BACTERIOLOGY AND THE ROLE OF GLYCOCALYX IN FOOT INFECTIONS

Foot infections were the most common septic problem requiring hospitalization of the diabetic patient, in 2005, with about 20-25% of diabetics developing foot infections. These infections tend to be polymicrobial, with greater than four species isolated per specimen from deep tissue infections. Aerobic organisms such as *Staphylococcus aureus*, coagulase-negative staphylococci, aerobic streptococci, and Gram (-) negative aerobic bacilli have been reported from foot lesions. Lambe and Ferguson cultured multiple types of lower extremity infections from 434 diabetic patients. They found that the most common aerobic species included *S. aureus*, *Staphylococcus epidermidis*, *Enterococcus*, *Pseudomonas aeruginosa*, *Proteus mirabilis*, Group B *Streptococcus*, *Escherichia coli*, *Klebsiella pneumoniae*, and *Acinetobacter calcoaceticus*.

The association of a putrid odor and soft tissue gas with foot lesions has long been observed, suggesting that anaerobic bacteria play a role in the etiology of diabetic foot lesions. With improvement in methods of specimen collection, transport and culture in recent years, numerous investigators have shown that anaerobes indeed play a major role in these infections.

In reviewing reports of bacteria in specimens from foot infections in diabetic patients, the numbers and species of aerobes and anaerobes varied greatly because each study sampled a slightly different patient population and used different specimen sources. For example, specimens obtained by scraping the base of the foot ulcer or the deep portion of a wound edge were cultured at the bedside in one study of 20 patients.

Another retrospective study reviewed 30 diabetic patients who had undergone surgery for lower limb infections. Specimens were taken during surgery or deep wound aspirates, taken within the first 48 hours of hospitalization, were analyzed. This study yielded a low incidence of *Clostridium*, also observed by Wheat et al., and Lambe and Ferguson. However, specimens from 13 diabetic patients scheduled for lower limb amputations yielded a high percentage of *Clostridium* species. Wheat evaluated 131 infections in 103 diabetic patients over a two year period, culturing specimens from ulcers, abscesses, bullae, bone and soft tissue infections.

This group divided specimens into two categories, which were called "unreliable", if the specimen had come into contact with the ulcer or other open draining lesion.
It was "reliable" if the specimen was not so contaminated. Differences in the incidence of various species of bacteria isolated from unreliable versus reliable sources were noted.

Deep surgical specimens from 250 patients with diabetic foot ulcers and bone biopsies from 250 patients with osteomyelitis of one of the bones of the foot were cultured by Klainer and Bisaccia. Many other species of aerobes and anaerobes have been isolated and the importance of these species should not be underestimated.

The variation in species identification procedures from different laboratories and changes in taxonomic classification contribute to confusion in comparing different studies. Some authors grouped organisms belonging to *Peptostreptococcus*, *Peptococcus*, *Porphyromonas* (formerly *Bacteroides*), *Prevotella* (formerly *Bacteroides*), or *Clostridium* genera without speciating. *Peptostreptococcus magnus*, *Peptostreptococcus prevotii*, *Peptostreptococcus asaccharolyticus*, and *Peptostreptococcus anaerobius* represented 58% of anaerobes isolated by Lambe and Ferguson. *Porphyromonas asaccharolytica* (formerly *Bacteroides asaccharolyticus*), and *Prevotella intermedia* (formerly *Bacteroides intermedius*) were reported only by Lambe and Ferguson; these two species plus *Prevotella melaninogenica* (formerly *Bacteroides melaninogenicus*) comprised 12% of anaerobes isolated.

More recent studies that speciated anaerobes reported a high incidence of anaerobes, including *P. asaccharolytica*, *P. intermedia*, *P. melaninogenica*, *Bacteroides fragilis*, other *Bacteroides* species, members of the genus *Peptostreptococcus* including *P. magnus*, *P. prevotii*, *P. asaccharolyticus*, *P. anaerobius*, other *Peptostreptococcus* species, anaerobic *Streptococcus* species, *Peptococcus* species, *Veillonella parvula*, *Propionibacterium* species, and various species of *Clostridium*, including *C. perfringens*. The ten most common anaerobic species isolated by Lambe included *P. magnus*, *P. prevotii*, *P. asaccharolyticus*, *P. asaccharolytica*, *Propionibacterium acnes*, *B. fragilis*, *P. anaerobius*, *P. melaninogenica*, *V. parvula*, and *Eubacterium lentum*.

Two important points are noteworthy. Black pigmented anaerobes, including *P. asaccharolytica*, *P. melaninogenica*, and *P. intermedia*, the twelfth most common anaerobe isolated by Lambe, were isolated with very high frequency from these infections. These extremely fastidious anaerobes were notably absent in reports of many studies. These, and other fastidious, organisms require freshly prepared, reduced or pre-reduced, anaerobically sterilized (PRAS) agar media and an incubation period of up to ten days for growth. Many strains of the genera *Prevotella* and *Porphyromonas* may take seven to ten days of incubation to grow and produce black pigment. Secondly, *E. lentum*, another slow-growing anaerobe, may take seven or more days to produce visible colonies. The identification of the black pigmenting anaerobic bacilli (*P. asaccharolytica*, *P. melaninogenica*, and *P. intermedia*) is important because this group of bacilli produce important toxins such as endotoxin, collagenase, fibrinolysin, deoxyribonuclease and ribonuclease. This group of bacilli are important pathogens in head and neck infections as well as in diabetic foot infections.

Anaerobic bacteria are particularly prevalent in more serious infections, such as necrotizing infections and those involving osteomyelitis. Sapico reported that anaerobes outnumbered aerobes 10:1 in a study in which deep tissue samples were cultured.

**Mode of Bacterial Growth in Diabetic Infections and Osteomyelitis**
In about one-third of diabetic patients hospitalized with foot infections, the soft tissue infection spreads to the medullary cavity of the bone, causing osteomyelitis. Osteomyelitis generally requires longer term antibiotic therapy than soft tissue infections and surgical intervention is frequently necessary.

Numerous reports have reviewed the microbiology of osteomyelitis. Generally, there are two types of osteomyelitis, with different organisms involved. Acute osteomyelitis is frequently found in children with associated skin or soft tissue infections, or in adults associated with intravenous drug abuse. These infections usually involve one organism, frequently \textit{S. aureus}, and respond well to antimicrobial therapy. Chronic osteomyelitis is usually associated with an older age group, and may follow trauma or skeletal surgery, frequently with the implantation of a prosthetic device. Lower extremities are usually involved. The microbiology of this type of osteomyelitis is quite different from acute osteomyelitis, and is much more difficult to treat. There is usually a polymicrobial infection, involving anaerobes and aerobes.

Until several years ago, the incidence of anaerobes in bone infection was underestimated. An early report by Taylor and Davies demonstrated that anaerobes were common in long bones of patients secondary to war injuries. Their techniques of anaerobic isolation and identification were primitive compared with those of today, and clostridia were primarily isolated. Although sporadic reports of anaerobic involvement in osteomyelitis appeared throughout the century, the importance of anaerobes was not emphasized until Ziment's 1968 report in which he described features of osteomyelitis involving anaerobes, including foul odor, Gram stain results, failure to isolate aerobic pathogens, soft tissue gas, and the presence of black discharge and necrotic tissue. Specimens from seventeen patients with foot infections (eight cases), skull infections (five cases) and long bone infections (four cases) all yielded anaerobes, including \textit{B. fragilis}, \textit{Peptostreptococcus}, \textit{Peptococcus}, \textit{Sphaerophorus}, \textit{Fusobacterium} and \textit{Bifidobacterium}. With the improvement of transport and culture techniques for anaerobes, the importance of these organisms in osteomyelitis became apparent. Lewis' review of anaerobes in bone infections, through 1975, cites over 700 cases of anaerobic osteomyelitis reported in the literature. The anaerobes most frequently isolated have been \textit{B. fragilis}, black pigmented anaerobic Gram negative rods, and anaerobic cocci, although numerous species of anaerobes have been reported. The longer duration of chronic disease, the more anaerobic species are isolated.

Generally, surgery is required, with removal of necrotic tissue and any implanted foreign body, along with long-term antibiotic therapy. Gristina has emphasized the importance of adherent bacteria in the persistence of osteomyelitis.

\textbf{Bacterial Glycocalyx}

The role of bacterial growth on a bone surface contributes to development and persistence of chronic diseases such as osteomyelitis. The mode of growth involves the glycocalyx in a network of exopolysaccharide polymers elaborated by bacteria. The term glycocalyx includes bacterial capsule and "slime" which detaches from the cell, floats away and adheres to surrounding tissue.

This glycocalyx mediates attachment of the bacterial cells to each other, forming microcolonies, and to a wide variety of surfaces including substrates in the natural environment, bone, and artificial
prostheses. The microcolonies form thick biofilms composed of bacteria and glycocalyx, which render the bacteria resistant to surfactants, antibiotics, and to destruction by host defense mechanisms such as antibodies and phagocytosis.

Bacterial glycocalyces are present in chronic diseases such as cystic fibrosis, and in foreign body related infections. The glycocalyx therefore plays a role in the persistence of many chronic diseases, including osteomyelitis. Ultrastructural studies in animal models of experimentally induced osteomyelitis have shown that glycocalyces are formed by Staphylococcus aureus, Prevotella bivia (formerly Bacteroides bivius), Prevotella disiens (formerly Bacteroides disiens), Bacteroides thetaotaomicron, B. fragilis, and P. intermedia. The same mode of growth observed in animal models is seen in human Osteomyelitis. Foreign bodies such as surgically implanted biomaterials and prosthetic devices, are reported to enhance infection rates, presumably by serving as substrates for sessile, glycocalyx-enclosed microorganisms.

Osteomyelitis is a disease that is recalcitrant to medical and surgical forms of treatment, although reasons for this were unknown until the role of the glycocalyx was elucidated. We established several rabbit models of osteomyelitis which demonstrated that the mode of bacterial growth and the production of glycocalyx were important factors in treating osteomyelitis. A description of these animal models and the mode of bacterial growth follow.

**Staphylococcus aureus** Rabbit Model of Osteomyelitis

Osteomyelitis was induced in the rabbit using **S. aureus**. A piece of silicone-coated rubber catheter (foreign body) was surgically implanted in the marrow cavity of a rabbit tibia, with the introduction of a sclerosing agent and bacterial cells. Osteomyelitis developed in 60% of animals infected with **S. aureus**, as determined by radiologic and histologic examination. Organisms were recovered from infected samples of animals with osteomyelitis. Samples of bone marrow, bone chips and foreign bodies were examined by transmission (TEM) and scanning electron microscopy (SEM). Infection with the accompanying production of glycocalyx advanced from the injection site into nearby bone and the foreign body.

The model of osteomyelitis in the rabbit shares many similarities with the human disease. Animals which developed osteomyelitis showed weight loss and radiological evidence of progressive disease. Histological samples of rabbit tibia showed striking morphological changes in infected animals consistent with a diagnosis of osteomyelitis. This model showed a high rate of induction of osteomyelitis, with recovery of **S. aureus** in pure culture from infected tibia, and gram (+) positive cocci were seen histologically.

Ultrastructural studies performed using this model showed that large quantities of **S. aureus** glycocalyx was present in the infection. TEM of samples from bone scrapings and bone marrow showed microcolonies of Gram-positive cocci surrounded by glycocalyx either condensed on the bacterial cell surface or in a fibrous intracellular network. Material from the interior of the foreign body showed masses of Gram (+) positive cocci embedded in glycocalyx. It appears that this mode of growth, that is, large amounts of glycocalyx surrounding masses of bacterial microcolonies, is typical of osteomyelitis. Thus, it is not surprising that this disease is so difficult to treat with antibiotics which fail
to penetrate the glycocalyx.

Normal topographical features of the bone were altered dramatically by copious material adhering to the bone. Further magnification of this material revealed coccoid forms embedded in a matrix of accreted material. The accretions were observed on the interior and exterior surfaces of the foreign body. The accreted material observed for the rabbit model of osteomyelitis was far more extensive than has been previously described for the adhesion of uropathogens to polypropylene surfaces.

*S. aureus* cells in this rabbit model grew in thick, adherent, glycocalyx-enclosed biofilms. This aggregated mode of growth of the pathogen presented a fibrous exopolysaccharide bacterial cell surface to the host defense mechanisms and a thick anionic barrier to the penetration of antibiotics. This mode of growth helps to explain the persistence of these pathogens in osteomyelitis.

### Additional Rabbit Models of Osteomyelitis with Gram-Negative Anaerobes and *Staphylococcus*

Following the development of the *S. aureus* osteomyelitis rabbit model, other bacteria were studied in this animal model to determine if a similar mode of growth was occurring with certain Gram (-) negative anaerobes and staphylococci. The organisms examined included *B. fragilis*, *B. thetaiotaomicron*, *P. bivia*, *P. disiens*, *S. aureus*, and *S. epidermidis*, alone or in combination. The mode of growth and the formation of glycocalyx found with these bacteria in osteomyelitis was similar to that observed with *S. aureus* alone. A description of the bacterial growth on the foreign body, in bone marrow and on the bone surface follows.

The pathogenicity of the organisms tested in the rabbit model varied. *B. fragilis* induced osteomyelitis at high rates, alone or in combination with *S. epidermidis*. The *B. fragilis* isolate when the rabbit model came from a patient with osteomyelitis, and such strains are more virulent than strains isolated from other types of clinical infection. *B. thetaiotaomicron* and *S. epidermidis* can induce alone or in combination. However, the incidence of osteomyelitis is higher in polymicrobial infection (95%) than in animals infected with either species alone. In addition, the presence of foreign body enhanced the incidence of osteomyelitis monoinfected in rabbits. However, when in combination with *S. epidermidis*, 73% of rabbits developed osteomyelitis, compared with 25% infected with *S. epidermidis* alone. Thus, there is an important synergistic relationship between *S. epidermidis* and certain anaerobic negative bacilli.

### Treatment of Staphylococcal Osteomyelitis with Clindamycin

After induction of experimental osteomyelitis with *S. aureus*, animals were treated with clindamycin phosphate for one, two or three week periods. SEM of infected bone samples showed masses of coccoid profiles embedded in a matrix of condensed glycocalyx. This material was present in untreated animals and those treated with clindamycin for one week. After two to three weeks of clindamycin treatment, SEM revealed few coccoid profiles adhering to the bone and marrow. Cultures of bone
marrow and bone were negative. Many aspects of the rabbit model of osteomyelitis are quite similar to human osteomyelitis. Roentgenologic evidence of progressive disease was seen in animals with osteomyelitis, and histologic observations showed changes in the tissues consistent with human osteomyelitis. Infecting microorganisms can also be recovered from sites of infection in the animal model, as with human osteomyelitis.

**Human Osteomyelitis**

Ultrastructural studies of specimens from patients with osteomyelitis show that the mode of bacterial growth is the same in human and experimentally induced osteomyelitis. A 69-year-old diabetic male developed osteomyelitis in the right hallux, which was amputated. A pre-surgical specimen cultured by the hospital clinical laboratory yielded beta-hemolytic Streptococcus and Staphylococcus. A portion of bone from the amputated toe cultured in the hospital laboratory was negative for anaerobes and aerobes. Another portion of the bone was placed in a sterile Petri plate in an anaerobe jar, and transported to an anaerobe chamber within 20 minutes of removal from the patient. Culture of this bone yielded no aerobes, but the anaerobes B. fragilis, P. asaccharolytica, P. anaerobius, P. magnus, and P. acnes were found. SEM of the bone surface showed bacilli connected by strands of glycocalyx. Another bone surface at a greater magnification showed a bacillus surrounded by a profusion of glycocalyx strands.

**Influence of Glycocalyx on Infection**

In an in vitro study to examine the effect of sub-inhibitory concentrations of clindamycin on the adherence of bacteria to bone, S. aureus and P. intermedia were grown in broth media containing discs of rabbit tibia in the absence or presence of varying concentrations of clindamycin.

In the absence of clindamycin, S. aureus colonized the bone surface extensively, and formed large microcolonies surrounded by glycocalyx. When grown in 0.1 minimal inhibitory concentration (MIC) of clindamycin, S. aureus adherence to bone surfaces decreased 80% with fewer and smaller microcolonies present. At a concentration of 0.25 MIC clindamycin, only a rare microcolony was seen, with most of the bone surface uncolonized. No colonization occurred at 0.5 MIC clindamycin.

Another SEM shows colonization of the bone surface by P. intermedia in the absence of antibiotic. Unlike S. aureus, 0.1 MIC clindamycin had little effect upon adherence of P. intermedia. A concentration of 0.25 MIC clindamycin was required to dislodge 80% of P. intermedia from the bone. At a concentration of 0.5 MIC clindamycin, single cells and small microcolonies were observed, with bone surface visible. With 1.0 MIC clindamycin no colonization was observed.

**Inhibition of Phagocytosis by Glycocalyx**

Veringa showed that subinhibitory concentrations of clindamycin and trospectomycin significantly enhanced the phagocytosis of glycocalyx-enclosed Bacteroides. Without antibiotic treatment, few bacterial cells were phagocytized. The bacterial cells that were phagocytized contained little or no glycocalyx. A moderate amount of glycocalyx was observed surrounding B. thetaiotaomicron, but a thick layer surrounded B. fragilis clindamycin treated cells. Homologous, or heterologous glycocalyx
added to preopsonized, preadhered B. fragilis, B. thetaiotaomicron, or S. epidermidis prior to incubation with polymorphonuclear leukocytes has no effect upon phagocytosis of untreated bacteria, because the untreated bacterial cells already had glycocalyx that was antiphagocytic. However, when isolated glycocalyx from B. thetaiotaomicron or B. fragilis was added to clindamycin or trospectomycin treated bacteria, phagocytosis was significantly reduced.

This effect of glycocalyx on phagocytosis was quantitative, the more glycocalyx added, the less phagocytosis. The antibiotic altered the glycocalyx in some unknown way so that the glycocalyx was no longer antiphagocytic. The decrease in phagocytosis caused by the addition of glycocalyx to antibiotic treated cells demonstrates that glycocalyx per se is antiphagocytic. Glycocalyx preparations from Bacteroides and Staphylococcus significantly inhibited the chemiluminescence and chemotactic responses of viable polymorphonuclear leukocytes, without toxicity to the polymorphonuclear leukocytes. These studies showed that the glycocalyx specifically inhibits phagocytosis, chemiluminescence, and chemotactic responses of viable PMNs. Certain other antibiotics such as amdinocillin, ceftriazone and ciprofloxacin did not alter the glycocalyx, thus they did not enhance phagocytosis.

Inhibition of Lymphocyte Proliferation by Glycocalyx

Ten to 100 μg/ml glycocalyx isolated from Staphylococcus lugdunensis or S. epidermidis and partially purified under endotoxin-free conditions inhibited the phytohemagglutinin-stimulated proliferation of peripheral blood mononuclear cells.

When peripheral blood mononuclear cells were depleted of adherent monocytes, the phytohemagglutinin-stimulated proliferation of nonadherent cells (PBL) occurred in the presence or absence of glycocalyx. Therefore, the mechanism of glycocalyx mediated inhibition required the presence of adherent monocytes. Culture supernatants of monocytes stimulated by glycocalyx contained a soluble factor which inhibited proliferation of monocyte depleted peripheral blood mononuclear cells.

The soluble factor was not produced in the absence of glycocalyx. Nor was it produced when glycocalyx was added to peripheral blood mononuclear cells along with indomethacin, which inhibits the synthesis of prostaglandin E2, a product of adherent blood monocytes. Therefore, the glycocalyx does not have a direct effect upon T lymphocytes, but rather inhibits proliferation indirectly by stimulating monocyte production of prostaglandin E2, which in turn inhibits lymphocyte proliferation.

Concluding Remarks

The role of bacteria in diabetic foot infections, and the number and species of aerobic and anaerobic microorganisms, were reviewed in this section. The role of glycocalyx and several models of animal and human osteomyelitis were also presented. Finally, the treatment of staphylococcal osteomyelitis with the antibiotic clindamycin, was elucidated. It may be important that you be aware of this material for board certification examinations.

References:


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Additional References:


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