orthopedic implants or prosthodontic materials, or residual monomers, chemical initiators, inhibitors, plasticizers, antioxidants, etc., from polymer implants and dental materials. Other degradation products from inorganic, organic, and composite devices also "rub off" to the surrounding tissues. The kinetic mechanisms for biomaterials components are in part the same as those of xenobiotics introduced by food or environmental exposure, i.e., the released components are subject to oxidation, reduction, and hydrolysis followed by conjugation mechanisms. All metabolic changes are in their nature intended to eliminate them by way of the urine, bile, lungs, and to a certain degree in salivary, sweat, and mammary glands and hair (deBruin, 1981).

A key question is, do the released components or their metabolites have any systemic toxic effect on the host and/or could they induce unwanted immunological reactions?

TOXICODYNAMIC CONSIDERATIONS

Systemic toxicity depends on toxic substances hitting a target organ with high sensitivity to a specific toxicant. Target organs are the central nervous system, the hematopoietic system, the circulatory system, and visceral organs such as liver, kidney, and lungs, in that order. The toxicity is based on interference with key cell functions and depends on the dose and the duration of the exposure. Serious effects may be incompatible with continued life, but most effects are local and reversible cell damage. However, some sublethal effects may include somatic cell mutation expressed as carcinogenesis, or germinal cell mutation, resulting in reproductive toxicity.

The key word in the evaluation of general toxicity is the dose, defined as the amount of a substance an organism is exposed to, usually expressed as mg per kg body weight. Adverse effects of foreign substances are often the result of repeated, chronic exposure to small doses that over a prolonged period of time may have deleterious effects similar to one large, short time exposure, provided that the repeated doses exceed a certain threshold level. This level is determined by the capacity of metabolism and elimination. Another important factor is the possibility of synergistic potentiating effects when several toxicants are present simultaneously. Whatever mechanism is involved, the principle of systemic toxicity presupposes a dose-dependent reaction that may be measured and described, and that may be explained by specific reactions at distinct molecular sites (Eaton and Klaassen, 1996).

The components derived from biomaterials represent a large series of widely different foreign substances with few characteristics in common and with a largely unknown concentration. Most of them have to be characterized as toxic per se, with large variations regarding their place on a ranking list of potential toxicity. Metal ions and salts derived from biomaterials devices, such as mercury, nickel, and chromium, are classified as toxicants. A similar statement could be made for components associated with polymeric materials. However, clinically relevant data on the concentration of degradation products are scarce, e.g., phthalate additives and degradation products from chemical additives derived from poly(methyl methacrylate) dental prostheses have been quantified in saliva (Lygre *et al.*, 1993). *In vitro* experiments have shown that chromium and nickel are released from base metal orthodontic appliances, although the amounts are not comparable with the amounts calculated in food intake (Park and Shearer, 1983). In addition, the proportion of uptake by mucosa is unknown. The presence of leachable substances has also been demonstrated in the surrounding tissues of implants, but quantification is difficult. Information is available on the release and uptake of mercury derived from dental amalgam. For example a series of studies has shown the presence mercury in plasma and urine after inhalation of metallic mercury released from dental amalgam (Mackert and Berglund, 1997). Accumulation of mercury in tissues belonging to the central nervous system has been shown after occupational exposure (Nylander *et al.*, 1989). Reproductive toxicity has been of specific concern. However, similar to other metals such as chromium and nickel, mercury exposure also takes place through food and through respiratory air. Careful scrutiny of the large number of partly controversial data by national and international scientific committees has not resulted in a consensus conclusion that the application of mercury amalgam should be discontinued as a dental biomaterial, although mercury is a significant environmental concern (The European Commission, 1998).

When occupational exposure is disregarded, the possibility of systemic toxicity or reproductive toxicity has not been seriously considered for other biomaterial components or metabolites, because of their low concentration as compared with their toxic potential. A fair conclusion at this point would be that there are no data indicating any systemic toxicity caused by biomaterials-derived xenobiotics. However, this field of interest is characterized by the increasing number of synthetic biomaterials on the market. Despite the premarketing testing programs it is difficult to predict single or synergistic toxic effects of leachable components and degradation products in the future.

ADVERSE EFFECTS OF DEFENSE MECHANISMS

The low probability of direct systemic adverse effects on target organs caused by biomaterial products does not rule out deleterious effects by other, dose-independent mechanisms. All substances not recognized as natural components of the tissues are subject to possible clearance by several mechanisms, e.g., phagocytic cells such as polymorphonuclear leukocytes, macrophages, and monocytes attempt to degrade and export the components. Larger foreign components are subject to more aggressive reactions by giant cells causing an inflammatory foreign-body reaction. Enzymes and other bioactive molecules associated with the phagocytosis and foreign-body reaction may cause severe local tissue damage. In addition, phagocytic cell contact and the contact with the circulatory

system of lymph and blood opens up another way of neutralizing foreign substances by way of the immune system, introducing a biologic memory of previously encountered foreign substances and an enhancement system for their neutralization.

HYPERSENSITIVITY AND IMMUNOTOXICITY

The immune system is an indispensable biologic mechanism to fight potentially adverse invaders, most commonly of microbial origin. However, the immune system occasionally strikes invading molecules—adverse or not—with an intensity that stands in contrast to the sometimes minute amounts of foreign substances, and with the ability to cause host tissue damage. This phenomenon is called hypersensitivity. The resulting injury is part of a group of adverse reactions classified as immunotoxic.

In principle, immunologic hypersensitivity comprises two different mechanisms: allergy and intolerance. Allergy is a acquired condition resulting in an overreaction upon contact with a foreign substance, given a genetic disposition and previous exposure to the substance. Allergic reactions may include asthma, rhinitis, urticaria, intraoral and systemic symptoms, and eczema. Intolerance is an inherited reaction that resembles allergy and has common mediators and potentiating factors, such as complement activation, and histamine release, but is not dependent on a previous sensitization process. The intolerance reactions have been associated with drugs such as acetylsalicylic acid, whereas intolerance to leachable biomaterial components such as benzoic acid is conceivable but not known.

ALLERGY AND BIOMATERIALS

A foreign substance able to induce an allergic reaction is called an allergen. There is no acceptable way of predicting whether a substance or a compound is potentially allergenic only on the basis of its chemical composition and/or structure. However, experimental evidence and years of empirical results after testing substances causing allergic reactions have given some leads, e.g., large foreign molecules such as proteins and nucleoproteins are strong allergens, whereas lipids are not. However, the strongest chemical allergens associated with biomaterials are often chemically active substances of low molecular weight, often less than 500 Da, such as lipid-soluble organic substances derived from polymer materials or metal ions and metal salts. These are called haptens, i.e., they become full allergens only after reaction or combination with proteins that may be present in macrophages and Langerhans cells of the host.

TYPES OF ALLERGIES

The allergies are most often categorized into four main groups (type I–IV) according to the reaction mechanisms. The types I to III are associated with humoral antibodies initiated by B-lymphocytes that develop to immunoglobulin-producing plasma cells. The immunoglobulins are classified into five different classes, Ig E, A, D, G, and M, according to their basic structure and size. A variable portion of the immmunoglobulin is specific for the antigen that induced its production (Roitt *et al.*, 1997). The type IV reaction is a cell-mediated reaction caused by T-lymphocytes. These interactions are also discussed in Chapter 4.3.

The types II and III allergies comprise antigen/antibody encounters including complement activation, cell lysis, release of vasoactive substances, inflammatory reaction, and tissue damage. Necrosis of periimplant tissue with histologic appearance and serum complement analyses consistent with Type III hypersensitivity has been observed in cases of atypical loosening of total hip prostheses (Hensten-Pettersen, 1993). However, an FDA document (Immunotoxicity Testing Guidance, 1999) omits the type II and III reactions for reasons of being "relatively rare and less likely to occur with medical devices/materials" leaving the types I and IV as relevant in the present context.

Type I Hypersensitivity

The type I reaction is based on an interaction between an intruding allergen and IgE immunoglobulins located in mast cells, basophils, eosinophils, and platelets, resulting in release of active mediators such as histamine and other vasoactive substances. The results are local or systemic reactions seen within a short time (minutes). The symptoms depend on the tissue or organ subject to sensitization, e.g., (1) inhaled allergens such as pollen or residual proteins associated with surgical latex gloves or other natural latex products that may result in asthmatic seizures, swelling of the mucosa of the throat, or worse; or (2) decreased blood pressure and anaphylactic shock. Food allergies may also give systemic symptoms. This type of host reaction is usually associated with full antigens. Since the potential allergens associated with biomaterials are small molecular haptens, the probablility of IgE-based allergic reactions is low, although IgE antibodies to chromium and nickel have been reported (Hensten-Pettersen, 1993). Reports on adverse reactions to orthopedic devices describe patients with urticarial reactions. Contact urticaria is a wheal and flare response to compounds applied on intact skin. The role of immunological contact urticaria in relation to medical devices is not clear.

Type IV Hypersensitivity

The cell-mediated hypersensitivity is referred to as "delayed" because it takes more than 12 hours to develop, often 24–72 hours. Prolonged challenges of macrophage-resistant allergens, usually of microbial origin, may result in persistant immunological granuloma formation. The T-lymphocytes producing the response have been sensitized by a previous encounter with an allergen and act in concert with other lymphocytes and mononuclear phagocytes to create four histologically different types characterized by skin-related tissue reactions. The reactions are elicited by interaction of cells and mediators that comprise (1) swelling (the

Jones–Mote type); (2) induration (the granulomatous type); (3) swelling and induration and possibly fever (the tuberculin type); and (4) eczema (the contact type) (Roitt *et al.*, 1997). The latter form of delayed hypersensitivity has been of specific importance in relation to biomaterials. Most information on this reaction has been obtained by studying the reaction patterns following exposure to external environmental and occupation-related chemicals.

Allergic contact dermatitis is acquired through previous sensitization with a foreign substance. The hapten is absorbed by the skin or mucosa and binds to certain proteins associated with the Langerhans cells, forming a complete antigen. The antigen is brought in contact with the regional lymph nodes, resulting in the formation of activated, specialized T cells that are brought into circulation. Upon new exposure, the allergen may again be transported from the site of entrance. The new contact between the allergen and the activated, specialized T cells releases inflammatory mediators, resulting in further production and attraction of T cells causing tissue damage. The reactions are not necessarily limited to the exposure site.

The presence of allergic contact dermatitis is evaluated by allergologists or dermatologists by applying the suspected haptens using epidermal or intradermal skin tests and reading the dermal or epidermal reaction after specified amounts of time. Commecial test kits for epidermal testing are available for a series of chemical substances related to different occupations. A vast amount of information on the allergenic characteristics of biomaterials-related substances has been obtained in this way, especially as regards dental materials (Kanerva *et al.*, 1995).

Many biomaterials employed in dentistry such as metal alloys and resin-based materials have medical counterparts, and both categories of biomaterials have materials counterparts met with in everyday life. The sensitization process therefore often has taken place before the biomaterialsc contact.

ATOPY

Atopic individuals have a constitutional predisposition for IgE-based hypersensitive reactions caused by environmental and food allergens. The reactions include histamine-mediated hay fever, asthma, gastrointestinal symptoms, or skin rashes and are more pronounced at an early age. Atopics have an increased risk of acquiring irritant contact dermatitis to external biomaterial devices such as orthodontic appliances. The relation to allergic contact dermatitis is unclear (Lindsten and Kurol, 1997); so also is the relationship between atopy and allergens or haptens from biomaterials exposed parenterally.

IMMUNOLOGIC TOXICITY OF MEDICAL DEVICES

Immunologic toxicity to surface medical devices and external communicating devices (dialyzers, laparoscopes, etc.) may represent mechanisms of sensitization and hypersensitive reactions similar to those of orally exposed biomaterials. Hypersensitivity reactions to implants in clinically inobservable locations are difficult to recognize unless they have dermal or systemic expressions. In addition, such reactions may be part of local toxic and/or mechanically induced inflammatory reactions using similar mediators for tissue response. For lack of more distinct discriptions, such reactions have been referred to as "deep tissue" reactions of type IV hypersensitivity.

A vast battery of *in vitro* and *in vivo* experimental studies have been performed to study potential adverse effects of biomaterial devices such as artificial joints, heart valves, and breast prostheses (Rodgers *et al.*, 1997). Aseptic loosening of metallic hip prostheses have been associated with "biologic" causes in addition to biomechanical factors and wear debris. However, it is currently unclear whether metal sensitivity is a contributing factor to implant failure (Hallab *et al.*, 2001). In fact, it is argued that the loosening process enhances the immunological sensitization, indicating that the cause/effect relation may be reversed (Milavec-Puretic *et al.*, 1998). What is clear is that local and general eczematous reactions have been observed following the insertion of metallic implants in patients subsequently shown to be allergic to cobalt, chromium, and nickel. Many case reports also describe the immediate healing of dermal reactions associated with metal implants (Al-Saffar and Revell, 1999). Metal allergy has also been discussed as a possible contributing factor in the development of in-stent coronary restenosis, although there is little evidence for this effect (Hillen *et al.*, 2002). However, established metal allergy in a patient does not as a rule seem be accompanied by clinical reactions to implant alloys containing the metal. If this statement is true, it is in line with clinical observations made in surveys on the use of metallic alloys in prosthodontics and orthodontics. Inhomogeneiety or mixture of alloys appear to determine the efflux of potentially hypersensitive metal ions, and hence increase the possibility of eliciting hypersensitive reactions (Grimsdottir *et al.*, 1992).

Methyl methacrylate bone cement is another potential allergenic factor in orthopedic surgery parallell with reactions in dentistry and cosmetics (Kaplan *et al.*, 2002), and immune-mediated disease and silicone based implants has been a matter of discussion for some time. However, a scientifically valid cause and effect relationship between immune based disease and silicone based implants has not been established (Rodgers *et al.*, 1997).

An extensive literature reflects clinical surveys and research activities related to natural latex used as barrier material by the health professions. It is accepted that residual latex proteins and chemicals associated with the production process may cause immediate and delayed reactions in patients and health personnel (Turjanmaa *et al.*, 1996).

OTHER INTERACTIONS

The FDA testing guidance referred to above also lists other interactions of medical devices, extracts of medical devices, or adjuvants with the immune system such as impairment of the normal immunologic protective mechanisms (immunosuppression), and long-term immmunological activity (immunostimulation) that may lead to harmful autoimmune responses. The autoimmune reaction is explained by

the biomaterials-associated agent acting as an adjuvant that is stimulating to antibody/complement-based tissue damage by cross reactions with human protein. Chronic inflammatory, immune-related granuloma may take part in the development of autoimmune reactions.

CONCLUDING REMARKS

Biocompatibility issues related to medical devices form a multidimensional crossroad of technology and biology. One dimension is the various classes of biomaterials, such as plastics and other polymers, metals, ceramics, and glasses, depending on expert design to obtain maximal mechanical properties and minimal chemical dissolution. Another is the mode of application, ranging from skin and mucosal contact to totally submerged implants, with external communicating devices in between. A third dimension is the duration of contact, ranging from minutes to the expected lifetime, and the fourth, and decisive, is the biological reactions that can be expected. These circumstances prevent general statements on biomaterials. The present overview is aimed at students and limited to focus on collective mechanisms determining systemic toxicity and discuss hypersensitivity reactions documented by clinical reports.

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4.6 Blood Coagulation and Blood–Materials Interactions

Stephen R. Hanson

The hemostatic mechanism is designed to arrest bleeding from injured blood vessels. The same process may produce adverse consequences when artificial surfaces are placed in contact with blood. These events involve a complex set of interdependent reactions between (1) the surface, (2) platelets, and (3) coagulation proteins, resulting in the formation of a clot or thrombus that may subsequently undergo removal by (4) fibrinolysis. The process is localized at the surface by opposing activation and inhibition systems, which ensure that the fluidity of blood in the circulation is maintained. In this chapter, a brief overview of the hemostatic mechanism is presented. Although a great deal is known about blood responses to injured arteries and blood-contacting devices, important relationships remain to be defined in many instances. More detailed discussions of hemostasis and thrombosis have been provided elsewhere (Colman *et al.*, 2001; Esmon, 2003; Forbes and Courtney, 1987; Gresle *et al.*, 2002; Stamatoyannopoulos *et al.*, 1994).

PLATELETS

Platelets ("little plates") are nonnucleated, disk-shaped cells having a diameter of 3–4 μm and an average volume of 10×10^{-9} mm^3. Platelets are produced in the bone marrow, circulate at an average concentration of about 250,000 cells per microliter of whole blood, and occupy approximately 0.3% of

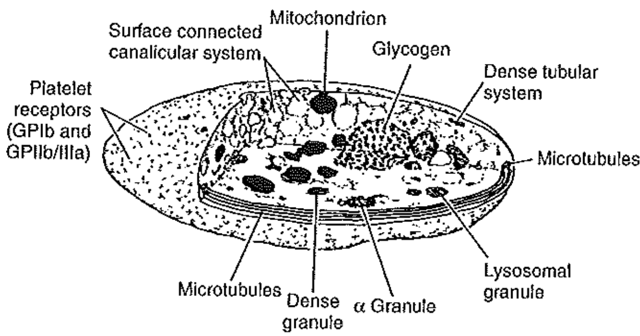

FIG. 1. Platelet structure.

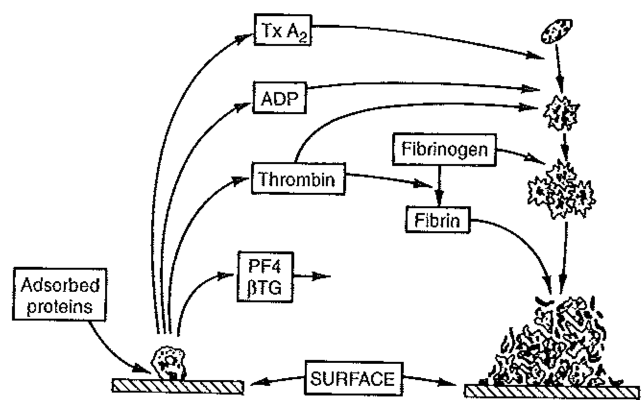

FIG. 2. Platelet reactions to artificial surfaces. Following protein adsorption to surfaces, platelets adhere and release α-granule contents, including platelet factor 4 (PF4) and β-thromboglobulin (β-TG), and dense granule contents, including ADP. Thrombin is generated locally through coagulation reactions catalyzed by procoagulant platelet surface phospholipids. Thromboxane A_2 (TxA_2) is synthesized. ADP, TxA_2, and thrombin recruit additional circulating platelets into an enlarging platelet aggregate. Thrombin-generated fibrin stabilizes the platelet mass.

the total blood volume. In contrast, red cells typically circulate at 5×10^6 cells per microliter and may make up 40–50% of the total blood volume. As discussed later, platelet functions are designed to (1) initially arrest bleeding through formation of platelet plugs, and (2) stabilize the initial platelet plugs by catalyzing coagulation reactions leading to the formation of fibrin.

Platelet structure provides a basis for understanding platelet function. In the normal (nonstimulated) state, the platelet discoid shape is maintained by a circumferential bundle (cytoskeleton) of microtubules (Fig. 1). The external surface coat of the platelet contains membrane-bound receptors (e.g., glycoproteins Ib and IIb/IIIa) that mediate the contact reactions of adhesion (platelet–surface interactions) and aggregation (platelet–platelet interactions). The membrane also provides a phospholipid surface that accelerates important coagulation reactions (see below), and forms a spongy, canal-like (canalicular) open network that represents an expanded reactive surface to which plasma factors are selectively adsorbed. Platelets contain substantial quantities of muscle protein (e.g., actin, myosin) that allow for internal contraction when platelets are activated. Platelets also contain three types of cytoplasmic storage granules: (1) α-granules, which are numerous and contain the platelet-specific proteins platelet factor 4 (PF-4) and β-thromboglobulin (β-TG), and proteins found in plasma (including fibrinogen, albumin, fibronectin, and coagulation factors V and VIII); (2) dense granules that contain adenosine diphosphate (ADP), calcium ions (Ca^{2+}), and serotonin; and (3) lysosomal granules containing enzymes (acid hydrolases).

Platelets are extremely sensitive cells that may respond to minimal stimulation. Activation causes platelets to become sticky and change in shape to irregular spheres with spiny pseudopods. Activation is accompanied by internal contraction and extrusion of the storage granule contents into the extracellular environment. Secreted platelet products such as ADP stimulate other platelets, leading to irreversible platelet aggregation and the formation of a fused platelet thrombus (Fig. 2).

Platelet Adhesion

Platelets adhere to artificial surfaces and injured blood vessels. At sites of vessel injury, the adhesion process involves the interaction of platelet glycoprotein Ib (GP Ib) and connective tissue elements that become exposed (e.g., collagen) and requires plasma von Willebrand factor (vWF) as an essential cofactor. GP Ib (about 25,000 molecules per platelet) acts as the surface receptor for vWF (Colman *et al.,* 2001). The hereditary absence of GP Ib or vWF results in defective platelet adhesion and serious abnormal bleeding.

Platelet adhesion to artificial surfaces may also be mediated through platelet glycoprotein IIb/IIIa (integrin $\alpha_{IIb}\beta_3$) as well as through the GP Ib-vWF interaction. GP IIb/IIIa (about 80,000 copies per resting platelet) is the platelet receptor for adhesive plasma proteins that support cell attachment, including fibrinogen, vWF, fibronectin, and vitronectin (Gresle *et al.,* 2002). Resting platelets do not bind these adhesive glycoproteins, events which normally occur only after platelet activation causes a conformational change in GP IIb/IIIa. Platelets that have become activated near artificial surfaces (for example, by exposure to factors released from already adherent cells) could adhere directly to surfaces through this mechanism (e.g., via GP IIb/IIIa binding to surface-adsorbed fibrinogen). Also, normally unactivated GP IIb/IIIa receptors could react with surface proteins that have undergone conformational changes as a result of the adsorption process (Chapter 3.2). The enhanced adhesiveness of platelets toward surfaces preadsorbed with fibrinogen supports this view. Following adhesion, activation, and release reactions, the expression of functionally competent GP IIb/IIIa receptors may also support tight binding and platelet spreading through multiple focal contacts with fibrinogen and other surface-adsorbed adhesive proteins.

Platelet Aggregation

Following platelet adhesion, a complex series of reactions is initiated involving (1) the release of dense granule ADP, (2) the formation of small amounts of thrombin (see later discussion), and (3) the activation of platelet biochemical processes leading to the generation of thromboxane A_2. The release of ADP, thrombin formation, and generation of thromboxanes all act in

concert to recruit platelets into the growing platelet aggregate (Fig. 2). Platelet stimulation by these agonists causes the expression on the platelet surface of activated GP IIb/IIIa, which then binds plasma proteins that support platelet aggregation. In normal blood, fibrinogen, owing to its relatively high concentration (Table 1), is the most important protein supporting platelet aggregation. The platelet–platelet interaction involves Ca^{2+}-dependent bridging of adjacent platelets by fibrinogen molecules (platelets will not aggregate in the absence of fibrinogen, GP IIb/IIIa, or Ca^{2+}). Thrombin binds directly to platelet thrombin receptors and plays a key role in platelet aggregate formation by (1) activating platelets, which then catalyze the production of more thrombin; (2) stimulating ADP release and thromboxane A_2 formation; and (3) stimulating the formation of fibrin, which stabilizes the platelet thrombus.

Platelet Release Reaction

The release reaction is the secretory process by which substances stored in platelet granules are extruded from the platelet. ADP, collagen, epinephrine, and thrombin are physiologically important release-inducing agents and interact with the platelet through specific receptors on the platelet surface. Alpha-granule contents (PF-4, β-TG, and other proteins) are readily released by relatively weak agonists such as ADP. Release of the dense granule contents (ADP, Ca^{2+}, and serotonin) requires platelet stimulation by a stronger agonist such as thrombin. Agonist binding to platelets also initiates the formation of intermediates that cause activation of the contractile–secretory apparatus, production of thromboxane A_2, and mobilization of calcium from intracellular storage sites. Elevated cytoplasmic calcium is probably the final mediator of platelet aggregation and release. As noted, substances that are released (ADP), synthesized (TxA_2), and generated (thrombin) as a result of platelet stimulation and release affect other platelets and actively promote their incorporation into growing platelet aggregates. *In vivo*, measurements of plasma levels of platelet-specific proteins (PF-4, β-TG) have been widely used as indirect measures of platelet activation and release.

Platelet Coagulant Activity

When platelets aggregate, platelet coagulant activity is produced, including expression of negatively charged membrane phospholipids (phosphatidylserine) that accelerate two critical steps of the blood coagulation sequence: factor X activation and the conversion of prothrombin to thrombin (see below). Platelets may also promote the proteolytic activation of factors XII and XI. The surface of the aggregated platelet mass thus serves as a site where thrombin can form rapidly in excess of the neutralizing capacity of blood anticoagulant mechanisms. Thrombin also activates platelets directly and generates polymerizing fibrin, which adheres to the surface of the platelet thrombus.

Platelet Consumption

In man, platelets labeled with radioisotopes are cleared from circulating blood in an approximately linear fashion over time with an apparent life span of approximately 10 days. Platelet life span in experimental animals may be somewhat shorter. With ongoing or chronic thrombosis that may be produced by cardiovascular devices, platelets may be removed from circulating blood at a more rapid rate. Thus steady-state elevations in the rate of platelet destruction, as reflected in a shortening of platelet life span, have been used as a measure of the thrombogenicity of artificial surfaces and prosthetic devices (Hanson *et al.*, 1980, 1990).

COAGULATION

In the test tube, at least 12 plasma proteins interact in a series of reactions leading to blood clotting. Their designation as Roman numerals was made in order of discovery, often before their role in the clotting scheme was fully appreciated. Their biochemical properties are summarized in Table 1. Initiation of clotting occurs either intrinsically by surface-mediated reactions, or extrinsically through factors derived from tissues. The two systems converge upon a final common pathway that leads to the formation of thrombin, and an insoluble fibrin gel when thrombin acts on fibrinogen.

Coagulation proceeds through a "cascade" of reactions by which normally inactive factors (e.g., factor XII) become enzymatically active following surface contact, or after proteolytic cleavage by other enzymes (e.g., surface contact activates factor XII to factor XIIa). The newly activated enzymes in turn activate other normally inactive precursor molecules (e.g., factor XIIa converts factor XI to factor XIa). Because this sequence involves a series of steps, and because one enzyme molecule can activate many substrate molecules, the reactions are quickly amplified so that significant amounts of thrombin are produced, resulting in platelet activation, fibrin formation, and arrest of bleeding. The process is localized (i.e., widespread clotting does not occur) owing to dilution of activated factors by blood flow, the actions of inhibitors that are present or are generated in clotting blood, and because several reaction steps proceed at an effective rate only when catalyzed on the surface of activated platelets or at sites of tissue injury.

Figure 3 presents a scheme of the clotting factor interactions involved in both the intrinsic and extrinsic systems and their common path. Except for the contact phase, calcium is required for most reactions and is the reason why chelators of calcium (e.g., citrate) are effective anticoagulants. It is also clear that the *in vitro* interactions of clotting factors, i.e., clotting, is not identical with coagulation *in vivo*, which may be triggered by artificial surfaces and by exposure of the cell-associated protein, tissue factor. There are also interrelationships between the intrinsic and extrinsic systems, such that under some conditions "crossover" or reciprocal activation reactions may be important (Colman *et al.*, 2001; Bennett *et al.*, 1987).

MECHANISMS OF COAGULATION

In the intrinsic clotting system, contact activation refers to reactions following adsorption of contact factors onto a negatively charged surface. Involved are factors XII, XI,

TABLE 1 Properties of Human Clotting Factors

Clotting factor	Apparent molecular weight (number of chains)	Approximate normal plasma concentration (μg/ml)	Active Form
Intrinsic clotting system			
Prekallikrein	100,000 (1)	50	Serine protease
High molecular weight kininogen	120,000 (1)	80	Cofactor
Factor XII	80,000 (1)	30	Serine protease
Factor XI	143,000 (2)	3–6	Serine protease
Factor IX	57,000 (1)	3–5	Serine protease
Factor VIII[a]	330,000 (1)	0.2	Cofactor
Von Willebrand factor[a]	250,000 (1)	10	Cofactor for platelet adhesion
Extrinsic clotting system			
Tissue factor	44,000 (1)	0[b]	Cofactor
Factor VII	50,000 (1)	1	Serine protease
Common pathway			
Factor X	59,000 (2)	5	Serine protease
Factor V	330,000 (1)	5–12	Cofactor
Prothrombin	72,000 (1)	140	Serine protease
Fibrinogen	340,000 (6)	2500	Fibrin polymer
Factor XIII	320,000 (4)	10	Transglutaminase

[a] In plasma, factor VIII is complexed with von Willebrand factor which circulates as a series of multimers ranging in molecular weight from about 600,000 to 2×10^6.

[b] The tissue factor concentration in cell free plasma is low since tissue factor is an integral cell membrane–associated protein expressed by vascular and inflammatory cells, although a role in coagulation and thrombosis for a circulating form of soluble tissue factor has recently been postulated.

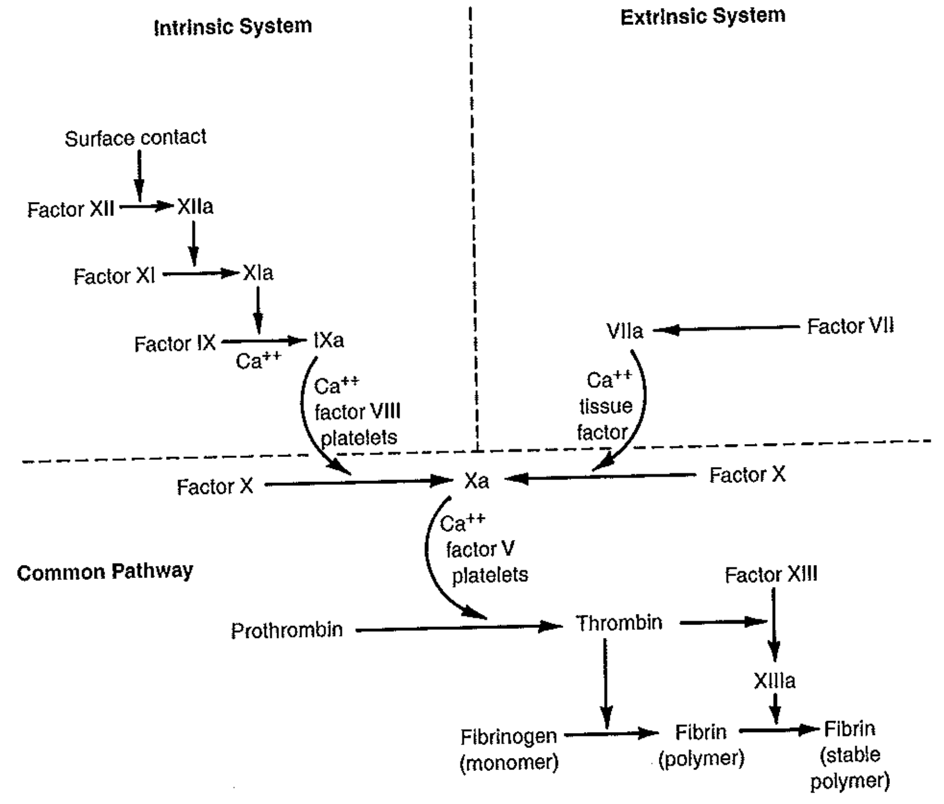

FIG. 3. Mechanisms of clotting factor interactions. Clotting is initiated by either an intrinsic or extrinsic pathway with subsequent factor interactions that converge upon a final, common path.

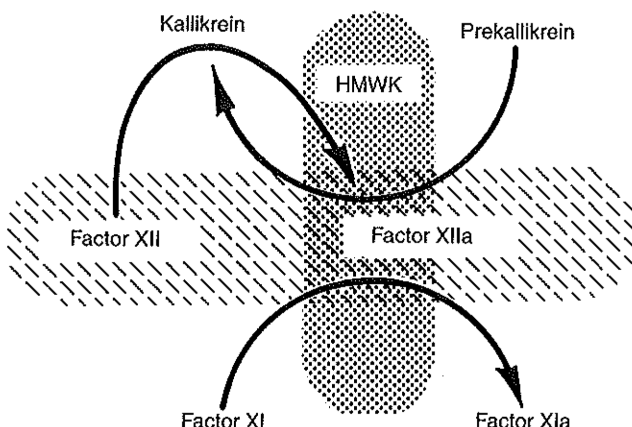

FIG. 4. Contact activation. The initial event *in vitro* is the adsorption of factor XII to a negatively charged surface (hatched, horizontal ovoid) where it is activated to form factor XIIa. Factor XIIa converts prekallikrein to kallikrein. Additional factor XIIa and kallikrein are then generated by reciprocal activation. Factor XIIa also activates factor XIa. Both prekallikrein and factor XI bind to a cofactor, high-molecular-weight kininogen (HMWK; dotted, vertical ovoid), which anchors them to the charged surface.

prekallikrein, and high-molecular-weight kininogen (HMWK) (Fig. 4). All contact reactions take place in the absence of calcium. Kallikrein also participates in fibrinolytic system reactions and inflammation (Bennett *et al.*, 1987). Although these reactions are well understood *in vitro*, their pathologic significance remains uncertain. For expample, in hereditary disorders, factor XII deficiency is not associated with an increased bleeding tendency, and only a marked deficiency of factor XI produces abnormal bleeding.

A middle phase of intrinsic clotting begins with the first calcium-dependent step, the activation of factor IX by factor XIa. Factor IXa subsequently activates factor X. Factor VIII is an essential cofactor in the intrinsic activation of factor X, and factor VIII first requires modification by an enzyme, such as thrombin, to exert its cofactor activity. In the presence of calcium, factors IXa and VIIIa form a complex (the "tenase" complex) on phospholipid surfaces (expressed on the surface of activated platelets) to activate factor X. This reaction proceeds slowly in the absence of an appropriate phospholipid surface and serves to localize the clotting reactions to the surface (versus bulk fluid) phase. The extrinsic system is initiated by the activation of factor VII. When factor VII interacts with tissue factor, a cell membrane protein that may also circulate in a soluble form, factor VIIa becomes an active enzyme which is the extrinsic factor X activator. Tissue factor is present in many body tissues; is expressed by stimulated white cells and endothelial cells; and becomes available when underlying vascular structures are exposed to flowing blood upon vessel injury.

The common path begins when factor X is activated by either factor VIIa–tissue factor or by the factor IXa–VIIIa complex. After formation of factor Xa, the next step involves factor V, a cofactor, which (like factor VIII) has activity after modification by another enzyme such as thrombin. Factor Xa–Va, in the presence of calcium and platelet phospholipids, forms a complex ("prothrombinase" complex) that converts prothrombin (factor II) to thrombin. Like the conversion of factor X, prothrombin activation is effectively surface catalyzed. The higher plasma concentration of prothrombin (Table 1), as well as the biologic amplification of the clotting system, allows a few molecules of activated initiator to generate a large burst of thrombin activity. Thrombin, in addition to its ability to modify factors V and VIII and activate platelets, acts on two substrates: fibrinogen and factor XIII. The action of thrombin on fibrinogen releases small peptides from fibrinogen (e.g., fibrinopeptide A) that can be assayed in plasma as evidence of thrombin activity. The fibrin monomers so formed polymerize to become a gel. Factor XIII is either trapped within the clot or provided by platelets and is activated directly by thrombin. A tough, insoluble fibrin polymer is formed by interaction of the fibrin polymer with factor XIIIa.

CONTROL MECHANISMS

Obviously, the blood and vasculature must have mechanisms for avoiding massive thrombus formation once coagulation is initiated. At least four types of mechanisms may be considered. First, blood flow may reduce the localized concentration of precursors and remove activated materials by dilution into a larger volume, with subsequent removal from the circulation following passage through the liver. Second, the rate of several clotting reactions is fast only when the reaction is catalyzed by a surface. These reactions include the contact reactions, the activation of factor X by factor VII–tissue factor at sites of tissue injury, and reactions that are accelerated by locally deposited platelet masses (activation of factor X and prothrombin). Third, there are naturally occurring inhibitors of coagulation enzymes, such as antithrombin III, which are potent inhibitors of thrombin and other coagulation enzymes (plasma levels of thrombin–antithrombin III complex can also be assayed as a measure of thrombin production *in vivo*). Another example of a naturally occuring inhibitor is tissue factor pathway inhibitor (TFPI), a protein that in association with factor Xa inhibits the tissue factor/factor VII complex. Fourth, during the process of coagulation, enzymes are generated that not only activate coagulation factors, but also degrade cofactors. For example, the fibrinolytic enzyme plasmin (see below) degrades fibrinogen and fibrin monomers and can inactivate cofactors V and VIII. Thrombin is also removed when it binds to thrombomodulin, a protein found on the surface of blood vessel endothelial cells. The thrombin–thrombomodulin complex then converts another plasma protein, protein C, to an active form that can also degrade factors V and VIII. *In vivo*, the protein C pathway is a key physiologic anticoagulant mechanism (Colman *et al.*, 2001; Esmon, 2003).

In summary, the platelet, coagulation, and endothelial systems interact in a number of ways that promote localized hemostasis while preventing generalized thrombosis. Figure 5 depicts some of the relationships and inhibitory pathways that apply to blood reactions following contact with both natural and artificial surfaces.

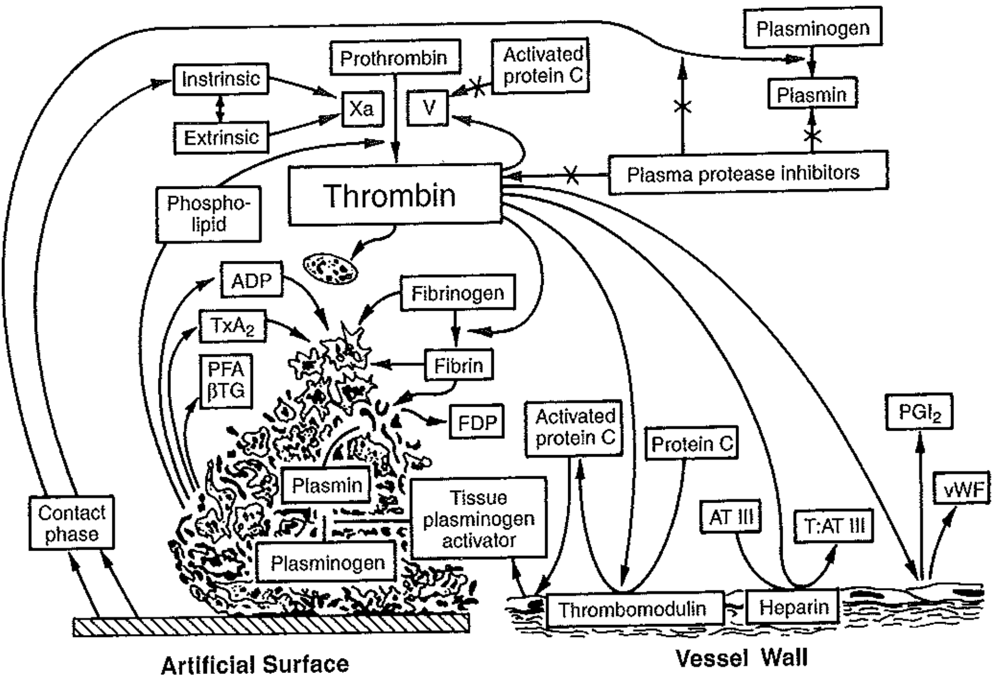

FIG. 5. Integrated hemostatic reactions between a foreign surface and platelets, coagulation factors, the vessel endothelium, and the fibrinolytic system.

Fibrinolysis

The fibrinolytic system removes unwanted fibrin deposits to improve blood flow following thrombus formation, and to facilitate the healing process after injury and inflammation. It is a multicomponent system composed of precursors, activators, cofactors and inhibitors, and has been studied extensively (Colman *et al.*, 2001; Forbes and Courtney, 1987). The fibrinolytic system also interacts with the coagulation system at the level of contact activation (Bennett *et al.*, 1987). A simplified scheme of the fibrinolytic pathway is shown in Fig. 6.

The most well-studied fibrinolytic enzyme is plasmin, which circulates in an inactive form as the protein plasminogen. Plasminogen adheres to a fibrin clot, being incorporated into the mesh during polymerization. Plasminogen is activated to plasmin by the actions of plasminogen activators that may be present in blood or released from tissues, or that may be administered therapeutically. Important plasminogen activators occurring naturally in man include tissue plasminogen activator (tPA) and urokinase. Following activation, plasmin digests the fibrin clot, releasing soluble fibrin–fibrinogen digestion products (FDP) into circulating blood, which may be assayed as markers of *in vivo* fibrinolysis (e.g., the fibrin D-D dimer fragment). Fibrinolysis is inhibited by plasminogen activator inhibitors (PAIs), and by a thrombin-activated fibrinolysis inhibitor (TAFI) that promotes the stabilization of fibrin and fibrin clots (Colman *et al.*, 2001).

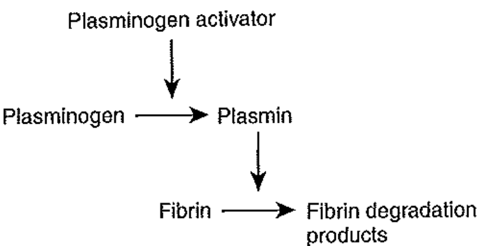

FIG. 6. Fibrinolytic sequence. Plasminogen activators, such as tissue plasminogen activator (tPA) or urokinase, activate plasminogen to form plasmin. Plasmin enzymatically cleaves insoluble fibrin polymers into soluble degradation products (FDPs), thereby effecting the removal of unnecessary fibrin clot.

Complement

As detailed in Chapter 4.4, the complement system is primarily designed to effect a biologic response to antigen–antibody reactions. Like the coagulation and fibrinolytic systems, complement proteins are activated enzymatically through a complex series of reaction steps (Bennett *et al.*, 1987). Several proteins in the complement cascade function as inflammatory mediators. The end result of these activation steps is the generation of an enzymatic complex that causes irreversible damage (by lytic mechanisms) to the membrane of the antigen-carrying cell (e.g., bacteria).

Since there are a number of interactions between the complement, coagulation, and fibrinolytic systems, there has been considerable interest in the problem of complement activation by artificial surfaces, prompted in part by observations that devices having large surface areas (e.g., hemodialyzers) may cause (1) reciprocal activation reactions between complement

enzymes and white cells, and (2) complement activation that may mediate both white-cell and platelet adhesion to artificial surfaces. Further observations regarding the complement activation pathways involved in blood–materials interactions are likely to be of interest.

Red Cells

Red cells are usually considered as passive participants in processes of hemostasis and thrombosis, although under some conditions (low shear or venous flows) red cells may form a large proportion of total thrombus mass. The concentration and motions of red cells have important mechanical effects on the diffusive transport of blood elements. For example, in flowing blood, red-cell motions may increase the effective dissusivity of platelets by several orders of magnitude. Under some conditions, red cells may also contribute chemical factors that influence platelet reactivity (Turitto and Weiss, 1980). The process of direct attachment of red cells to artificial surfaces has been considered to be of minor importance and has therefore received little attention in studies of blood–materials interactions.

White Cells

The various classes of white cells perform many functions in inflammation, infection, wound healing, and the blood response to foreign materials. White-cell interactions with artificial surfaces may proceed through as-yet poorly defined mechanisms related to activation of the complement, coagulation, fibrinolytic, and other enzyme systems, resulting in the expression by white cells of procoagulant, fibrinolytic, and inflammatory activities. For example, stimulated monocytes express tissue factor, which can initiate extrinsic coagulation. Neutrophils may contribute to clot dissolution by releasing potent fibrinolytic enzymes (e.g., neutrophil elastase). White cell interactions with devices having large surface areas may be extensive (especially with surfaces that activate complement), resulting in their marked depletion from circulating blood. Activated white cells, through their enzymatic and other activities, may produce organ dysfunction in other parts of the body. In general, the role of white-cell mechanisms of thrombosis and thrombolysis, in relation to other pathways, remains an area of considerable interest.

CONCLUSIONS

Interrelated blood systems respond to tissue injury in order to quickly minimize blood loss, and later to remove unneeded deposits after healing has occurred. When artificial surfaces are exposed, an imbalance between the processes of activation and inhibition of these systems can lead to excessive thrombus formation and an exaggerated inflammatory response. Whereas many of the key blood cells, proteins, and reaction steps have been identified, their reactions in association with artificial surfaces have not been well defined in many instances. Therefore, blood reactions that might cause thrombosis continue to limit the potential usefulness of many cardiovascular devices for applications in man. Consequently, these devices commonly require the use of systemic anticoagulants, which present an inherent bleeding risk.

Acknowledgments

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4.7 TUMORIGENESIS AND BIOMATERIALS

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The possibility that implant materials could cause tumors or promote tumor growth has long been a concern of surgeons and biomaterials researchers. This chapter describes general concepts in neoplasia, the association of tumors with implants in human and animals, and the pathobiology of tumor formation adjacent to biomaterials.

GENERAL CONCEPTS

Neoplasia, which literally means "new growth," is the process of excessive and uncontrolled cell proliferation (Cotran *et al.,* 1999; Kumar *et al.,* 1997). The new growth is called

TABLE 1 Characteristics of Benign and Malignant Tumors

Characteristics	Benign	Malignant
Differentiation	Well defined; structure may be typical of tissue of origin	Less differentiated with bizarre (anaplastic) cells; often atypical structure
Rate of growth	Usually progressive and slow; may come to a standstill or regress; cells in mitosis are rare	Erratic and may be slow to rapid; mitoses may be absent to numerous and abnormal
Local invasion	Usually cohesive, expansile, well-demarcated masses that neither invade nor infiltrate the surrounding normal tissues	Locally invasive, infiltrating adjacent normal tissues
Metastasis	Absent	Frequently present; larger and more undifferentiated primary tumors are more likely to metastasize

a neoplasm or tumor (i.e., a swelling, since most neoplasms are expansile, solid masses of abnormal tissue). Tumors are either benign (when their pathologic characteristics and clinical behavior are relatively innocent) or malignant (harmful, often deadly). Malignant tumors are collectively referred to as cancers (derived from the Latin word for crab, to emphasize their obstinate ability to adhere to adjacent structures and spread in many directions simultaneously). The characteristics of benign and malignant tumors are summarized in Table 1. Benign tumors do not penetrate (invade) adjacent tissues, nor do they spread to distant sites. They remain localized and surgical excision can be curative in many cases. In contrast, malignant tumors have a propensity to invade contiguous tissues. Moreover, owing to their ability to gain entrance into blood and lymph vessels, cells from a malignant neoplasm can be transported to distant sites, where subpopulations of malignant cells take up residence, grow, and again invade as satellite tumors (called metastases).

The primary descriptor of any tumor is its cell or tissue of origin. Benign tumors are identified by the suffix "oma," which is preceded by reference to the cell or tissue of origin (e.g., adenoma—from an endocrine gland; chondroma—from cartilage). The malignant counterparts of benign tumors carry similar names, except that the suffix "carcinoma" is applied to cancers derived from epithelium (e.g., squamous- or adeno-carcinoma, from protective and glandular epithelia, respectively) and "sarcoma" (e.g., osteo- or chondro-sarcoma, producing bone and cartilage, respective) to those of mesenchymal origin. Malignant neoplasms of the hematopoietic system, in which the cancerous cells circulate in blood, are called leukemias; solid tumors of lymphoid tissue are called lymphomas. The major classes of malignant tumors are illustrated in Fig. 1.

Cancer cells express varying degrees of resemblance to the normal precursor cells from which they derive. Thus, neoplastic growth entails both abnormal cellular proliferation and modification of the structural and functional characteristics of the cell types involved. Malignant cells are generally less differentiated than normal cells. The structural similarity of cancer cells to those of the tissue of origin enables specific diagnosis (source organ and cell type); moreover, the degree of resemblance usually predicts prognosis of the patient (i.e., expected outcome based on biologic behavior of the cancer). Therefore, poorly differentiated tumors generally are more aggressive (i.e., display more malignant behavior) than those that are better differentiated. The degree to which a tumor mimics a normal cell or tissue type is called its grade of differentiation. The extent of spread and other effects on the patient determine its stage.

Neoplastic growth is unregulated. Neoplastic cell proliferation is therefore unrelated to the physiological requirements of the tissue and is unaffected by removal of the stimulus which initially caused it. These characteristics differentiate neoplasms from (1) normal proliferations of cells during fetal development or postnatal growth, (2) normal wound healing following an injury, and (3) hyperplastic growth that adapts to a physiological need, but that ceases when the stimulus is removed.

All tumors, benign and malignant, have two basic components: (1) proliferating neoplastic cells that constitute their parenchyma, and (2) supportive stroma made up of connective tissue and blood vessels. Although the parenchyma of neoplasms is characteristic of the specific cells of origin, the growth and evolution of neoplasms are critically dependent on the nonspecific stroma, usually composed of blood vessels, connective tissue, and inflammatory cells.

ASSOCIATION OF IMPLANTS WITH HUMAN AND ANIMAL TUMORS

Neoplasms occurring at the site of implanted medical devices are unusual, despite the large numbers of implants used clinically over an extended period of time. Nevertheless, cases of both human and veterinary implant-related tumors have been reported (Black, 1988; Jennings *et al.*, 1988; Pedley *et al.*, 1981; Schoen, 1987). In all, more than 50 cases of tumors associated with foreign material have been reported, of which approximately half were adjacent to therapeutic implants. The remainder include tumors related to bullets, shrapnel, other metal fragments, sutures, bone wax, and surgical sponge. Implant-related tumors have been reported both short and long term following implantation. More than 25% of tumors associated with foreign bodies have developed within 15 years, and more than 50% within 25 years (Brand and Brand, 1980).

The vast majority of malignant neoplasms associated with clinical fracture fixation devices, total joint replacements, mechanical heart valves, and vascular grafts and experimental foreign bodies in both animals and humans are sarcomas. They comprise various histologic subtypes, including fibrosarcoma,

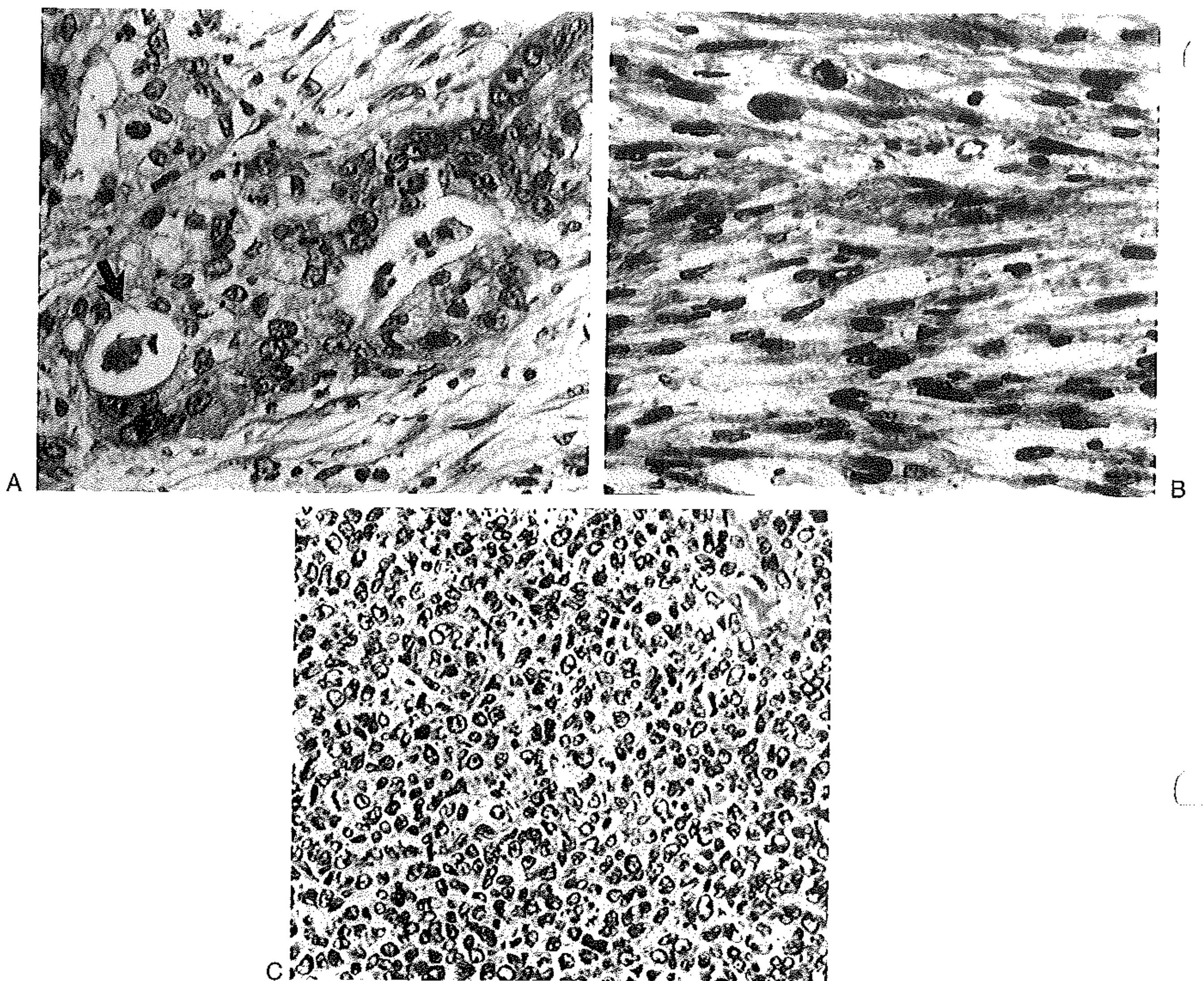

FIG. 1. Types of malignant tumors. (A) Carcinoma, exemplified by an adenocarcinoma (gland formation noted by arrow). (B) Sarcoma (composed of spindle cells). (C) Lymphoma (composed of malignant lymphocytes). All stained with hematoxylin and eosin; all × 310.

osteosarcoma (osteogenic sarcoma), chondrosarcoma, and angiosarcoma, and are characterized by rapid and locally infiltrative growth. Carcinomas, reported far less frequently, have usually been restricted to situations where an implant has been placed in the lumen of an epithelium-lined organ. Illustrative reported cases are noted in Table 2; descriptions of others are available (Goodfellow, 1992; Jacobs *et al.*, 1992; Jennings *et al.*, 1988). Lymphomas have been reported in association with the capsules surrounding breast implants (Gaudet *et al.*, 2002; Keech and Creech, 1997; Sahoo *et al.*, 2003). A tumor forming adjacent to a clinical vascular graft is illustrated in Fig. 2. A non-implant-related primary tumor (gastric cancer) with a metastasis to a total knee replacement has also been reported (Kolstad and Hgstorp, 1990).

Whether there is a causal role for implanted medical devices in local or distant malignancy remains controversial. In an individual case, caution is necessary in implicating the implant in the formation of a neoplasm; demonstration of a tumor occurring adjacent to an implant does not necessarily prove that the implant caused the tumor (Morgan and Elcock, 1995). Large-scale epidemiological studies and reviews of available data have concluded that there is no evidence in humans for tumorgenecity of non-metallic and metallic surgical implants (McGregor *et al.*, 2000). Indeed, the risk in populations must be low, as exemplified by recent cohorts of patients with both total hip replacement and breast implants who show no detectable increases in tumors at the implant site (Berkel *et al.*, 1992; Deapen and Brody, 1991; Mathiesen *et al.*, 1995; Brinton and Brown, 1997). A clinical and experimental study even suggested that the evidence of breast carcinoma may be decreased in women with breast implants (Su *et al.*, 1995). However, one study suggested a small increase in the number of lung and vulvar cancers in patients with breast implants (Deapen and Brody, 1991). Importantly, the presence of an implant does

TABLE 2 Tumors Associated with Implant Sites in Humans—Representative Reports

Device (adjacent material)[a]	Tumor[b]	References	Postimplantation (years)
Fracture fixation			
Intramedullary rod (V)	L	McDonald (1980)	17
Smith–Petersen (V)	OS	Ward *et al.* (1987)	9
Total hip			
Charnley–Mueller (UHMWPE, PMMA)	MFH	Bago-Granell *et al.* (1984)	2
Mittlemeier (Al_2O_3)	STS	Ryu *et al.* (1987)	1+
Charnley–Mueller (UHMWPE)	OS	Martin *et al.* (1988)	10
Charnley–Mueller (SS, PMMA)	SS	Lamovec *et al.* (1988)	12
Unknown (porous Ti–cobalt alloy)	OS	Adams *et al.* (2003)	3
Total knee			
Unknown (V)	ES	Weber (1986)	4
Vascular graft			
Abdominal aortic graft (D)	MFH	Weinberg and Maini (1980)	1+
Abdominal aortic graft (D)	AS	Fehrenbacker *et al.* (1981)	12
Heart valve prosthesis			
St. Jude Medical (Carbon, Sizone-coated Dacron sewing cuff)	RS	Grubitzsch *et al.* (2001)	<1

[a] Materials: D, Dacron; PMMA, poly(methyl methacrylate) bone cement; SS, stainless steel; Ti, titanium; UHMWPE, ultrahigh-molecular-weight polyethylene; V, Vitallium.
[b] Tumor types: AS, angiosarcoma; ES, epithelioid sarcoma; L, lymphoma; MFH, malignant fibrous histiocytoma; OS, osteosarcoma; RS, rhabdomyosarcoma; SS, synovial sarcoma; STS, soft tissue sarcoma.

not impair the diagnosis of breast cancer (Brinton and Brown, 1997).

Moreover, neoplasms are common in both humans and animals and can occur naturally at the sites at which biomaterials are implanted. Most clinical veterinary cases have been observed in dogs, a species with a relatively high natural frequency of osteosarcoma and other tumors at sites where orthopedic devices are implanted. Moreover, spontaneous human musculoskeletal tumors are not unusual. However, since sarcomas arising in the aorta and other large arteries are rare, the association of primary vascular malignancies with clinical polymeric grafts may be stronger than that with orthopedic devices.

Clinically benign but exuberant foreign-body reactions may simulate neoplasms. For example, fibrohistiocytic lesions resembling malignant tumors may occur as a reaction to silica, previously injected as a soft-tissue sclerosing agent (Weiss *et al.*, 1978). Moreover, regional lymphadenopathy (i.e., enlargement of lymph nodes) may result from an exuberant foreign-body reaction to material that has migrated from a prosthesis. This has been documented in cases of silicone emanating from both finger joints (Christie *et al.*, 1977) and breast prostheses (Hausner *et al.*, 1978), as well as in association with polymeric replacements of the temporomandibular joint, and with conventional metallic, ceramic, and polymeric total replacements of large joints (Jacobs *et al.*, 1995). A mass lesion caused by foreign-body granuloma in a lymph node can masquerade as a neoplasm on physical examination (sometimes called a pseudotumor). Potentially, it could evolve into a lymphoma owing to chronic stimulation of the immune system.

PATHOBIOLOGY OF FOREIGN BODY TUMORIGENESIS

Considerable progress has been made over the past several decades in the understanding of the molecular basis of cancer (Cotran *et al.*, 1999; Kumar *et al.*, 1997). Four principles are fundamental and well accepted: (1) Neoplasia is associated with and often results from nonlethal genetic damage (or mutation), either inherited or acquired by the action of environmental agents such as physical effects (e.g., radiation, fibers or foreign bodies; Fry, 1989), chemicals or viruses. (2) The principal targets of the genetic damage are cellular regulatory genes (normally present and necessary for physiologic cell function, inducing cellular replication, growth and repair of damaged DNA). (3) The tumor mass evolves from the clonal expansion of a single progenitor cell that has incurred the genetic damage. (4) Tumorigenesis is a multistep process, generally owing to accumulation of successive genetic lesions. After a tumor has been initiated, the most important factors in its growth are the kinetics (i.e., balance of replication or loss) of cell number change and its blood supply. The formation of new vessels (angiogenesis) is essential for enlargement of tumors and for their access to the vasculature and, hence, metastasis (Carmeliet, 2003).

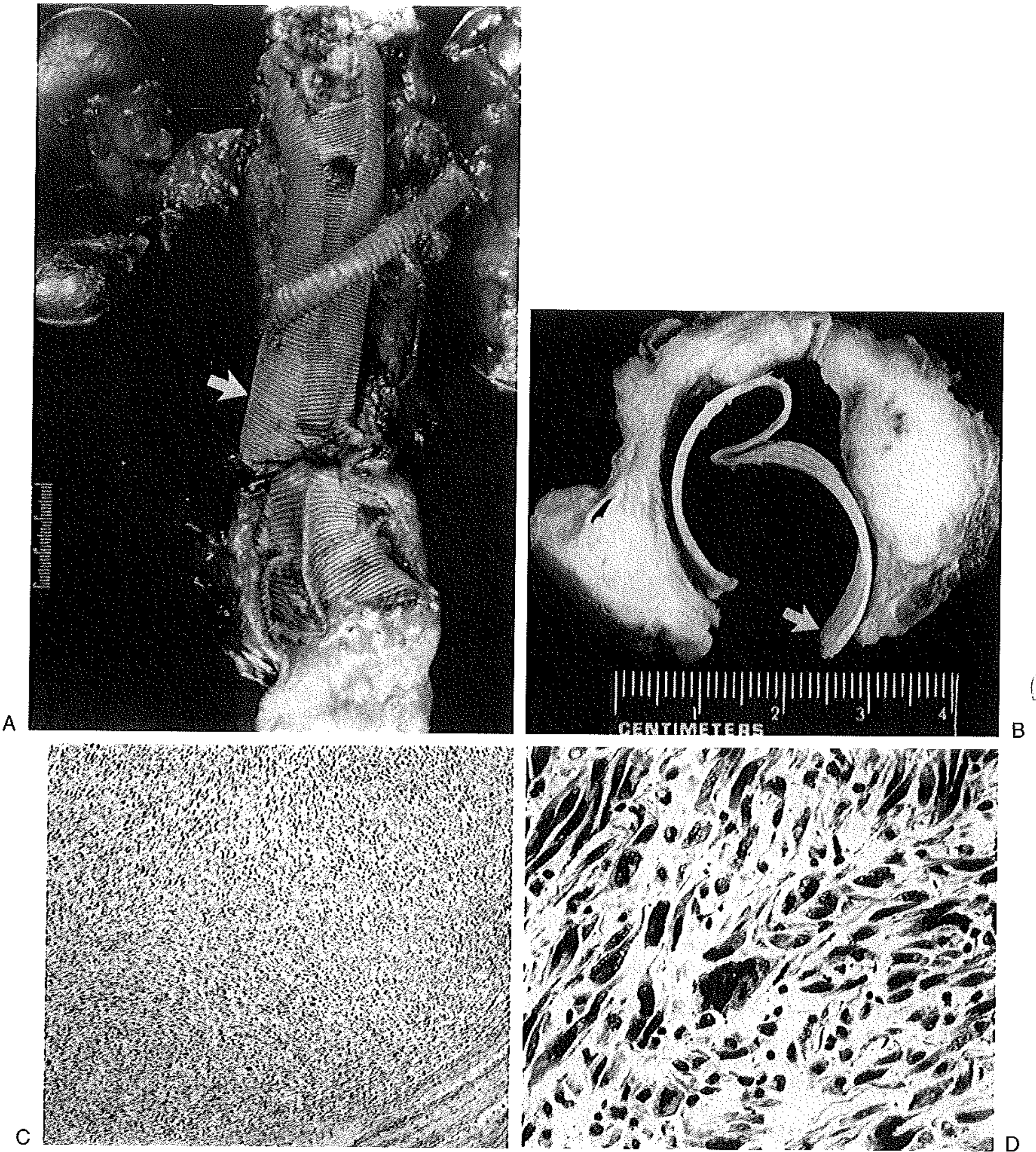

FIG. 2. Sarcoma arising 1 year following and in association with Dacron graft repair of abdominal aortic aneurysm. (A) and (B) Gross photographs (graft designated by arrow, surrounded by tumor mass). (C) and (D) Histologic appearance of tumor. (C) and (D) Stained with hematoxylin and eosin. (C) × 49, (D) × 300. [(A), (C), and (D) reproduced by permission from D. S. Weinberg and B. S. Maini, *Cancer* 46: 398–402, 1980.]

The pathogenesis of implant-induced tumors is not well understood, yet most experimental data indicate that physical effects rather than the chemical characteristics of the foreign body are the principal determinants of tumorigenicity (Brand *et al.*, 1975). Tumors are induced experimentally by a wide array of materials of diverse composition, including those that could be considered essentially nonreactive, such as certain glasses, gold or platinum, and other relatively pure metals and polymers. Indeed, one surgeon performed a much-maligned experiment in which dimes inserted in rats yielded a rate of 60% sarcomas in 16 months (prompting the suggestion that dimes and probably all metallic coins were carcinogenic and should be discontinued!) (Moore and Palmer, 1997). Solid materials implanted in a configuration with high surface area are most tumorigenic. Materials lose their tumorigenicity when implanted in pulverized, finely shredded or woven form, or when surface continuity is interrupted by multiple perforations. This trend is often called the Oppenheimer effect. Thus, foreign-body neoplasia is generally considered to be a transformation process mediated by the physical state of implants; it is largely independent of the composition of the materials, unless specific carcinogens are present.

Solid-state tumorigenesis depends on the development of a fibrous capsule around the implant. Tumorigenicity corresponds directly to the extent and maturity of tissue encapsulation of a foreign body and inversely with the degree of active cellular inflammation. Thus, an active, persistent inflammatory response inhibits tumor formation in experimental systems. Host (especially genetic) factors also affect the propensity to form tumors as a response to foreign bodies. Humans are less susceptible to foreign-body tumorigenesis than are rodents, the usual experimental model. In rodent systems, tumor frequency and latency depend on species, strain, sex, and age. Concern has recently been raised over the possibility that foreign-body neoplasia can be induced by the release of wear debris or needlelike elements from composites in a mechanism that is analogous to that of asbestos-related mesothelioma (Brown *et al.*, 1990; Jaurand, 1991). However, animal experiments suggest only particles with very high length-to-diameter ratios (>100) produce this effect. Particles with this high aspect ratio are highly unlikely to arise as wear debris from orthopedic implants.

Nevertheless, cancer at foreign-body sites may be mechanistically related to that which occurs in diseases in which tissue fibrosis is a prominent characteristic, including asbestosis (i.e., lung damage caused by chronic inhalation of asbestos), lung or liver scarring, or chronic bone infections (Brand, 1982). However, in contrast to the mesenchymal origin of most implant-related tumors, other cancers associated with scarring are generally derived from adjacent epithelial structures (e.g., mesothelioma with asbestos).

Chemical induction effects are also possible. With orthopedic implants, the stimulus for tumorigenesis could be metal particulates released by wear of the implant (Harris, 1994). Indeed, implants of chromium, nickel, cobalt, and some of their compounds, either as foils or debris, are carcinogenic in rodents (Swierenza *et al.*, 1987), and the clearly demonstrated widespread dissemination of metal debris from implants (to lymph nodes, bone marrow, liver, and spleen, particularly in subjects with loose, worn joint prostheses) not only could cause damage to distant organs, but also could be associated with the induction of neoplasia (Case *et al.*, 1994). Although unequivocal cases of metal particles or elemental metals provoking the formation of malignant tumors are not available, continued vigilance and further study of the problem in animal models is warranted (Lewis and Sunderman, 1996).

"Nonbiodegradable" and "inert" implants have been shown to contain and/or release trace amounts of substances such as remnant monomers, catalysts, plasticizers, and antioxidants. Nevertheless, such substances injected in experimental animals at appropriate test sites (without implants), in quantities comparable to those found adjacent to implants, are generally not tumorigenic. Moreover, chemical carcinogens such as nitrosamines or those contained in tobacco smoke may potentiate scar-associated cancers.

A chemical effect has been considered in the potential carcinogenicity of polyurethane biomaterials (Pinchuk, 1994). Under certain conditions (i.e., high temperatures in the presence of strong bases), diamines called 2,4-toluene diamine (TDA) and 4,4′-methylene dianiline (MDA) can be produced from the aromatic isocyanates used in the synthesis of polyurethanes. TDA and MDA are carcinogenic in rodents. However, it is uncertain whether (1) TDA and MDA are formed *in vivo*, and (2) these compounds are indeed carcinogenic in humans, especially in the low dose rate provided by medical devices. Although attention has been focused on polyurethane foam-coated silicone gel-filled breast implants, one type of which contained the precursor to TDA, the risk is considered zero to negligible (Expert Panel, 1991).

Foreign-body tumorigenesis is characterized by a long latent period, during which the presence of the implant is required for tumor formation. Available data suggest the following sequence of essential developmental stages in foreign-body tumorigenesis (summarized in Table 3): (1) cellular proliferation in conjunction with tissue inflammation associated with the foreign-body reaction (specific susceptible preneoplastic cells may be present at this stage); (2) progressive formation of a well-demarcated fibrotic tissue capsule surrounding the implant; (3) quiescence of the tissue reaction (i.e., dormancy and phagocytic inactivity of macrophages attached to the foreign body), but direct contact of clonal preneoplastic cells with the foreign body surface; (4) final maturation of preneoplastic cells; and (5) sarcomatous proliferation. Support for this

TABLE 3 Steps in Implant-Associated Tumorigenesis: A Hypothesis[a]

1. Cellular foreign-body reaction
2. Fibrous capsule formation
3. Preneoplastic cells contact implant surface during quiescent tissue reaction
4. Preneoplastic cell maturation and proliferation
5. Tumor growth

[a]Following K. G. Brand and colleagues.

multistep hypothesis for foreign body tumorigenesis comes from an experimental study by Kirkpatrick (2000) in which premalignant lesions were frequently found in implant capsules. A spectrum of lesions was observed, from proliferative lesions without atypical calls to atypical proliferation to incipient sarcoma.

The essential hypothesis is that initial acquisition of neoplastic potential and the determination of specific tumor characteristics does not depend on direct physical or chemical interaction between susceptible cells and the foreign body, and, thus, the foreign body per se probably does not initiate the tumor. However, although the critical initial event occurs early during the foreign-body reaction, the final step to neoplastic autonomy (presumably a genetic event) is accomplished only when preneoplastic cells attach themselves to the foreign-body surface. Subsequently, there is proliferation of abnormal mesenchymal cells in this relatively quiescent microenvironment, a situation not permitted with the prolonged active inflammation associated with less inert implants.

Thus, the critical factors in sarcomas induced by foreign bodies include implant configuration, fibrous capsule development and remodeling, and a period of latency long enough to allow progression to neoplasia in a susceptible host. The major role of the foreign body itself seems to be that of stimulating the formation of a fibrous capsule conducive to neoplastic cell maturation and proliferation. The rarity of human foreign body–associated tumors suggests that cancer-prone cells are infrequent in the foreign-body reactions to implanted human medical devices.

CONCLUSIONS

Neoplasms associated with therapeutic clinical implants in humans are rare; causality is difficult to demonstrate in any individual case. Experimental implant-related tumors are induced by a large spectrum of materials and biomaterials, dependent primarily on the physical and not the chemical configuration of the implant. The mechanism of experimental tumor formation, as yet incompletely understood, appears related to the implant fibrous capsule.

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4.8 BIOFILMS, BIOMATERIALS, AND DEVICE-RELATED INFECTIONS

Bill Costerton, Guy Cook, Mark Shirtliff, Paul Stoodley, and Mark Pasmore

INTRODUCTION

Tens of millions of medical devices are used each year and, in spite of many advances in biomaterials, a significant proportion of each type of device becomes colonized by bacteria and becomes the focus of a device-related infection. Topical devices (e.g., contact lenses) are colonized as soon as they are placed on tissue surfaces, transcutaneous devices (e.g., vascular catheters) are progressively colonized by skin organisms, and even surgically implanted devices regularly become foci of infection. Implanted devices may be colonized by bacteria at the time of surgery, or they may be colonized by organisms that gain access to their surfaces by a hematogenous route, from a distant source. The most significant factor in the development of device-related infections appears to be the skill of the surgical team, with prosthetic hips being infected in less than 0.2% of cases in large specialized clinics, and as many as 4% in less proficient facilities. Generally, large and complex medical devices that require long and complicated surgery for their placement are at high risk of bacterial infection, and transcutaneous devices in this category (e.g., the Jarvik heart) automatically become infected. In many areas of medicine, the risk of infection limits the use of devices that constitute the epitome of the engineer's skill and imagination and incorporate the finest and most sophisticated biomaterials available in this fast-moving field.

As medical devices came into more regular use, the surgeons who placed them used their well-developed observation skills to define the "classic" device-related infection. These infections were often very slow to develop, with overt symptoms sometimes being seen almost immediately and sometimes being seen months or even years after the device was installed. Inflammation and pus formation were often local, especially in transcutaneous devices, but a certain proportion of patients with device-related infections suddenly developed acute disseminated infections caused by the same species that had colonized the device. These acute exacerbations of device-related infections responded well to antibiotic therapy. However, this treatment almost never reversed the local symptoms, and colonized devices often gave rise to a predictable series of acute exacerbations, so that good medical management usually dictated their removal. The bacteria that caused device-related infections were common skin biota (e.g., *Staphylococcus epidermidis*) and common environmental organisms (e.g., *Pseudomonas aeruginosa*), and certain species predominated in infections of certain devices. Because the infecting bacteria, and occasional fungi (e.g., *Candida albicans*), were so ubiquitous in the modern human environment, device recipients always had good immunity against these low-level pathogens, but these antibodies failed to prevent infection. It was the "front-line" medical specialists (e.g., orthopedic surgeons) who gradually persuaded medical microbiologists

and infectious disease specialists that device-related infections differed from acute bacterial infections in several important respects.

The biofilm concept was developed and articulated (Costerton *et al.*, 1978) in environmental microbiology, and it was introduced into medical microbiology when Tom Marrie *et al.* (1982) examined the surfaces of devices that had failed because of bacterial infection. This concept states that bacteria, in all but the most nutrient-deprived ecosystems, grow preferentially in matrix-enclosed communities attached to surfaces (Costerton *et al.*, 1987). Electron microscopy proved to be useful in the examination of the surfaces of failed medical devices, because both scanning (SEM) and transmission (TEM) electron microscopy involve dozens of washing steps that remove floating or loosely adherent bacteria. Therefore any bacterial or fungal cells that remained on the surfaces of the device, after processing, were *bona fide* biofilm organisms. With medical colleagues leading the search (Khoury *et al.*, 1992; Marrie and Costerton, 1984; Nickel *et al.*, 1985), our morphological team examined hundreds of types of failed medical devices and found biofilms on all of their surfaces. Biofilms were seen on the surfaces of contact lenses that had been worn by volunteers (McLaughlin-Borlace *et al.*, 1998), and very extensive sessile communities were seen on the surfaces of lenses that had been stored overnight in storage cases (Gray *et al.*, 1995; McLaughlin-Borlace *et al.*, 1998). Some of the most extensive biofilms we ever saw on a medical device were found on the surfaces of intrauterine contraceptive devices (Marrie *et al.*, 1982), and teeth and dental devices were equally heavily colonized. It was in this area of topical medical devices that the distinction was made between colonization, which is the simple presence of microbial biofilms on a surface, and the infections that occur when this presence of a biofilm elicits a pathogenic response.

The surface of skin is colonized by a wide variety of bacteria and fungi, most of which are removed or killed by surgical preparations, but the deeper layers are also colonized by bacteria (mostly *S. epidermidis*) that escape skin sterilants. This cutaneous biota rapidly colonizes the surfaces of any transcutaneous device, and the biofilm moves along any device that is placed in a subcutaneous "tunnel," until the entire surface of the device is colonized. In this manner a microbial biofilm is introduced into the normally sterile environment of the peritoneum, by the Techkhoff catheter (Dasgupta *et al.*, 1987), or into the normally sterile environment of the heart, by devices like the Hickman (Tenney *et al.*, 1986) and the Swan-Ganz (Mermel *et al.*, 1991) catheters. The inevitable colonization of transcutaneous devices, which is usually complete in 3–4 weeks, does not automatically lead to infection. All of the Hickman catheters in our National Cancer Institute study (Tenney *et al.*, 1986) were seen to be colonized, and one was even partially blocked by a very exuberant biofilm, but only four of the 81 patients experienced overt infection and bacteremia. Chronic ambulatory peritoneal dialysis (CAPD) patients all have well-developed biofilms on their Tenckhoff catheters, but many do not develop peritonitis if their humoral and cellular immune mechanisms can "keep up" (Dasgupta *et al.*, 1990) with the planktonic (floating) cells that are released from these sessile communities.

When implanted medical devices become colonized, the presence of these microbial biofilms always triggers pathogenic changes in the surrounding tissues, but symptoms are often slow to develop. Mechanical heart valves and vascular grafts can fail because biofilms on stitches that hold them in place cause inflammation, weaken the tissues involved, and lead to their detachment and displacement (Hyde *et al.*, 1998). Orthopedic devices may develop "aseptic loosening" in that the device is loosened by bone dissolution, but there are no signs of inflammation. The biofilms of the causative pathogens are so coherent that routine cultures of the device and the tissues are almost always negative. Biofilms elicit few symptoms, because their matrix-enclosed cells produce few toxins and stimulate only cursory immune responses and inflammation, but local symptoms will be produced when planktonic cells are released from these sessile communities.

The examination of failed medical devices frequently reveal microbial biofilms. Therefore, the unique characteristics of device-related infections can be explained in terms of the characteristics of biofilms (Costerton *et al.*, 1999). The slow development and asymptomatic nature of many device-related infections can be explained by the observation that biofilm bacteria produce few toxins and elicit little inflammatory response. Many device-related infections are negative in routine microbiological cultures because biofilms release a limited number of planktonic cells, large biofilm fragments grow up as a single colony on plates, and sessile cells do not grow well on agar surfaces. Common bacterial species predominate in device-related infections because they form biofilms very effectively in their natural environments (e.g., skin), and this biofilm mode of growth protects them from the immune responses that occur in all potential hosts. The biofilm mode of growth protects the causative agents of device-related infections from both humoral and cell-mediated immunity (see Chapter 4.3) (Leid *et al.*, 2002), so these infections occur in healthy individuals, and they are never resolved by even the most active host defense mechanisms. Exacerbations of device-related infections are caused by the release of planktonic cells, and antibiotics can kill these floating cells and reverse the symptoms of acute infection, but the infection persists because the causative biofilm is resistant to these antibacterial agents. Most, if not all, of the characteristics of device-related infections can be explained in terms of the characteristics of biofilms, so it may be useful to examine the burgeoning field of biofilm microbiology, as an early step in the search for new biomaterials that will control these infections.

BIOFILM MICROBIOLOGY

Many of the concepts and techniques that have served microbiologists well, in the virtual conquest of epidemic bacterial diseases caused by planktonic organisms, now serve us only poorly in the study of device-related and other chronic bacterial diseases. This section on biofilm microbiology will focus on the central fact that biofilm bacteria differ from their planktonic counterparts in so many ways that they are as different as spores are from vegetative bacteria, and it is imperative

that special biofilm methods be used in studies of the bacterial colonization of biomaterials.

Bacterial Adhesion to Surfaces

Often, the DLVO theory is applied to the study of bacterial adhesion to surfaces (van Loosdrecht *et al.*, 1990). This classic concept of colloid behavior visualizes a planktonic bacterial cell as a smooth colloid particle that interacts with the surface in a manner based on the charges on both surfaces, which overcome the basic repulsion of individual particles. Examinations of the surfaces of planktonic bacteria, using special preparations and electron microscopy, have clearly shown that these surfaces are not smooth. In addition to proteinaceous appendages (flagella and pili) that project 2–6 μm from the cell, the entire surfaces of planktonic cells of natural strains of bacteria are covered by a matrix of hydrophobic exopolysaccharide (EPS) fibers, and sometimes by a highly structured protein "coat." The external EPS layer of planktonic cells is anchored to the polysaccharide O antigen fibers that project from the lipopolysaccharide (LPS) of the outer membrane of gram-negative cells, and to the polysaccharide teichoic acid fibers that project from the cell walls of gram-positive cells. Elegant freeze-substitution microscopy preparations have shown that the actual surface of planktonic bacterial cells that would be capable of interacting with the surface to be colonized is a 0.2 to 0.4-μm-thick forest of protein and polysaccharide fibers. The planktonic bacterial cell is not a smooth-surfaced colloid particle, and the actual interaction of these cells with surfaces is based on the bridging of bacterial fibers with fibers adsorbed to the surface being colonized. Thus, DLVO theory is of limited application in the study of bacterial adhesion.

Another conventional microbiology method, the reliance on pure cultures of bacteria isolated from the system of interest, but subcultured hundreds of times in rich media, also does not serve us well in biofilm studies relevant to medical devices. This method, which dates from Robert Koch in the 1850s, produces lab-adapted strains of bacteria that are selected in favor of planktonic growth, because the simple act of subculturing leaves adherent cells behind in the old culture and transfers only free-floating cells. These lab-adapted strains lack many surface structures that would be necessary for their survival in a hostile "wild" environment, but they are not challenged by antibacterial agents, so they survive in the test tube, but perish if they are released into natural ecosystems. When these lab-adapted strains are used in studies of bacterial adhesion to biomaterials, they come close to the smooth-surfaced colloidal particles visualized in the DLVO theory, and data that are misleading for the understanding of medical-device-centered infection are generated. Several companies have spent millions of dollars on novel biomaterials to which lab-adapted strains of bacteria would adhere to only very poorly, only to have these biomaterials heavily colonized by "wild" natural bacteria, and to find that they performed unsatisfactorily in clinical tests. Most microbiologists who focus on biofilms never do adhesion experiments on strains that are more than one transfer from an infected patient, if their objective is to assess the propensity of a biomaterial for colonization by a putative pathogen. Scientists at the FDA and EPA are aware of this necessity to use "wild" bacteria in biomaterials testing.

When planktonic cells adhere to a surface, which they do with considerable avidity, they exhibit behaviors that have been divided into "reversible" and "irreversible" patterns (Marshall *et al.*, 1971). The most actively motile organisms (e.g., *P. aeruginosa*) may use their flagella as landing mechanisms, and then may use their type IV pili to produce a twitching motility that allows them to pile up into elaborate structures, some of which resemble the fruiting bodies of the myxobacteria. Other less mobile organisms produce "windrows" of cells (Korber *et al.*, 1995) following adhesion, while cells that have neither flagella nor pili simply stay in place if the location is favorable, and detach if it is not. Movies showing these post-adhesion behaviors of bacteria are available on the Center for Biofilm Engineering (CBE) Web site (www.erc.montana.edu). Biofilm engineers have generated surprising data (Stoodley *et al.*, 2001a, b) showing that many cells that adhere to surfaces also detach and leave the area, before they make the genetic switch to attach irreversibly and initiate the process of biofilm formation. Many people in the biomaterials field have speculated, intuitively, that key surface characteristics must favor (or inhibit) bacterial adhesion, and almost every possible combination of these characteristics has been tried in the search for colonization-resistant biomaterials. Wild bacteria adhere equally well to very hydrophobic (e.g., Teflon) and to very hydrophilic (e.g., PVC) surfaces, they colonize smooth surfaces as well as they adhere to rough surfaces (Marrie and Costerton, 1984; Sottile *et al.*, 1986), and they colonize smooth surfaces in very high shear flow systems (Characklis, 2003). Thus, we have no perfect biomaterial surface that resists bacterial colonization by virtue of its inherent surface properties, but nonfouling surfaces show limited potential for this application (Chapter 2.13).

Biofilm Formation on Surfaces

When a bacterial cell has "made the decision" to colonize a surface it sets in motion a pattern of gene expression that profoundly alters its previous planktonic phenotype, to produce a unique biofilm phenotype that may differ by as much as 70% in the proteins expressed (Sauer and Camper, 2001). Some of the first genes that are up-regulated in adherent cells are those involved in the production of the EPS material that will form the matrix of the biofilm and will also anchor the cell irreversibly to the surface. In *P. aeruginosa* the up-regulation of *algC*, which is a part of the alginate synthesis pathway, occurs within 18 minutes of initial cell adhesion (Davies and Geesey, 1995), and we see the secretion of matrix material by these cells within 30 minutes of adhesion. The genes that are up-regulated in the biofilm phenotype of many bacterial species are being analyzed by proteomics (Miller and Diaz-Torres, 1999; Oosthuizen *et al.*, 2002; Sauer and Camper, 2001; Sauer *et al.*, 2002; Svensater *et al.*, 2001; Tremoulet *et al.*, 2002a, b) and by microarray analysis (Schembri *et al.*, 2003; Schoolnik *et al.*, 2001; Stoodley *et al.*, 2002; Wagner *et al.*, 2003; Whiteley *et al.*, 2001), and individual genes involved in this profound phenotype shift are being identified daily. Sauer and her colleagues

have reported that the phenotype of planktonic cells of both *P. aeruginosa* and *P. putida* differ from that of their biofilm counterparts more than they differ from that of planktonic cells of other species in the same genus. The inherent resistance of biofilm bacteria to antibiotics, all of which were selected on the basis of their ability to kill planktonic cells, is now largely attributed to the altered gene expression pattern of the biofilm phenotype. Scientists at Microbia Ltd (Boston) have identified one specific gene (fmt C) that is responsible for this inherent antibiotic resistance in biofilms formed by all staphylococcal species, and the deletion or blockage of this gene produces biofilms that are susceptible to conventional antibiotics.

Once attached cells have triggered the conversion to the biofilm phenotype, the multicellular community on the colonized surface begins to accrete larger numbers of cells by binary fission and by further recruitment of planktonic cells from the bulk water phase. As they increase in numbers and produce large amounts of EPS matrix material, the attached cells form microcolonies in which they constitute approximately 15% of the volume and the matrix occupies approximately 85% of the volume. The microcolonies assume tower-like and mushroom-like shapes (Fig. 1) in most natural and cultured biofilms, but many other morphologies may be dictated by species characteristics and by nutrient availability. The microcolonies may occupy the colonized surface, as discrete entities separated by open water channels (Fig. 1), or they may pile up in several layers to form thick sessile communities, but they always maintain their structural integrity and move independently under shear stress. As the biofilm matures and undergoes more phenotypic changes (Stoodley *et al.*, 2002), the processes of cell division and recruitment come into balance with programmed detachment of planktonic cells from the sessile community and sloughing. Most natural biofilms reach a mature thickness and

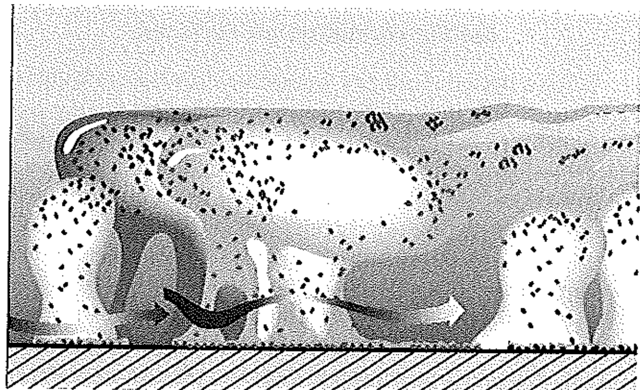


FIG. 1. Diagrammatic representation of the tower-like and mushroom-like microcolonies that are the basic structural units of the biofilms on colonized surfaces. Note that the matrix material occupies ±85% of the mass of these structures (while the cells comprise ±15%), that the microcolonies can be deformed into oscillating streamers by shear forces, and that water moves through these complex communities in a convective pattern. It was the complex differentiated structure of these microcolonies, and the maintenance of the open water channels, that stimulated speculation that some form of "hormone-like" cell–cell signaling must be involved in the formation of microbial biofilms.

a stable community structure within a week or two of their initiation of colonization, and many remain relatively stable for years. Stoodley *et al.* (2002) have concluded that the biofilm phenotype would favor bacterial survival in the harsh environment of the primitive earth, and they have suggested that the planktonic phenotype may have developed considerably later, as a mechanism for dissemination.

Natural Control of Biofilm Formation on Surfaces

The complex structure of biofilm communities (Fig. 1) stimulated a lively discussion of what signals (e.g., hormones or pheromones) must be operative to allow the development of shaped microcolonies and sustained water channels. In 1998 the first demonstration that biofilm development in *P. aeruginosa* is controlled by an acyl homoserine lactone (AHL) quorum-sensing signal was published (Davies *et al.*, 1998), and it has subsequently been reported that agr, sar, and RAP (Balaban *et al.*, 1998) signals control this same process in gram-positive organisms. Additionally, it was shown that the autoinducer II signal (furanone) controls biofilm formation, and many other processes, in virtually all bacterial species (Schauder *et al.*, 2001; Xavier and Bassler, 2003). Most biofilm specialists agree that these signals are simply the tip of the iceberg, that many more signals will be discovered, and that specific blockage of many of these simple chemical signals offers a practical way to control virtually any bacterial "behavior." It has already been shown that specific signal inhibitors can block toxin production in *S. aureus*, and can even render specific bacteria essentially nonpathogenic in animal models (Balaban *et al.*, 2000).

The manipulation of biofilm formation is a very attractive target for new agents to prevent device-related infections. If bacteria that make contact with biomaterials were "locked" in the planktonic phenotype and were unable to assume the protected biofilm phenotype, they would be readily killed by host defense mechanisms and/or by antibiotic therapy. Several chemical analogs that block signal activity by interfering with the binding of the signal to its cognate receptor have been shown to be effective in inhibiting biofilm formation in specific pathogens (Balaban *et al.*, 1998). One such analog (RIP) prevents the binding of a biofilm control signal (RAP) to its receptor (TRAP). This signal blocker has been shown to prevent biofilm formation in an animal model of a device-related infection, and to allow complete eradication of the bacteria with conventional antibiotic therapy (Balaban *et al.*, 2003a, b). The researchers involved in the search for biofilm-control signal inhibitors are acutely aware of the subtle nature of the signal network in bacterial cells. It is highly unlikely that we will find a single ON/OFF switch for biofilm formation, and blockers that prevent biofilm formation may up-regulate invidious bacterial behaviors (e.g., toxin production), but we are encouraged by several observations made in natural ecosystems. Marine plants and animals control biofilm formation on their surfaces, presumably because biofilm/silt accretion would bury them and preclude photosynthesis in the plants, and at least one of the compounds that they use for this purpose is a signal inhibitor (de Nys *et al.*, 1995). In these natural systems, plant and animal surfaces are ideal locations for biofilm formation and growth,

but no bacterial mutant capable of thwarting the action of these natural biofilm control agents has emerged in millions of years of evolution.

Novel Engineering Approaches to Biofilm Control

The current therapy for device-related infections consists of trying to kill a biological entity (the bacteria) with a chemical (the antibiotic), with the only variable parameters being concentration and time of contact. Engineers have suggested that a number of physical forces could be harnessed to deliver higher concentrations of the antibiotic to the infecting organisms, or to compromise the bacteria in ways that make them more susceptible to the agents concerned. Two technologies that offer considerable promise involve the use of ultrasonic energy (Nelson *et al.*, 2002; Rediske *et al.*, 1998), and the imposition of a very weak sustained DC field (Costerton *et al.*, 1994) across the biofilm, and both have been shown to render sessile microbial populations susceptible to conventional antibiotics. Practical research is currently underway in the modification of biomaterials, and of device design, to harness the potential of these physical biofilm control technologies in our general strategies for the control of device-related infections.

BIOFILM-RESISTANT BIOMATERIALS

Biofilm-related complications have cost many lives in clinical settings. This unfortunate outcome may be reduced if the concepts and methods of modern biofilm microbiology can be inculcated into the development process for antibiofilm biomaterials. We will discuss some of the new agents that may give us effective control over the colonization of biomaterials and the incidence of device-related infections, and then we will discuss new methods for the release/delivery of these agents at the surfaces of biomaterials.

Testing for Antibacterial and Antibiofilm Properties of Biomaterials

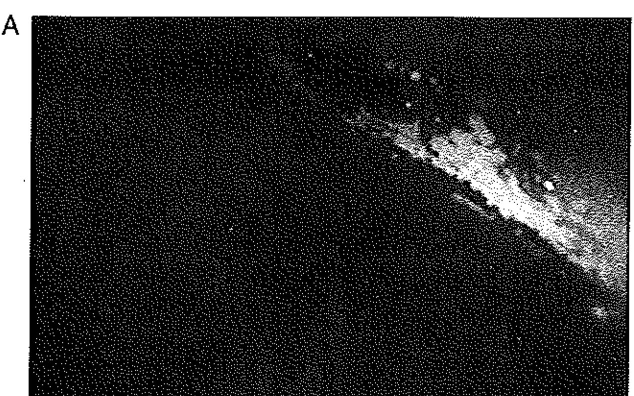
There are serious concerns with the utility and information content of some of the methods used to assess the biofilm resistance of biomaterials. If a biomaterial gives a positive zone of inhibition test, what does it mean? It means that the biomaterial contained an antibacterial agent, which it released in the moist environment of the surface of an agar plate, and the agent killed the planktonic bacteria that had been deposited on this same surface to produce a "lawn." The major parameter operative in the test is the diffusion of the antibacterial agent through the agar, or in the fluid on the agar surface, more than the effectiveness of the agent. A very effective agent would have a very small zone, if it moves slowly through agar, and a weak agent would have an enormous zone if it diffused well through agar, or if it diffused well through water and the plate was wet. The release kinetics of the agent from the biomaterial are those of a biomaterial on a moist agar surface, which is not a common use target for biomaterials. Flask tests, in which candidate biomaterials are suspended in a medium that is simultaneously inoculated with planktonic bacteria, are equally naive. If the biomaterials release enough of an antibacterial agent in the first few minutes of the test, all of the planktonic cells will be killed, the medium will be sterile, and there will be no organisms to colonize the biomaterial. So an antibiotic-releasing biomaterial that "dumps" all of its antibacterial agents in a few minutes will emerge from this test with flying colors! If the bacteria used in these relatively crude tests are lab-adapted by repeated subculturing, and thus defective in both antibacterial resistance and adhesion to surfaces, the biomaterials will be seen as promising. Yet both these tests are inappropriate and tend to lead to biomaterials that fail in biofilm resistance in animal and clinical trials.

The most appropriate tests are ones that mimic the conditions in the systems in which the biomaterial is targeted for use. If the biomaterial will be subjected to flow, or even to fluid exchange, the test should include these parameters. If the biomaterial will be used in a body fluid, such as blood or urine, an accurate simulation of that fluid should be used in the test, and the bacteria supplying the challenge should be adapted to the fluid. Bacteria used to challenge the putative biomaterial should be "wild" strains, recently isolated from clinical sources, and the challenge should come from fast-growing exponential-phase cells supplied by a chemostat, and not from variable cells from a "batch" culture. All of these parameters are best delivered using a flow cell, fed by a chemostat, and one of the most popular designs for such a system is given in Stoodley *et al.* (2001a). The flow cell also allows direct observation of large areas of the surface of the biomaterial, especially if the flow cell is mounted on the stage of a confocal scanning laser microscope (CSLM), and surface colonization can be monitored continuously (Cook *et al.*, 2000). Because the confocal microscope can resolve bacteria on opaque surfaces, and because this microscope allows us to examine living hydrated preparations, we can actually see the first microbial cells that adhere to biomaterial surface (Fig. 2A). If the adherent cells survive, they will initiate biofilm formation, and the adherent cells will gradually form matrix-enclosed communities (Fig. 2B) within which the cells will be separated by 3–5 μm of slime-filled space. The formation of biofilms requires that the adherent cells must be alive, so the observation of structured biofilms (Fig. 2C) on a surface that makes antibacterial and antibiofilm claims could have unfortunate clinical consequences (Cook *et al.*, 2000).

The observation of cells on the surface of a biomaterial is not necessarily negative data, especially if the cells are not very numerous and have not formed biofilms, because some antibacterial agents kill "incoming" planktonic cells and the dead cells remain on the surface (Fig. 2D). Even though we prefer biomaterials whose active agents kill "incoming" bacteria and do not retain these dead cells on the surface, agents that kill and retain bacterial cells are of some interest. For this reason, one of several available live/dead probes to ascertain the viability of adherent bacterial cells on biomaterials can be used. All of these methods give "snapshot" data, in that they necessitate the termination of colonization and the removal of biomaterial from the test system, but they yield accurate and useful data. The BacLite Live/Dead probe (Cook *et al.*, 2000) distinguishes live cells on the basis of membrane integrity, and live cells stain green while dead cells stain red (Fig. 2B). Living cells can also

be distinguished from dead cells on the basis of their respiration using tetrazolium salts that produce an orange color when they are reduced by metabolically active organisms. In very practical terms, biomaterials set up in flow cells can be exposed to realistic flowing fluid containing active cells of a "wild" strain of potential pathogen, and the resultant colonization of its surface can be monitored by CSLM. When adherent bacterial cells are few and intermittent, live/dead data are not germane and the test can run for days without interruption. When the biomaterial becomes heavily colonized, by biofilm-forming bacteria that stain as living cells in the live/dead assay, the material is designated as having exceeded its period of colonization resistance. Because a layer of surviving cells provides

A



B

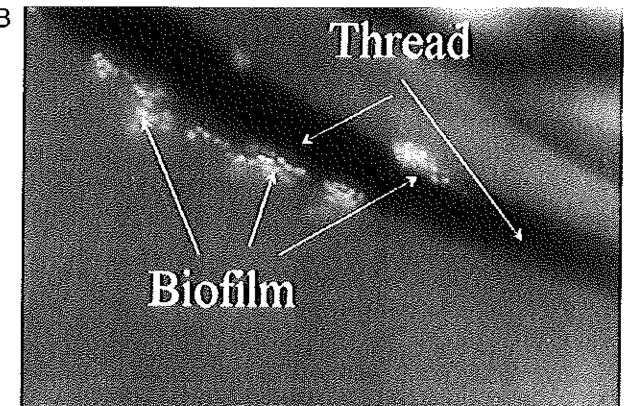
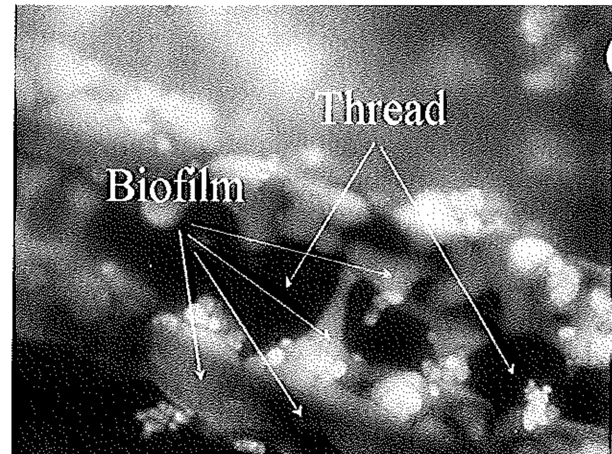


FIG. 2. These micrographs are confocal scanning laser microscope (CSLM) images of living unfixed biofilms formed on individual fibers of the clothlike material used to form the sewing cuffs of mechanical heart valves. (A–C) Images of biofilms formed when fibers of a silver-coated medical device were exposed to planktonic cells of *Staphylococcus epidermidis*, in a flow cell, as described in Cook *et al.* (2000). The biofilm seen in (A) was formed on the silver-coated fiber after 24 hours exposure, and special staining with the Live/Dead BacLite probe (B) showed that live cells (green) outnumbered dead cells (red) by a wide margin. When these same fibers were exposed to this planktonic challenge for 48 hours, as seen in (C), a mature biofilm had formed and individual bacterial cells were seen to be buried in large aggregates of matrix material. (D) Large numbers of dead cells (red) as seen in a Live/Dead stain of an effective antibacterial biomaterial that killed sessile cells of *S. epidermidis* as they attempted to colonize its surface. This putative biomaterial was, unfortunately, too toxic for clinical use.

C



D

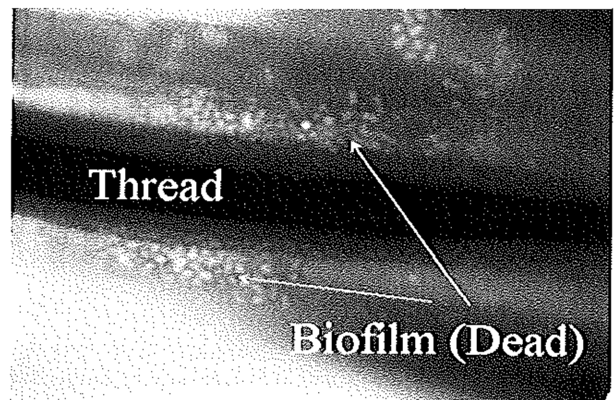


FIG. 2.—continued

an optimum surface for further bacterial colonization, biomaterials that have exceeded their colonization-resistant period tend to accrete biofilms and fail very rapidly, as in the case of the materials seen in (Fig. 2A–C).

Although direct observations are obviously the "gold standard" in tests of the resistance of biomaterials to bacterial colonization, CSLM microscopes are complex and relatively expensive. Many groups, including the CBE, have struggled with the inherent difficulties of removing sessile biofilm bacteria from colonized surfaces, and of enumerating these cells by standard microbiological techniques. The technique usually used is called "scraping and plating," and it involves breaking up the clumps of bacteria in the biofilm fragments and spreading these dispersed cells on the surface of an agar plate so that each cell gives rise to one colony when the plate is incubated. Many difficulties can contribute to the inaccuracy of this method, but all can be resolved or rationalized if all of the steps are monitored by microscopy. First, some cells may be left on the surface by the scraping method, which must be calibrated by microscopic examination of the scraped surface to see how many cells remain. Second, the scraped material must be homogenized or sonicated to break up clumps of bacteria in the scraped biofilm fragments to ensure that each living bacterium

gives rise to one colony on the agar plate. Sonication may kill some cells, so it is important to calibrate the sonication time for each type of biofilm, until microscopy shows that the resultant suspension is mostly single cells, and most of these cells are alive. Cells that have assumed the biofilm phenotype may not grow well on the surfaces of agar plates, when they have been removed from the sessile community and suspended in an unfamiliar milieu, so that "committed" biofilm cells may not grow well on plates. When scraping and plating are used without the calibrations discussed above, this method can yield data that are 4 log values lower than the bacterial numbers seen by direct microscopy. However, the scrape and plate method can yield accurate and consistent data when it is properly calibrated by microscopy, and the first biofilm method using this enumeration technique has now been accepted as ASTM Method E 2196-02.

Potential Agents for the Control of Microbial Colonization of Biomaterials

The continued search for biomaterials that resist microbial colonization by virtue of their inherent surface properties may still give us valuable information on minimizing adhesion, but we should use modern biofilm methods to conduct this research. The search may be somewhat quixotic, because the large sums of money spent to date have yielded only a handful of materials of questionable utility. Thus, candidate materials should be subjected to *sine qua non* testing in realistic systems early in their development cycles.

The strategy most commonly used in current antibacterial biomaterials is the incorporation of conventional antibiotics into the material, with the objective of killing incoming planktonic cells, before they can adhere and initiate biofilm formation. Although there are some successful applications of this basically sound approach, the problem lies in the typical release kinetics of such materials. Most of the agent is liberated in the first short time period, while the remainder is made available slowly and over a long period of time, thus exposing the bacteria to sublethal antibiotic concentrations that may stimulate the development of resistant strains. Antibiotics with very specific targets, such as penicillin (penicillin binding protein) and ciprofloxacin (DNA gyrase), may induce bacterial mutations that render the mutants dramatically more resistant to the agent in question. For this reason many biomaterial designers have chosen to use multitarget antimicrobial agents (e.g., chlorhexidine), because mutants are only marginally more resistant, but most of these nonspecific compounds are not approved for systemic use in humans.

This quandary of balancing efficacy against the danger of acquired bacterial resistance does not affect the large cohort of bacterial manipulation molecules that is currently moving briskly toward the biomaterials market. Some of these biofilm control molecules are specifically targeted on quorum-sensing mechanisms, such as RIP on the TRAP two-component system in gram-positives and the brominated furanones on the AHL systems of gram-negatives, but others are simply known to affect biofilm formation. It is now obvious that signal control of bacterial behavior is a subtle process, in which many factors interact to control a network of activities, so we do not expect to find a simple ON/OFF switch that controls biofilm development. Nonetheless, we have found several signal blockers that inhibit biofilm formation and sharply reduce pathogenicity in animal models. The pivotal concept is that bacteria in contact with a biomaterial would be prevented from forming a biofilm on its surface, would be "locked" in the planktonic phenotype, and would be killed by host defenses (antibodies and activated leukocytes) and any antibiotics that might be present. Balaban *et al.* (2003b) have shown that the RIP analog of the RAP signal, which controls biofilm formation in all species of the *Staphylococcus* genus, prevents biofilm formation by these organisms on subcutaneous Dacron implants in rats. When specific antibiotics were administered to these test animals, while the challenging bacteria were locked in the planktonic phenotype, no live cells could be recovered from the biomaterial surfaces of the surrounding tissues. This approach to the control of device-related infections is rational and much more focused than conventional antibiotic therapy, and its proponents visualize a whole new series of species- and genus-specific agents that will control both biofilm formation and toxin production. Biofilm specialists take comfort from new observations that plants protect themselves from pathogenic biofilm colonization by the use of similar signals and signal blockers, and millions of years of coevolution have not produced resistant bacterial strains (Stoodley *et al.*, 2002).

Delivery of Biofilm Control Agents at Biomaterial Surfaces

The more candid among the surgeons who install medical devices have confided that some operations proceed perfectly, and the device slides into place rapidly and smoothly, while others take longer and "just don't feel right." It is these later cases that sometimes develop biofilms and device-related infections because the skin and environmental bacteria present near the biomaterial surfaces will have time to adhere and to initiate biofilm formation. Killing the planktonic bacteria before they have time to initiate biofilm formation is the objective of many programs in this area, and this can be accomplished by three general strategies:

1. Systemic antibiotic therapy that produces bactericidal concentrations in the body fluids in the operative field
2. Release of antibiotics and other bacterial manipulation molecules from the biomaterials to produce high and sustained concentrations of the agent in the immediate vicinity of the device
3. Irrigation and other techniques that deliver antibiotics to the biomaterial surface after the device is installed, before the operative wound is closed

Most surgeons use systemic antibiotic therapy in the perioperative time fame, and most also use this strategy in subsequent operations (including dental procedures) if a device has recently been installed and might not be fully epithelialized. The simplest manifestation of the antibiotic-releasing biomaterial strategy is a class of materials that can be "loaded," like a sponge, by soaking them in a solution of the antibiotic in

question immediately before the device is installed. This tactic has backfired in many cases in which bacteria resistant to the antibiotic have started to grow in the fluids of the vessel in which the device is being soaked, have formed a biofilm on its surface, and have caused serious infections. We must be fastidious in the installation of medical devices, in that no preformed biofilms must ever be implanted, because preformed biofilms automatically give rise to biofilm infections (Ward *et al.*, 1992). It is equally important that the surfaces of biomaterials be absolutely clean, because any residue of dead biofilm or other organic accretion radically increases the colonization of that surface by planktonic bacteria and increases the chance of a biofilm infection. Also, some gram-negative bacterial cell-wall residues (endotoxin) can lead to inflammatory reactions.

The most commonly employed strategy in infection prevention is the impregnation of biomaterials with recognized antibacterial ions or molecules, with the intent of killing planktonic bacteria before they can colonize the material concerned. Whenever an ion or molecule is loaded into or onto a polymer, Fick's laws dictate that large amounts will be released in the early time frame, and that the release will taper off during the long period in which the concentration in this reservoir is being depleted (see Chapter 7.14). Many biomaterial designers have become adept at manipulating the initial concentration of the agent and the release rate, but we are always left with certain "spectra" of concentrations and polymer configurations that require choices of the "Hobson's" variety (that is, no real choice). If we put a large amount of ionic silver on a surface and release it quickly, we are flirting with silver toxicity. If we put a very stable form of silver on a surface and silver ions are released very slowly, bacteria will grow all over the silver coating (Fig. 2C) just as they colonize metallic copper (McLean *et al.*, 1993) if few copper ions are present. Westaim Biomedical, Inc., has introduced an exciting new silver coating for burn bandages that uses a galvanic combination of silver and copper and releases silver and copper ions at a steady rate that control bacterial colonization for a useful period of time. The galvanic potentials set up by these side-by-side "lakes" of copper and silver may also have an inhibitory effect on bacterial adhesion, biofilm formation, and the inherent resistance of biofilm bacteria to antibacterial agents.

Because many modern antibiotics are much less toxic than metal ions, the release patterns of these agents from biomaterials pose a different problem. We can obtain high and very effective concentrations of antibiotics, in the immediate vicinity of devices, for lengths of time that have already been found to be effective in certain clinical situations. These biomaterials are useful, but we cannot control the low-level release of the agent for months or years after this effective time frame. This produces a prolonged period in which the agent is present at a sublethal concentration, near the device and sometimes in the whole body, and raises the specter of the development of acquired antibiotic resistance in many potentially dangerous bacterial species. A new development at the University of Washington Engineered Biomaterials (UWEB) Engineering Research Center has addressed this problem. Biomaterials can be coated with a molecular "skin," a self-assembled surface layer, that completely contains molecules loaded into an underlying plastic and can be temporarily deranged (by ultrasonic energy) to yield a controlled release of the molecule in question (Kwok *et al.*, 2001). This coating has been used to deliver insulin, in controlled pulses, and the UWEB and the CBE are currently adapting this ultrasonic-sensitive coating for the controlled release antibacterial agents (and bacterial manipulation agents) from implanted biomaterials. High concentrations of these agents could be released at the surfaces of medical devices, perioperatively or at any preliminary signs of device-related infection, and no further release would occur if the coating was not stimulated ultrasonically.

Many surgeons have expressed an interest in being able to sterilize a medical device *in situ*, after it has been installed and before the operative wound is closed. This strategy is rational, because the device is accessible, and any planktonic bacteria present in the operative field will initiate colonization of the surface of the device if they are not killed or manipulated to preclude biofilm formation. Irrigation with antibiotics is presently used, biofilm-inhibiting signals and signal blockers are being developed, and this *in situ* procedure may be the perfect opportunity for the use of ultrasonic energy and/or DC electric fields to enhance the killing of bacteria in nascent biofilms. We can readily contaminate sham animal operations with bacteria and determine the efficacy of several possible procedures for *in situ* sterilization by using the live/dead probe to examine the surfaces of devices recovered at intervals after the procedure. The final proof of the efficacy of the *in situ* sterilization of medical devices would be obtained in clinical tests, in which significant reductions in device-related infections could be documented.

SUMMARY

The concept that has been distilled from decades of clinical experience with device-related infections has now been fused with the biofilm concept, which states that bacteria live predominantly in matrix-enclosed protected communities. This fusion is reassuring, and intellectually satisfying, because it asserts that bacteria employ the same strategy for survival in the human body that they use in all other ecosystems. The mental image that is invoked is one in which a biofilm forms on the surface of a biomaterial, and that it has all of the properties of the sessile communities that predominate in industrial and environmental systems. Its cells express the distinct biofilm phenotype: They are resistant to antibacterial agents and to uptake by phagocytes, most of them grow slowly and adopt many different metabolic strategies, and they detach planktonic cells and biofilm fragments in a programmed manner. The clinical consequences of this mode of bacterial growth are that antibiotics are useful in treating acute planktonic exacerbations, but that these agents cannot clear the biofilm reservoir on the biomaterial, and the device must usually be removed to resolve the infection.

As we fuse the device-related infection concepts with biofilm concepts, we can discard several older methods that have been used to assess the efficacy of putative antibacterial biomaterials. New biofilm methods allow us to visualize bacteria on opaque surfaces, to determine the viability of these organisms, and even to identify the cells by genus and species. We realize that freshly

isolated "wild" bacteria adhere avidly to plastic and metal surfaces that have been "conditioned" by exposure to body fluids, and we are more sanguine about claims that biomaterials can resist colonization by virtue of their surface properties alone. New technologies that deliver antibiotics in controlled and effective doses, at the surfaces of novel biomaterials, offer a solution to the problem of bacterial resistance induced by sublethal concentrations of these agents from "exhausted" biomaterials. The discovery that cells in biofilms communicate with each other by means of chemical signals brings to the medical biomaterials area a whole set of new molecules that can manipulate bacterial behaviors, such as toxin production and biofilm formation. These bacterial manipulation agents, many of which control biofilms in natural environments, have already been shown to "lock" targeted bacteria in the planktonic phenotype and to make them susceptible to conventional antibiotics and host defense factors. Physical treatments (e.g., ultrasonic radiation and DC electric fields) that make biofilm cells susceptible to antibacterial agents are also made available for use in medical systems, because of the fusion of the biofilm field with the study of device-related infections. This synthesis of concepts may accelerate the development of biomaterials that truly resist bacterial colonization, and these materials may allow us to build medical devices that will be substantially less susceptible to device-related infections.

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