

Chapter 7

Osteomyelitis

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7.1 Introduction

Osteomyelitis is defined as an infection of the bone. The pathogenesis of osteomyelitis has been delineated clinically and several types of can be distinguished and classified according to the source of the infecting microorganism (i.e., hematogenous or contiguous focus) and the vascular capability of the infected individual (i.e., with or without generalized vascular insufficiency) (Lew and Waldvogel 2004).

7.1.1 Anatomy and Function of Bone

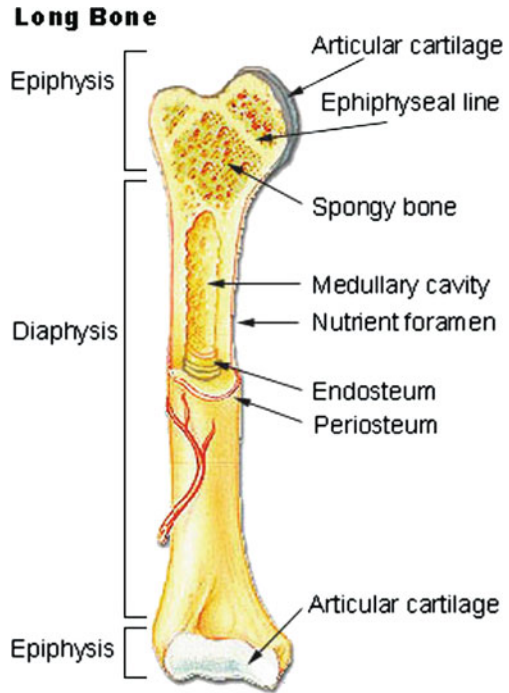
In order to fully understand the pathogenesis, treatment, and prevention of osteomyelitis, knowledge of the structure and function of bone is necessary. The functions of bone in the body are (i) to support the body's mass against gravity, (ii) act as a shield against blunt or penetrating trauma for certain vital areas of the body (notably the heart (sternum) and brain (skull)), and (iii) to provide a solid frame against which muscles can pull in order to provide mobility. These functions have, through the action of natural selection, dictated the structure of bone. Bone must be hard and yet not so much that it is brittle; a little flexibility is necessary before breakage occurs.

The long, straight section of a long bone is called the *diaphysis*; the two ends are termed *epiphyses* (Fig. 7.1). When bones are growing, the junction between the epiphyses and diaphysis contains an actively growing cartilage plate called the *epiphysial plate*; this is where most bone growth and elongation occurs. Such elongation occurs through the generation of additional cartilage, forcing the two ends apart; this new cartilage will be replaced eventually by new bone. The outer surface

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Fig. 7.1 Gross anatomy of a long bone. Taken from <http://training.seer.cancer.gov/anatomy/skeletal/classification.html>



of the epiphyses – where they meet other bones in a joint – are covered with *articular cartilage*, which functions to provide an almost frictionless bearing surface for joint movement.

The diaphysis of a long bone is hollow. Its outer layer is composed of *compact bone*, a hard layer of bone. A hollow cavity within this outer layer is known as the *marrow cavity*. The contents of the marrow cavity alter with age: in children it contains red marrow, a site of red blood cell production, whilst in adults this has been replaced with yellow marrow. Yellow marrow is a fatty tissue which no longer supports production of red blood cells. Epiphyses also are covered with a layer of compact bone, albeit a thinner layer than the diaphysis. Underlying this in the epiphyses is *spongy bone*, a network of strengthening crossbeam-like bony plates and rods called *trabeculae*. Within and formed by this network are a multitude of small spaces which in some bones contain red marrow. The interior of many irregular (short or flat) shaped bones also contains spongy marrow.

Microscopically, compact bone has a layered structure consisting of, directly beneath the outer surface, several rings known as the *circumferential lamellae*. These extend around the entire circumference of the bone. Deeper into the lumen of the bone are located cylinder-shaped structural units known as the *Haversian systems* (Fig. 7.2). Each of these systems is centered around a *Haversian canal*, within which are located nerves and blood vessels. Running perpendicular to the Haversian canal are *Volkman's canals*; these provide a conduit for nerves and blood vessels

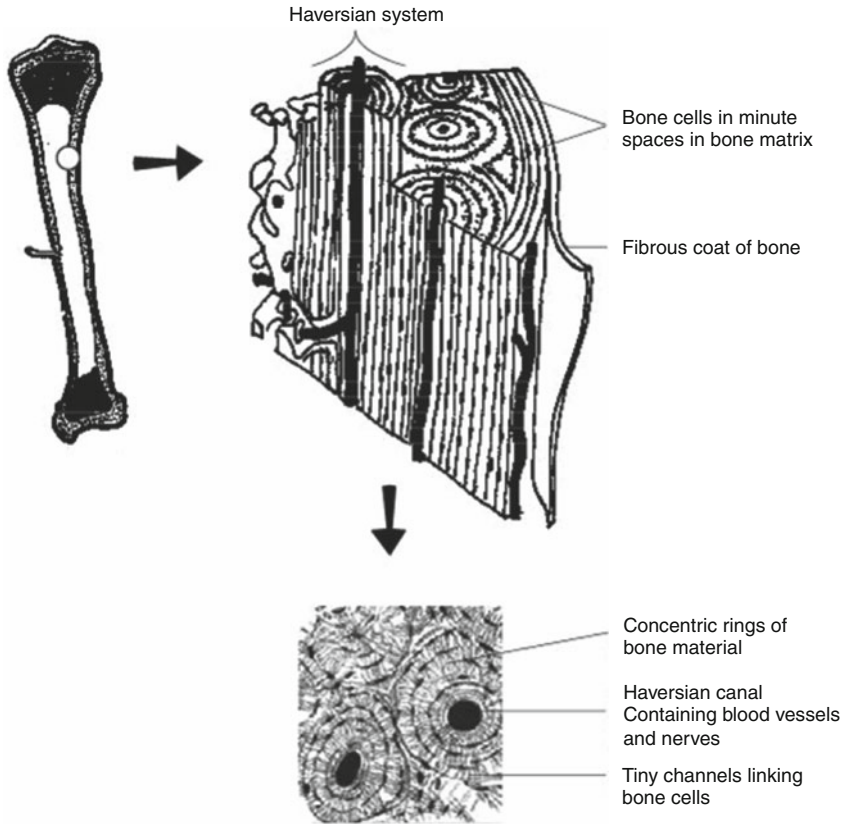


Fig. 7.2 Haversian systems of the compact bone in long bones. http://en.wikibooks.org/wiki/Anatomy_and_Physiology_of_Animals/The_Skeleton

going to and from the periosteum to the Haversian canal. The *Haversian lamellae* lie around each Haversian canal; between each lamellum lies the lacunae within which are located the *osteocytes*, inactive bone-producing cells trapped after they laid down bone. *Canaliculi* link each lacunae to its neighbors and function to transport nutrients and waste materials. The cylindrical shape of the Haversian systems renders the gaps between them triangular; these spaces are filled with *interstitial lamellae*, composed of material that previously formed Haversian systems. This material is continually being destroyed and rebuilt, giving rise to the interstitial lamellae.

Surrounding the bone is a sheet of connective tissue known as the *periosteum*. Its inner osteogenic layer gives rise to osteoblasts; these cells, after being trapped by the bone they have created are the source of the osteocytes. Osteoblasts reside on the surface of the bone where they manufacture the protein and mineral matrix of new circumferential lamellae. Other cell types within bone include osteoclasts; these large multinucleated cells, formed from monocytes, function to dissolve and

resorb bone. A balance between the action of osteoblasts and osteoclasts is vital for the continuing structural integrity of bone.

7.2 Types and Pathogenesis of Osteomyelitis

Two basic types are recognized, these being hematogenous and contiguous focus osteomyelitis. The primary difference between these is the source of the infecting microorganisms; in hematogenous osteomyelitis the infective agent originates in the bloodstream. Contiguous focus osteomyelitis can result from either direct introduction of the infective agent to the bone (as in traumatic injuries) or from an adjacent soft tissue infection (Brady et al. 2008).

7.2.1 Hematogenous Osteomyelitis

Hematogenous osteomyelitis accounts for *circa* 20% of the total cases of osteomyelitis. Primary hematogenous osteomyelitis is caused by direct seeding of bone from an infective agent present in the vasculature. This type of osteomyelitis is more predominant in infants and children; however, it is not unknown in the adult population (Lew and Waldvogel 2004). In adult individuals, hematogenous osteomyelitis is more usually caused by secondary infection; bacteria gain access to the bloodstream and colonize distal bone. Reactivation of a dormant focus of hematogenous osteomyelitis that an individual suffered during infancy or childhood and “arrested” can also be the source of a hematogenous osteomyelitis. Hematogenous osteomyelitis is most common in the distal tibia; the lesion is usually close to the metaphysis. This type of osteomyelitis is usually located in either a long bone (i.e., tibia, ulna, radius, etc.) or in a vertebra and is most often caused by a single etiologic agent. Symptoms at presentation are usually one or more of malaise, lethargy, fever, tenderness (at or above the site of infection) and a decreased range of motion in the affected limb (Carek et al. 2001).

The anatomy of the metaphyseal region, where the blood flow is sluggish and disordered, explains why the long bones (tibia, femur) are most frequently involved in osteomyelitis (Shirliff et al. 1999). This slowing of blood flow allows bacteria to settle and initiate colonization with a resultant inflammatory response. Minor trauma likely predisposes the infant or child to infection by producing a small hematoma, vascular obstruction, and a subsequent bone necrosis that is susceptible to inoculation from a transient bacteremia (Morrissy and Haynes 1989). Generally, an acute infection will develop within 2 weeks of disease onset (Carek et al. 2001); typically this results in local cellulitis and a breakdown of leukocytes, increased bone pressure, decreased pH, and decreased oxygen tension. The end result of the action of these physiologic factors is compromise of the circulation within the bone and further spread of infection. In infants, infection may spread to the joint surfaces through the vascularized growth plate (Jackson and Nelson 1982). However, in children greater than 1 year old, the growth plate lacks capillaries and the infection tends to be confined to the metaphysis and diaphysis. The joint is thus usually

spared unless the metaphysis is intracapsular. Infants and children suffering from hematogenous osteomyelitis usually have normal soft tissue enveloping the infected bone and are able to mount an efficient metabolic response to the infection. They also have the potential to absorb large sequestra and generate a significant response to the infection in the periosteal region. Because of this resorbing ability, if appropriate antimicrobial therapy is begun before the onset of significant bone necrosis, the younger patient has an excellent probability of halting or resolving the infection without surgical intervention (Berendt and Byren 2004).

Chronic hematogenous osteomyelitis can be said to occur beginning several weeks to months after onset of the disease (Carek et al. 2001). The existing cortex is usually viable. The *involucrum* – an area of live, encasing bone surrounding infected dead bone within a compromised soft tissue envelope – is the hallmark sign of chronic osteomyelitis (Mader et al. 1980). The involucrum contains necrotic marrow and endosteal bone. In normal bone, necrosis is a vital part of the cycle as it signals granulation tissue to resorb dead bone at the junction of living and dead tissue. Some of the dead cortex will usually detach from the living bone and form a sequestrum. After complete separation, or sequestration, the dead bone is eroded by granulation tissue and destroyed. However, in some cases the area of dead bone is too large to be resorbed, or the host response is compromised. This can lead the process of resorption to be inadequate, and may cause the formation of an involucrum. The involucrum affords mechanical continuity and assists in maintenance of function during healing. Involucra have an irregular surface and often have holes through which pus may move into the surrounding soft tissues and eventually drain to the skin surface, forming a draining sinus tract (Mader et al. 1996). The purpose of the involucrum is to isolate the infection from the remaining healthy bone. Involucrum development occurs upon establishment of the infection, after fibrous tissue and chronic inflammatory cells surround granulations and dead bone. New bone forms, as a result of the vascular reaction to the infection, from the periosteum, endosteum, and cortex. Involucra may continue to increase thickening for weeks or months and eventually form a portion of, or in some cases, all of a new bone shaft.

Though the involucrum functions to contain the infection, decreases in vascularity and low oxygen tension due to the presence of this structure can lead to decreased effectiveness of the host response; chronic disease can then ensue. Dead bone functions as an inert surface for the attachment of bacteria and the formation of biofilm. This form of infection, coupled with the host's inability to resorb the dead bone, results in a very complicated disease to treat because bacteria in a biofilm are 50–500 times more resistant to antimicrobial agents than their planktonic, free-floating counterparts. Therefore, debridement (surgical removal of infected bone and/or surrounding soft tissue) is often necessary for these infections to resolve.

Between 2 and 7% of total hematogenous osteomyelitis cases are located within a vertebra (Tyrrell et al. 1999). Incidences of this type of osteomyelitis are increasing due to the rising proportion of the population composed of aging adults, who possess both risk factors for bacteremia and deteriorating spinal pathology (Berendt and Byren 2004). In vertebral osteomyelitis (as well as all other locations), polymorphonuclear leukocytes (PMNs) are present due to the acute inflammatory response

(see also [Chapter 12](#)). Degradative enzymes released from disintegrating PMNs, together with vascular ischemia and release of bacterial products, can cause an extension of the infection into the cartilaginous end-plate, disc, and/or proximal regions. Posterior extension of the infection is an especial difficulty as it can in some cases lead to abscesses in either the epidural or subdural spaces; in particularly serious cases, meningitis can result. Extension of the focus of infection anteriorly or laterally can lead to paravertebral, retropharyngeal, mediastinal, subphrenic, or retroperitoneal abscesses. Additionally, the rich venous networks within the bones of the spinal column can lead to efficient and rapid spread to adjacent vertebrae.

7.2.2 Contiguous Focus Osteomyelitis

In the past several years there has been a marked decline in hematogenous osteomyelitis with a concurrent rise in contiguous disease (Espersen et al. 1991). Although the term “contiguous focus” implies that the infection stems from an adjacent soft tissue infection, chronic contiguous focus osteomyelitis can also begin as an acute infection, with the microbes being directly inoculated into the bone at the time of trauma (Healy and Freedman 2006). Infection can also be spread by nosocomial contamination during preoperative or intraoperative procedures. The age distribution of contiguous focus osteomyelitis prevalence peaks in both the young and the elderly; infections occurring in younger individuals are usually a result of trauma and related surgery whilst in older individuals it is secondary for surgical procedures and decubitus ulcers. Also, if the osteomyelitis is secondary to a penetrating trauma then multiple microorganisms may be involved; they are termed polymicrobial infections.

Trauma contributes to osteomyelitis infections in several ways besides the obvious direct inoculation of bacteria through the skin barrier and into the soft tissues and bone beneath. Damage of any sort to tissue tends to cause a decrease in blood supply to the affected area, which itself can cause formation of necrotic areas of inert tissue. Bacteria are then able to bind to this essentially inert tissue surface and infection can be the unfortunate end result. Indeed, trauma has been shown to depress the immune and inflammatory responses to bacterial invasion. Degree of severity of tissue injury is thought to be correlated with risk of infection; the presence of bacteria within the tissues in a wound is not always sufficient in and as of itself for establishment of osteomyelitis (Ziran 2007).

7.3 Etiology of Bacterial Osteomyelitis

A number of bacteria of diverse genera capable of causing – or more correctly – have been recovered from cases of osteomyelitis. *Staphylococcus* spp. cause the majority of cases and are fully capable of causing osteomyelitis in individuals of any age and with functioning immune systems. Of course, other pathogenic microorganisms

osteomyelitis; these include *Enterococcus* spp., *Streptococcus* spp., *Pseudomonas aeruginosa*, *Enterobacter* spp., *Mycobacterium* spp., and various anaerobic and mycoidal species (specifically *Candida* spp.). Each of these pathogenic genera individually represents a very small minority of infections when compared to that represented by *Staphylococcus* spp. The immature or compromised immune status of the host is the primary cause of initial infection and development into a persistent and chronic osteomyelitis.

Hematogenous osteomyelitis is generally monomicrobial in nature, i.e., a single bacterial taxon is isolated from the infected region. Polymicrobial hematogenous osteomyelitis is rare (Lew and Waldvogel 2004). In younger individuals, aged under 1 year, *Staphylococcus aureus*, *Streptococcus agalactiae*, and *Escherichia coli* are most frequently recovered from infected bone, while in the child (ages between 1 and 18), *S. aureus*, *Streptococcus pyogenes*, and *Haemophilus influenzae* are the most common organisms isolated. After the age of four, the incidence of osteomyelitis from which *H. influenzae* is recovered decreases. However, the overall incidence of *H. influenzae* as a cause of osteomyelitis is decreasing because of the *H. influenzae* vaccine now given to children (De Jonghe and Glaesener 1995). In adults, *S. aureus* is the most common organism isolated (Shirtliff et al. 1999). Other pathogenic microorganisms associated with osteomyelitis include *Enterococcus* spp., *Streptococcus* spp., *Pseudomonas aeruginosa*, *Enterobacter* spp., *Mycobacterium* spp., as well as anaerobic and mycoidal species (specifically *Candida* spp.). Each of these, individually represents a small minority of infections. The immature or compromised immune status of the host is the primary cause of both initial infection and development into a persistent and chronic osteomyelitis infection by these other species. In hematogenous vertebral osteomyelitis, aerobic Gram-negative rods are sometimes found, with the urinary tract or intravenous drug use as the source of infection (Berendt and Byren 2004). *P. aeruginosa* and *Serratia marcescens* have a high incidence in intravenous drug users (Holzman and Bishko 1971, Sapico 1996). It should be stressed, however, that while these varied species have been known to cause the disease, *S. aureus* produces the vast majority of osteomyelitis infections in all age groups.

Contiguous focus osteomyelitis located within a vertebra is usually a polymicrobial infection from which anaerobic or facultative anaerobic are often isolated. Alternative sources of infection include the genitourinary tract, adjacent skin and soft tissue, respiratory tract, an infected intravenous line site, endocarditis, dental infection (see also Chapter 4), as well as sources not known (Sapico and Montgomerie 1979, Berendt and Byren 2004). Positive cultures are at present thought to be very important for diagnosis, since other conditions such as trauma and vertebral collapse may simulate infection. Typically multiple organisms are isolated from individuals suffering osteomyelitis secondary to a diabetic foot infection. These are typically two or more of: *S. aureus*, coagulase-negative *Staphylococcus* spp., *Streptococcus* spp., *Enterococcus* spp., Gram-negative bacilli, and various anaerobes (Calhoun et al. 1988b, Berendt and Byren 2004, Rao and Lipsky 2007). Aerobic Gram-negative bacilli are commonly present in a mixed infection (Calhoun et al. 1988b).

In contrast to hematogenous osteomyelitis, multiple pathogenic species are usually isolated from the infected bone in cases of contiguous focus osteomyelitis. Once more, staphylococci are involved in a majority of cases, with *S. aureus* and coagulase-negative staphylococci accounting for 75% of bacteria recovered from such infections (Mader et al. 1996). These data further reinforce the critical importance of the genus *Staphylococcus* in the pathogenesis of osteomyelitis. However, Gram-negative bacilli and anaerobic bacteria of various genera are also found, albeit at a lower prevalence, in these situations. The infection usually manifests within 1 month after inoculation of the organisms from trauma, surgery, or a soft tissue infection. Patients usually present with a low-grade fever, pain local to the site of infection, and sinus tract drainage.

7.3.1 *Staphylococcus spp.*

Staphylococci are by far the most common etiologic agent recovered from cases of osteomyelitis (Shirliff et al. 1999). The most important pathogen of this genus is without doubt *S. aureus*.

S. aureus is a Gram-positive, ubiquitous bacterial species. *S. aureus* is a normal commensal of the human nostrils; ca 20% of the population are permanently colonized with this bacterium, while a further 60% are transient carriers (Kluytmans et al. 1997). The presence of *S. aureus* alone will not usually lead to illness; however, if the mucosal or skin surfaces are breached and the microorganism gains access to the tissues beneath, serious infection can ensue (Fitzpatrick et al. 2005b). Due to the increasing participation of *S. aureus* in osteomyelitis and other types of infection (see below), its swift development of multiple-antibiotic resistance, and its predilection to move from an acute infection to one that is biofilm-mediated, persistent, chronic and recurrent, this pathogen continues to receive considerable attention. The virulence mechanisms by which this pathogen colonizes the host, evades and destroys the immune response, and persists are outlined below.

7.3.1.1 Virulence

Staphylococcus spp. have been shown to be the causative agent of a plethora of infections [e.g., tropical pyomyositis, lower respiratory infections (pneumonia), superficial skin infections (boils, sties and carbuncles), localized abscesses, endocarditis, toxic shock syndrome, serious skin infections (furunculosis), food poisoning, bacteremia, empyema, pyopneumothorax, and exfoliative diseases] and are by far the etiologic agent isolated most commonly from cases of osteomyelitis. Therefore, it is unsurprising that the most important pathogen of the genus, *S. aureus*, has evolved a wide variety of virulence products and mechanisms in order to cause disease. The pathogenesis of staphylococcal infections is multifactorial and it is difficult to determine the precise role of any given factor in infection. Most of the virulence factors whose function is known appear specifically adapted to persistence, immune evasion, and infection within the host. Staphylococcal products

with a role in infection can be categorized as those responsible for (i) adherence, (ii) direct host damage, or (iii) immunoavoidance. There exist also a number of enzymes and extracellular proteins whose role in virulence is at present unclear. Staphylococcal virulence factors have a specific role in the colonization and infection process in osteomyelitis; their expression is coordinated throughout the various stages of infection. Therefore, the differential regulation of these virulence factors due to staphylococcal population levels and environmental factors is vital for successful colonization and establishment of infection.

S. aureus produces a large number of extracellular and cell-associated products that contribute to virulence and persistent infection. Most of these seem to be specifically adapted to survival and infection within the host. During early exponential growth when cell density is low, proteins that promote adherence and colonization (such as fibronectin binding protein, protein A, staphylokinase, and coagulase) are expressed. When cell growth reaches high densities, production of the adherence and colonization factors is suppressed, while secreted toxins and enzymes are expressed [such as enterotoxins B, C and D, epidermolytic (exfoliative) toxin A, α , β , and δ hemolysins, serine protease, nuclease, type 5 capsular polysaccharide, clumping factor, leukocidin, phosphatidyl-specific phospholipase C, fatty acid modifying enzyme, lipase, hyaluronate lyase (hyaluronidase), and toxic shock syndrome toxin (TSST) 1]. These proteins are produced after exponential growth in planktonic, batch culture has ceased (i.e., the culture has entered stationary phase), and are known to cause damage to host tissues, thus obtaining nutrients for pathogen growth and dissemination.

The expression of most of these staphylococcal products is under partial or complete control of the staphylococcal accessory regulator (*sar*) and the accessory gene regulator (*agr*) system. During early logarithmic growth, a protein encoded by *rot* (repressor of toxins) inhibits the expression of *agr*-activated virulence factors (McNamara et al. 2000). Once activation of the *agr* and *sar* regulatory loci occurs during late exponential phase, there is an increased transcription of an *agr* regulatory RNA molecule known as RNAPIII (Balaban and Novick 1995). RNAPIII blocks transcription of surface protein genes and upregulates transcription of genes encoding extracellular pathogenicity factors. In this way, *S. aureus* is able to sense when its population density has increased to the point where colonization has been successful. One of the major mechanisms by which *S. aureus* evades clearance by effector cells and molecules of the immune system is by formation of *biofilm*.

7.3.1.2 Adherence

For successful initiation of biofilm formation and infection, any pathogen must colonize the target tissue; the first step in this process is adherence. *Staphylococcus* spp. possesses a large number of adhesins for host proteins that allow adherence to the extracellular matrix in bone. These are known as “microbial surface components recognizing adhesive matrix molecules” (MSCRAMMS) (Herrmann et al. 1988, Yacoub et al. 1994, Ryden et al. 1997). Some host matrix proteins and their functions are fibronectin and laminin (adherence proteins), elastin (imparts elastic properties),

collagen (structural support), and hyaluronic acid (a glycosaminoglycan that is rich in the joints and the matrix and provides cushioning through hydration of its polysaccharides). A number of bone or joint-specific matrix proteins are recognized by MSCRAMMS. These include osteopontin (a soluble phosphoprotein that acts as a cytokine and osteoclast attachment protein and is needed for bone injury repair and remodeling), bone sialoprotein (interacts with osteoblasts and acts as a nucleator for calcium hydroxyapatite formation), and vitronectin (an adhesive glycoprotein involved in adhesion regulation and the coagulation, fibrinolytic, and complement cascades; also allows for bone resorption when bound to osteoclasts). Eight adhesin genes have been determined and include genes encoding fibrinogen binding proteins (*fib*, *cfIA*, and *fbpA*) (Boden and Flock 1994, McDevitt et al. 1994, Cheung et al. 1995), fibronectin binding proteins (*fnbA* and *fnbB*) (Jonsson et al. 1991), a collagen receptor (*cna*) (Patti et al. 1992), an elastin binding protein (*ebpS*) (Park et al. 1996), and a broad specificity adhesin (*map*) that mediates low level binding of several proteins including osteopontin, collagen, bone sialoprotein, vitronectin, fibronectin, and fibrinogen (McGavin et al. 1993). Also, this microorganism has been shown to possess a number of other host protein-binding receptors in which the genes have not yet been determined. These include a laminin (52 kDa) (Lopes et al. 1988), a lactoferrin (450 kDa) (Naidu et al. 1992), and a transferrin (42 kDa) (Modun et al. 1994) binding protein. The staphylococcal receptor that binds laminin may be used in extravasation (Lopes et al. 1985). These receptors were found in *S. aureus* but were absent from the noninvasive pathogen *S. epidermidis* (Lopes et al. 1985). The lactoferrin and transferrin receptors bind to these host iron acquisition proteins and may be used as adhesins and/or as iron acquisition mechanisms. In addition, *S. aureus* expresses a 42-kDa protein, Protein A, which is bound covalently to the outer peptidoglycan layer of their cell walls. This adherence protein binds to the host platelet gC1qR (a multifunctional, ubiquitously distributed cellular protein, initially described as a binding site for the globular heads of the complement complex C1q) (Nguyen et al. 2000). Therefore, Protein A may be able to promote adhesion to sites of vascular injury and thrombosis and has been implicated as an important colonization factor. Protein A production is repressed by the *sar* locus via both RNAIII-dependent and independent mechanisms during post-exponential phase growth (Cheung et al. 1997). This protein is also associated with *S. aureus* immunoavoidance (see below). Many of these and other staphylococcal cell wall proteins must be exported out of the bacterial cell in order to interact with the extracellular environment. This export can be either a targeting process (the protein is exported and has binding domains for cell wall secondary polymers such as teichoic acids) or a sorting process (a C-terminal conserved amino acid sequence, LPXTG, that directs the export and covalent attachment to the peptidoglycan) (Navarre et al. 1996).

Increasing evidence supports the importance of staphylococcal surface components as virulence determinants by enabling initial colonization. In a number of studies, mutants in these receptors strongly reduced the ability of staphylococci to produce infection. In addition, there was significant binding of *S. aureus* to bone sialoprotein, fibronectin, and collagen type I in a mouse model, indicating

that adherence remains a key phase in the early stages of infection (Bremell and Tarkowski 1995). Expression of adhesins permits the attachment of the pathogen to cartilage. Inoculation of mice with mutants of the collagen adhesin gene showed that septic arthritis occurred 43% less often than in the corresponding wild type (Switalski et al. 1993). Collagen adhesin positive strains were also associated with the production of high levels of IgG and interleukin-6 (Switalski et al. 1993). In a murine septic arthritis model, inoculation of mice with mutants of the collagen adhesin gene showed that septic arthritis occurred 43% less often than in the corresponding wild type (Switalski et al. 1993). Also, vaccination with a recombinant fragment of the *S. aureus* collagen adhesin was able to reduce the sepsis-induced mortality rate to 13%, compared with 87% in the control group (Nilsson et al. 1998). However, the role of collagen adhesion of *S. aureus* as a major virulence factor has been recently questioned since approximately 30–60% of clinical isolates do not display collagen binding in vitro or the *cna*-encoded collagen adhesin (Thomas et al. 1999). Staphylococcal fibronectin-binding proteins (FbpA and FbpB) may have a major role in colonization during musculoskeletal infections. In a recent study, all of the tested clinical isolates ($n = 163$) contained one or both of the coding regions for these binding proteins and 95% of these strains had a comparable fibronectin binding capacity to that seen in a staphylococcal reference strain known to efficiently bind fibronectin (Peacock et al. 2000). In addition, an in vivo study of endocarditis in a rat model showed that mutants deficient for fibronectin-binding protein were 250-fold less adherent to traumatized heart valves (Kuypers and Proctor 1989). Also, *S. aureus* adherence to miniplates from iliac bones of guinea pigs was three times higher than the adhesin-defective mutant strain (Fischer et al. 1996). It is likely that fibronectin-binding proteins play an important role in bone and joint infections, especially those associated with initial trauma or implanted medical devices (Patel et al. 1987).

7.3.1.3 Staphylococcal Biofilm

Staphylococcus spp. within the host produces a multilayered “biofilm” community (Fig. 7.3). A biofilm is a modular community of microbes embedded within a host- and/or microbe-derived hydrated matrix of exopolymeric substances that exists at a phase or density interface. This interface is, in most cases, between a solid or semi-solid support [e.g., soft tissue or bone (Fig. 7.4) and a liquid medium (e.g., extracellular fluid, blood, mucin, etc.)] (Wimpenny 2000). Biofilm thickness can vary from a single cell layer to (more commonly in infections) a thick community of cells embedded within a thick polymeric matrix. Structural analyses have demonstrated that in vitro biofilms possess a sophisticated architecture in which microcolonies exist in discrete pillar or mushroom-shaped structures (Costerton et al. 1995). Between these structures, an intricate channel network provides access to environmental nutrients. It has been hypothesized that the development and maintenance of this phenotype may be mediated through the action of quorum sensing systems in biofilm-producing microbes (McLean et al. 1997, Stickler et al. 1998, Parkins et al. 2000, Singh et al. 2000).

Fig. 7.3 Scanning electron micrograph showing *S. aureus* microcolony (*dark arrow*) growing within bone. Scale bar represents 2 μm

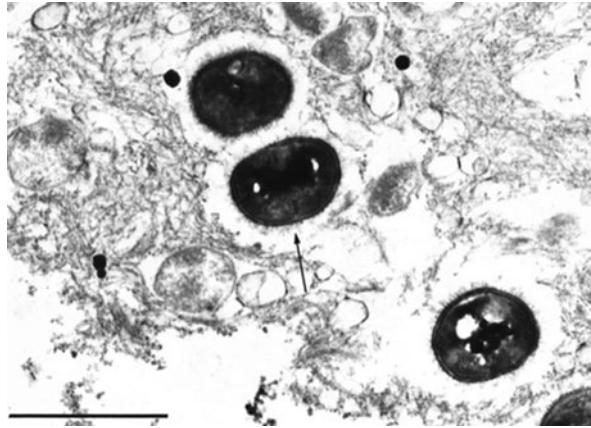
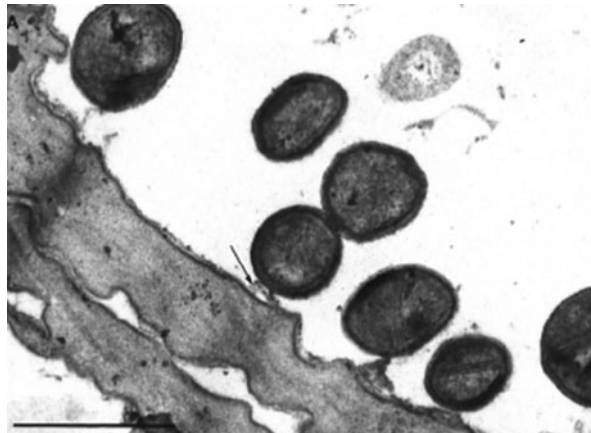


Fig. 7.4 Scanning electron micrograph showing *S. aureus* attaching to host bone, as indicated by the *dark arrow*. Scale bar represents 3 μm



By adopting this sessile mode of life, biofilm-embedded microbes benefit from a number of advantages over their planktonic counterparts. One such advantage is the capability of the extracellular matrix, or glycocalyx, to entrap and thus concentrate a number of environmentally derived nutrients, such as carbon, nitrogen, and phosphate (Beveridge et al. 1997). Another benefit to growing as a biofilm is facilitation of resistance to a number of removal mechanisms, for example, elimination by antimicrobial and/or antifouling agents, shear stress from fluid flow, host phagocytic clearance and opsonization by antibodies, and host oxygen radical and protease defenses (see also [Chapter 13](#)). This innate resistance to antimicrobial factors is mediated through a number of mechanisms including the very low metabolic levels and radically down regulated rates of cell division of the deeply entrenched microbes. While low metabolic rates may explain a great deal of the antimicrobial resistance properties of biofilms, other factors could add to the community's resistance capacity, adding to the cumulative effect. One such may be

the capability of the biofilm matrix to act as a “diffusion barrier” to slow down the penetration of some types of antimicrobial agent (Xu et al. 2000). For example, the reactive chlorine species (such as hypochlorite, chloramines, or chlorine dioxide) present in a number of antimicrobial/antifouling agents may be deactivated by reaction with the topmost parts of the biofilm before disseminating into the deeper community (De Beer et al. 1994). In another study, alginate (a component of *P. aeruginosa* exopolysaccharide) was shown to be able to induce α -helical conformation in antimicrobial peptides and likely entraps these peptides, preventing their diffusion into the biofilm (Chan et al. 2004).

Persistence in the host in the face of attack by the components of the immune system by clinical strains of *Staphylococcus* spp. is assisted through a number of properties of the glycocalyx. Since the normal phagocytic processes are devoted towards the removal of the glycocalyx and the implant, local immune deficiency and damage to adjacent host tissue can occur through an accumulation of immune effector molecules. Therefore, the energy and resources of the immune system that would normally be used to fight infection are subverted. Third, the glycocalyx may activate monocyte production of prostaglandin E2 to indirectly inhibit T-cell proliferation (Stout et al. 1992). Lastly, this glycocalyx has been shown to directly inhibit polymorphonuclear leukocytes (Ferguson et al. 1992).

An additional advantage conferred upon bacteria by the biofilm manner of growth is the potential for dispersion to distal sites via detachment from the community. Microcolonies, or parts of microcolonies, detach under the influence of mechanical fluid shear or through a genetically programmed response that mediates the detachment process (Boyd and Chakrabarty 1994). In the direction of fluid flow this detached population travels to distal regions of the host or water system to attach and begin anew biofilm formation in previously non-colonized areas. In addition, detachment and seeding of virgin surfaces may be accomplished by the migration of single, motile cells from the cores of attached microcolonies (Sauer et al. 2002). Therefore, this advantage allows an enduring bacterial source population that is resilient against antimicrobial agents and the host immune response, while simultaneously enabling continuous shedding to encourage bacterial spread. Thus a bacterial biofilm present within the body can act as a reservoir of pathogens that survive antibiotic administration and re-establish infection upon cessation of treatment.

The multilayered *S. aureus* biofilm is embedded within a polysaccharide glycocalyx (Gristina et al. 1985). This glycocalyx develops on devitalized bone (such as the involucrum) or medically implanted devices (Akiyama et al. 1993). The presence of implants are a predisposing factor in the development of infection since they are coated in host proteins soon after implantation, and this provides an excellent source of attachment for any bacteria remaining after debridement surgery (Herrmann et al. 1988, McDevitt et al. 1994, Gracia et al. 1997, Francois et al. 1998). Once attached, the bacteria can form the glycocalyx, or slime layer, which protects the bacteria from normal host defenses and systemic antibiotics (Gristina and Costerton 1984, Duguid et al. 1992, Darouiche et al. 1994, Oie et al. 1996). This pathogen usually grows in coherent microcolonies in the adherent biofilm that is often so extensive; the

underlying infected bone or implant surface is obscured. This layer slows the inward diffusion of a number of antimicrobials (Gristina and Costerton 1984, Duguid et al. 1992, Darouiche et al. 1994, Oie et al. 1996). In addition, the anaerobic nature of the deep layers of the biofilm results in a dramatically reduced growth rate and metabolic activity which is one of the main mechanisms allowing bacterial escape from the bactericidal and bacteriostatic effects of antimicrobial therapy (Anderl et al. 2003). The presence of persister cells also contribute to the biofilm mediated resistance (Lewis 2005). These cells are a metabolically inactive portion of the total population making this subset of the population resistant to antimicrobial agents. Upon cessation of antimicrobial therapy, the dormant state of persisters is reversible and the infection can be reactivated. Furthermore, those bacteria that survive antibiotic clearance often develop resistance to the impregnated antibiotic and regrow. This resistance has been clinically demonstrated by the isolation of small colony variants of *S. aureus* resistant to gentamicin from the wounds of patients treated with gentamicin-impregnated PMMA beads (von Eiff et al. 1997). Also, the glycocalyx displays antiphagocytic properties, thereby allowing the bacteria to evade clearance by the host's immune system (Ferguson et al. 1992, Dasgupta 1996, Shiau and Wu 1998), although more recent evidence has been contradictory (Leid et al. 2002). The glycocalyx is mainly composed of teichoic acids (80%) and staphylococcal and host proteins (Hussain et al. 1993). Host proteins such as fibrin are derived from the conversion of fibrinogen by the staphylococcal coagulase–prothrombin complex (see below) (Akiyama et al. 1997).

Another important component of biofilm produced by staphylococci is extracellular DNA (eDNA). eDNA as a component of the extracellular matrix of biofilms was noted first in *P. aeruginosa* (Whitchurch et al. 2002, Steinberger and Holden 2005, Allesen-Holm et al. 2006). Such is the importance of eDNA in *P. aeruginosa* infections that application of DNase concurrently with antibiotic therapy, is being used for treatment of cystic fibrosis patients (Shah et al. 1995, Gibson et al. 2003) (see also Chapter 14). The finding that DNase present on skin cells can lessen biofilm formation (Eckhart et al. 2007) reinforces the importance of eDNA to biofilm viability. Rice and co-workers have recently demonstrated that eDNA is important for biofilm formation and adherence in *S. aureus*, and that release of eDNA appears, at least in part, to be mediated by the function of *cidA*-encoded murein hydrolase (Rice et al. 2007). This gene product is a holin homologue and has been shown to play a role in cell lysis; thus it is believed that this gene allows lysis of *S. aureus* biofilm cells and release of their DNA into the extracellular milieu. Other cellular factors that may be involved in release of DNA include autolysins such as Atl or induction of prophages that lead to lysis (Webb et al. 2003). In *S. epidermidis*, the autolysin AtlE was shown to be important in release of chromosomal DNA and subsequent initial attachment during early biofilm formation, as an *atlE* mutant did not release DNA and had lower biofilm-forming capacity (Qin et al. 2007).

Biofilm produced by *S. epidermidis* contains both the capsular polysaccharide/adhesin (PS/A) that mediates cell adherence to biomaterials, and a polysaccharide intercellular adhesin (PIA) that may mediate bacterial accumulation into cellular aggregates (Heilmann et al. 1996, McKenney et al. 1998). PS/A is a

high-molecular-mass (>250 kDa) molecule composed of acid-stable polymers of β -1,6-linked glucosamine. PIA is a polymer of β -1,6-linked *N*-acetyl glucosamine residues with a molecular mass of less than 30 kDa that is synthesized through genes present on the intercellular adhesion locus (*ica*) (McKenney et al. 1998, Miyazaki et al. 1999). *S. aureus* and other *Staphylococcus* spp. also contain an *ica* locus and its deletion results in the loss of biofilm-forming ability (Miyazaki et al. 1999). The presence of glycocalyx was noted in 76% of *S. aureus*, 57% of *Staphylococcus epidermidis*, 75% of *Escherichia coli*, and 50% of *Pseudomonas aeruginosa* clinical osteomyelitis isolates (Alam et al. 1990).

In more recent work, however, the importance of PIA in biofilm formation and relevance to infection has been called into question. The *ica* gene cluster is not present in all *S. epidermidis* strains (Ziebuhr et al. 1997). Moreover, up to 30% of *S. epidermidis* biofilms have been found to be PIA-negative (Rohde et al. 2007); another study that focused on prosthetic hip infection found that only about one third of patients infected with *S. epidermidis* carried an *ica*-positive strain (Nilsson-Augustinsson et al. 2007). While the *ica* cluster is commonly found in *S. aureus* isolates, the importance of PIA production to virulence is uncertain. For example, in a guinea pig model of biofilm infection, deletion of *ica* and, thus, lack of PIA production caused no decrease in virulence (Francois et al. 2003), and deletion of *ica* in the clinical isolate UAMS-1 did not lead to lesser biofilm formation either in vitro or in an in vivo mouse model of catheter infection (Beenken et al. 2004). As well, several clinical isolates of MRSA (that were *ica*-positive) have been identified in which biofilm production is independent of *ica*, as increased transcription of the operon was not seen during glucose-mediated biofilm growth. As well, under NaCl-induced biofilm growth, though *ica* transcription was increased, levels of biofilm production were not similarly heightened (Fitzpatrick et al. 2005a). Deletion of the *ica* locus in one of these isolates did not lead to a lessened ability to form a biofilm; however, the same deletion in a laboratory strain of *S. aureus* did abrogate biofilm formation (Fitzpatrick et al. 2005a). Other studies support this idea and show that, in 114 MRSA clinical isolates, PIA production did not correlate with biofilm production, and deletion of the *ica* locus in six of these isolates did not lead to lessened biofilm formation (O'Neill et al. 2007). However, in methicillin-sensitive *S. aureus* (MSSA), there was a correlation between PIA production and biofilm formation, and deletion of *ica* abolished biofilm formation (O'Neill et al. 2007). Thus, it seems likely that the *ica* locus' contribution to biofilm development is strain- and environment-dependent, and that there are different mechanisms of biofilm development in MRSA vs. MSSA. In those *S. epidermidis* and *S. aureus* strains in which biofilm formation is not dependent on PIA expression, protein adhesin(s) seem to be the most important factors. For example, in *S. epidermidis*, the accumulation-associated protein (Aap) is found at elevated levels in the proteinaceous biofilm (Hennig et al. 2007, Rohde et al. 2007). Clearly, more work should be done to fully elucidate the alternative mechanisms that contribute to biofilm formation by these microorganisms.

A variety of other genes and their products have been demonstrated to be involved in the development of staphylococcal biofilms. There exists evidence that

attachment of bacterial cells to a polymer surface – of course necessary for biofilm formation – may be promoted by a *S. epidermidis* autolysin (Heilmann et al. 1996); *S. aureus* possesses a homologue of this gene (*atl*) which may have a similar function, perhaps through DNA release as discussed above. Teichoic acid structure is also crucial in the development of biofilms. Specifically, the addition of D-alanine esters to teichoic acids via the *dltA* gene product may be an important factor in imparting the proper charge balance on the Gram-positive cell surface, assisting in the physicochemical elements of initial attachment and biofilm formation. Another *S. aureus* gene product, known as the “biofilm associated protein” (Bap), was discovered via transposon mutagenesis to be required for biofilm formation on inert surfaces. However, the significance of this protein remains debatable since the *bap* gene was detected in only 5% of bovine mastitis isolates and none of the 75 clinical isolates evaluated. Gene expression in planktonic (shaken) versus biofilm (static) *S. aureus* cultures was evaluated by Becker and co-workers; five genes whose expression were increased in biofilms were identified (Becker et al. 2001). These included the genes encoding threonyl-tRNA synthetase (upregulated by amino acid starvation), three oxygen starvation response genes and a gene that encodes the ATPase ClpC. Microarrays are a powerful tool to investigate global gene expression. This technique, when used to study differential gene expression between these conditions, suggested 48 genes the transcription of which was increased at least twofold in the biofilm compared to planktonic conditions (Beenken et al. 2004). Taken together, these data suggest that genes involved in cell wall synthesis and pH balance are important in biofilms, whereas toxin and protease production is higher during planktonic growth (Beenken et al. 2004, Resch et al. 2006).

7.4 Properties of the Host Immune Response in the Development of Osteomyelitis

Biofilm formation by *S. aureus* makes eradication of the pathogen extremely difficult. Devitalized tissue induced by the staphylococcal toxins and the early inflammatory response not only present a suitable substrate for further bacterial adherence, but also interfere with the host's ability to mount an effective immune response against *S. aureus*, potentially leading to the development of a chronic infection (see also Chapter 11). One reason for this involves the functional impairment of phagocytic cells that are important during the early innate response to *S. aureus* infection. For example, a reduction in the amount of superoxide (a mediator of bacterial killing) produced within professional phagocytic blood cells of the infected host may occur (Roisman et al. 1983). Another mechanism by which dead bone can produce locally compromised immunity is through frustrated phagocytosis (Roisman et al. 1983), during which professional phagocytes undergo apoptosis when encountering a substrate of a size that is beyond its phagocytic capacity. The resulting release of reactive products may cause accidental host tissue damage and local vascular insufficiency, thereby increasing the predisposition to chronic infection development (Leid et al. 2002).

One theory behind the ineffectual phagocytosis of *S. aureus* growing in a biofilm has been that leukocytes are unable to penetrate into the depths of a biofilm to where the bacteria reside, thus leading to insufficient clearance and persistence. A study by Leid et al. (Leid et al. 2002), however, showed via time-lapse video microscopy that leukocytes do, in fact, attach to and enter a biofilm. Once inside the biofilm, however, these cells were unable to phagocytose the bacteria found there, but did produce inflammatory cytokines. This study supports the idea that there are other mechanisms of immune evasion at work besides frustrated phagocytosis, which prevent the proper engulfment of bacteria in a biofilm. One clue may be that the leukocytes that penetrated the biofilm in these studies were permeable to the large, intercalating dye, propidium iodide. Therefore, their phagocytic activity may have been disabled by the staphylococcal production of pore forming toxins, including α -hemolysin, γ -hemolysin, leukocidin, and the recently described phenol-soluble modulins-like peptides (Wang et al. 2007).

Besides recruitment of phagocytic cells to the site of infection, the innate immune system responds to peptidoglycan (via *N*-formyl methionine proteins and teichoic acids) by producing proinflammatory cytokines, such as IL-1, IL-6, and TNF- α , as well as C reactive protein. These factors enable the host to mount a protective inflammatory response that often contains and may resolve the infection. However, when the infection is not cleared by the innate immune system, *S. aureus* is well equipped to persist by a number of strategies. One such is elicitation of inadequate cell mediated (Th1) and humoral (Th2) adaptive immune responses (see also Chapter 12).

The timed expression of *S. aureus* virulence factors by its quorum-sensing system promotes host CD4⁺ helper T cells to release Th1 cytokines, including IL-12, IFN- γ , and TNF- α , resulting in a shift of the adaptive immune system to an ineffective Th1 cell-mediated immune response (Leid et al. 2002). Because this Th1 immune response is often inadequate for clearing the early biofilm form, it enables *S. aureus* to form a fully mature biofilm and, hence, a persistent infection. In a study using a murine model of acute *S. aureus* biofilm infection, the increase in central cytokines of cell mediated immunity (IL-2 and IFN- γ) appeared to be only transient, while inflammatory cytokines remained at elevated levels around biofilm-infected tissue (Yoon et al. 1999). This cytokine profile resulted in the initial expansion and activation of T-cell subsets followed by apoptosis. In this way, *S. aureus* seemed to interfere with the antibacterial immune response by down regulating T cell-mediated immunity and cytokine production. In addition, the Th1 response produced by staphylococcal infection is ineffective in the low oxygen partial pressures found in biofilms and in infected tissues where immune cell function is inhibited. A study performed in mice also found that high levels of IFN- γ (a Th1 cytokine) play a detrimental role in staphylococcal infection, and IL-4 and IL-10 (Th2 cytokines) are involved in host resistance to infection through regulation of IFN- γ (Sasaki et al. 2000).

The Th2 antibody-mediated response is also ineffective against a mature bacterial community. This Th2 response has, however, been previously shown to be readily effective at clearing a biofilm infection in the early phase of formation (Nayak et al.

2004, Shkreta et al. 2004, Sun et al. 2005). Unfortunately, this antibody-mediated response is down regulated by both the host cytokines associated with the initial response to *S. aureus* infection, most notably IFN- γ , as well as by *S. aureus* production of superantigens, capsule, and other toxins. Although the antibody-mediated immune response does eventually recover and is again able to mount a response against the biofilm by then the fully mature biofilm is resistant to antibody-mediated clearance.

7.5 Diagnosis, Treatment and Prevention of Osteomyelitis

7.5.1 Diagnosis

During acute infection, if the proper antibiotic is started early, the infection will usually clear after 2–4 weeks of treatment (Berendt and Byren 2004). However, diagnosing these infections during this early, clearable state is difficult. Radiographic changes during acute infections are usually not discernible until 1–1.5 weeks after inception of the disease. Magnetic resonance imaging (MRI) is effective in diagnosing acute infections in the absence of metal implants, but there is a lag time after previous surgery or infection (Berendt and Byren 2004).

In chronic osteomyelitis there exist large areas of devitalized cortical and cancellous bone within the wound. Because antibiotics do not penetrate well into devitalized bone (Healy and Freedman 2006), dead areas must be completely debrided, including devitalized scar tissue, marrow, and cortex. The soft tissue covering the area of bone trauma must heal; if this does not occur, the infection will persist and a new infection could form. Compromise of local soft tissue is a major reason for continued drainage. Diagnosis of chronic infection can often be made by radiography. Other techniques include radionuclide scans, though these lack specificity (Berendt and Byren 2004).

The presence of general vascular insufficiency makes suitable therapy and management of chronic contiguous osteomyelitis complicated. Most patients fitting this description have diabetes mellitus (Calhoun et al. 1988a), and range from 35 to 70 years of age. Due to the large increase in the diabetic population, osteomyelitis in the diabetic foot is now considered the most common bone infection (Berendt and Byren 2004). The small bones of the feet, as well as the talus, calcaneus, distal fibula, and tibia are commonly involved in this category of infection. Often, the infection is commenced by minor trauma to the feet, such as infected nail beds, cellulitis, or trophic skin ulceration. Neuropathy in these patients impairs the proper functioning of the foot as well as protective pain responses, leading to progression of soft tissue infections into underlying bone (Berendt and Byren 2004).

Osteomyelitis in those individuals with compromised vasculature can be difficult to diagnose. The patient may present with any of a large number of complaints, including ingrown toenails, a perforating foot ulcer, cellulitis, or a deep space infection. Examination shows decreased dorsal pedis and posterior tibia pulses, poor capillary refill, and decreased sensation; however, fever and systemic toxicity are

often absent. Although arrest of the infection is desirable, a more achievable treatment goal is to contain the infection and preserve the functional integrity of the involved limb. Debridement and ablation are often essential. The intractable character of this type of infection often leads to recurrent bone infections, even after suitable therapy. Partial removal of the infected bone is almost always necessary.

7.5.2 Antimicrobial Chemotherapy

Because staphylococci are by far the infectious agent most commonly recovered from cases of osteomyelitis, it is advisable to commence empirical therapy upon patient presentation with a regimen that includes an anti-staphylococcal agent (Berendt and Byren 2004). Following a definitive diagnosis by laboratory testing, the appropriate, more specialized antibiotic can be applied if it is found that the susceptibility profile of the infecting microorganism renders inappropriate the empirically selected antibiotic. Treatment usually lasts at least 4 weeks and is administered intravenously, but duration does vary markedly with age; length of treatment tends to be shorter in children (Jaberi et al. 2002). In adult individuals, *S. aureus* is generally treated with nafcillin or with cefazolin, clindamycin, vancomycin, ciprofloxacin, or levofloxacin being given as alternatives should treatment with the former drug fail (Lew and Waldvogel 2004).

7.5.3 Novel Treatments

Given the difficulties in treating osteomyelitis with conventional antimicrobial agents and the tendency of biofilm infections to resist clearance by such agents, it is apparent that novel treatments are necessary (see also Chapter 14). Recently the use of anti-PIA antibodies to prevent attachment or the formation of PIA in general has been investigated by various authors (McKenney et al. 1999, Maira-Litran et al. 2005, Kelly-Quintos et al. 2006). Another option is to coat medical devices prior to implantation. An enzyme produced by *Actinobacillus actinomycetemcomitans* known as “dispersin B” (DspB) is capable of cleaving PIA (Itoh et al. 2005). However, as mentioned above, many clinical isolates do not appear to express the PIA polysaccharide. Finally, Balaban and co-workers have advocated the approach of using the RIP heptapeptide, which is proposed to inhibit RNAIII-activated virulence factors, in the treatment of biofilm-associated infections (Balaban et al. 2003, Balaban et al. 2003, Giacometti et al. 2003, Balaban et al. 2005, Balaban et al. 2007). The suggested mechanism is inhibition of quorum sensing in *S. aureus* leading to reduced biofilm formation that is less recalcitrant to the action of antibiotics. However, the validity of this claim remains unclear since other authors have shown that the *agr* system works to increase levels of biofilm detachment and that disruption of the QS system leads to increased biofilm formation (Vuong et al. 2000, Vuong et al. 2003, Otto 2004, Vuong et al. 2004, Kong et al. 2006). Therefore, whether or not RIP will truly be an effective anti-biofilm agent is open to question. Thus it is apparent that the number of novel therapies that are under development

and could be effective against all clinical isolates of *S. aureus* is limited. To date, surgical intervention and debridement remains the most effective method of treatment of biofilm-associated infections. In osteomyelitis infections, this means debridement of the infected bone and, on occasion, the surrounding soft tissue.

7.5.4 Prevention

That prevention of a disease is always better than waiting for the disease to occur and then attempting a cure has been known since the time of Hippocrates. Of course, the best and most effective way of preventing an infectious disease is through vaccination; this is no less true in the case of osteomyelitis. Since vaccine development tends to focus on one microorganism at a time and *S. aureus* is one of the main causative agents of osteomyelitis, the following text will focus on anti-*S. aureus* vaccine development efforts to-date and particularly on those directed against biofilm infections.

A recent review of such efforts was published in late 2008 by Schaffer and Lee. A summary of recent vaccine efforts, adapted from (Schaffer and Lee 2008), is provided in Table 7.1 below. The majority of such efforts have focused upon identifying and testing staphylococcal antigens expressed during planktonic growth. Whilst a proportion of staphylococcal infections are undoubtedly caused by bacterial cells living planktonically within body fluids (probably the best example being septicemia), a significant proportion of staphylococcal infections are mediated by biofilms. Given the knowledge that bacteria residing within biofilms express markedly different proteomes than the same cells living planktonically, it seems likely that vaccines directed against planktonic antigens will be ineffective against biofilm-type infections. Therefore, selection of antigens for inclusion in a vaccine which targets biofilm infections must take into account the biofilm phenotype during antigen selection. Indeed, it is possible that multiple antigens will be required in order to protect against planktonic and biofilm-type *S. aureus* infections.

Efforts to identify *S. aureus* antigens expressed during biofilm growth have been undertaken recently. Brady et al. reported that a number of surface antigens are expressed uniquely by *S. aureus* during biofilm growth (Brady et al. 2006). In this study, rabbits were infected with experimental *S. aureus* osteomyelitis using

Table 7.1 Summary of anti-*S. aureus* vaccines currently in development

Name	Component details	Current status
StaphVax TM	Capsular polysaccharides types 5 and 8	Phase III failed
V710	IsdB; iron-regulated surface determinant	Phase II
PNAG	Poly- <i>N</i> -acetyl glucosamine	Experimental
ETI-211	Anti-protein A mAb linked to anti-CR1 mAb	Experimental
Alpha hemolysin	Secreted <i>S. aureus</i> protein; important virulence determinant	Experimental
SEB	Staphylococcal enterotoxin B toxoid; proteasome	Experimental

strain MRSA-M2 (isolated from a patient with osteomyelitis) (Mader and Shirtliff 1999) and sera drawn at early and late time-points post-infection. This sera was used to immunoblot two-dimensional SDS-PAGE gels upon which had been separated whole cell proteins of *S. aureus* grown in an in vitro biofilm model. In this way, the authors identified antigens that were both present during biofilm growth and immunogenic during osteomyelitis infection. The authors also fractionated cells and detected a number of cell-surface proteins expressed during biofilm growth and immunogenic in the rabbit model of osteomyelitis. Such proteins would be ideal candidates for inclusion in an anti-*S. aureus* biofilm infection vaccine.

The same group verified further the appropriateness of a number of these cell-wall antigens by direct immunovisualization of their presence and spatial distribution within intact *S. aureus* biofilm (Brady et al. 2007). Polyclonal antisera to each of five of the protein antigens discovered previously to be immunogenic in the animal model of osteomyelitis and expressed during biofilm growth (Brady et al. 2006) was raised in rabbits. Antibodies were then added to *S. aureus* biofilm in an in vitro flow model. A secondary goat anti-rabbit F(ab')₂ conjugated to Alexafluor-633 was added, followed by the DNA-interlocating stain SYTO9. Data suggested that all of the proteins investigated were present within the biofilm. Interestingly, however, expression of the proteins was not homogenous; each of the proteins was detected only in a proportion of the total community. The authors suggest that this spatially heterogeneous distribution of antigen expression within biofilm means that using only one biofilm-specific antigen in a vaccine will be ineffective as only a part of the whole community will be targeted by immune effectors. Therefore, the untargeted community will be unaffected and the infection able to persist.

To this end, the same group investigated the efficacy of a quadrivalent vaccine preparation containing four protein antigens (75 µg each) found to be both present in a biofilm in vitro and immunogenic in the rabbit model of osteomyelitis together with TiterMaxTM adjuvant (Brady et al. 2008, O'May et al. 2008). Four groups of animals were used: an untreated control group, one treated with vancomycin (40 mg/kg; 2/day) post-infection, one vaccinated pre-infection and one that received both pre-infection vaccination and post-infection vancomycin (40 mg/kg; 2/day) treatment. Animals who received both vaccination and vancomycin treatments showed significant differences in radiological, clinical (limping), and bacteriological signs of osteomyelitis when compared to the untreated controls, whilst those which received either vaccination or vancomycin alone did not. The authors postulate that this is because whilst vaccination is able to prevent establishment of a biofilm within the bone, planktonic (and, therefore, not targeted by the biofilm-specific vaccine) cells survive and are able to cause infection. Conversely, when only vancomycin was used, biofilm was able to form but the planktonic (and, therefore, vancomycin-sensitive) cells were killed. When the two were combined infection was prevented since both biofilm and planktonic cells were targeted. Work is now focusing upon both testing the efficacy of the vaccine in other models of biofilm infection and identifying an antigen expressed by planktonic *S. aureus* cells which will remove the need for vancomycin treatment.

7.6 Conclusion

The properties of bacterial biofilm are critical to the pathogenesis of osteomyelitis and, therefore, also to the development of novel methods of prevention and treatment. Bone provides a stable, non-ablative surface for biofilm formation, allowing invading bacteria to take refuge from the action of the immune system and any antimicrobial chemotherapy used in an attempt to clear the infection. The etiological agent responsible for the vast majority of cases of osteomyelitis, in both children and adults, *Staphylococcus aureus*, is a potent biofilm-forming bacterium, lending further weight to the assertion that biofilm is critical in the pathogenesis of osteomyelitis. Despite this, however, the majority of efforts in development of treatments and preventative therapies remain directed towards planktonic microorganisms. It seems likely that only a resolution of this dichotomy will lead to effective therapies for this highly debilitating infection.

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