Analysis of the Chronological Histo-Pathological Healing Events Occurring in Duodenal Tissue of a Porcine Model

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Introduction:

This paper will discuss the histo-pathological events that take place during three intervals of a ninety day time period in which the process of healing will occur in porcine duodenum samples after mechanical damage has occurred. The tissue has been implanted with barbs prior to investigation and the barbs were left in the living tissue for 3, 14, and 94 days. By using numerous techniques to examine and evaluate the three day, fourteen day, and ninety-four day tissue specimens obtained from mechanically damaged porcine duodenum, a rough outline of events can be materialized. The interpretations of JB4 sections, Masson's Trichrome stained slides, as well as scanning electron microscope and transmission electron microscope images provide pictorial evidence that can be integrated with present research on healing and foreign body response to provide a deeper understanding about chronic mechanical damage in the intestine.

The duodenum exists at the posterior end of the stomach where the pyloric sphincter meets the small intestine. It is a bulb-like structure that is composed of columnar epithelia with villi lining the lumen attached to lamina propria, followed by submucosa, musculara, and finally surrounded by serosa. The crypt zone of the mucosal layer, located between the villi, contains proliferative cells; while the surrounding villi contain differentiated cells (Kedinger *et al.* 1998). When mechanical damage occurs, the villi are often lost, as these differentiated cells cannot contribute to the rejuvenating wound site. The intestine is normally characterized by cell renewal since it is constantly in contact with the outside of the body. This renewal, which may be controlled by the mesenchymal cells differentiating, must be adjusted to accommodate the healing process (Kedinger *et al.* 1998). Of course, there are an abundance of other factors that are involved in the healing process of duodenal tissue. In order to adequately assess the healing

process in the obtained tissue samples, a comprehension of healing and all of its components must first be acquired.

The healing process is a complex progression of cellular and molecular actions that take place to restore the wound site to near normal function. This multifaceted response calls upon many different cell types, structural elements, growth factors and enzymes to primarily reestablish a barrier and secondarily to fully restore the injured tissue (Healing, Stadelmann *et al.* 1998). Classically, there are three phases of wound healing which include inflammation, fibroplasia, and maturation. The maturation stage can be split into remodeling and contraction phases (Healing). When these phases occur without flaw, there will be a resultant fine scar with modest variation from the surrounding tissue (Stadelmann *et al.* 1998). An overview of the biological healing response includes: the inflammation or "lag" phase which is characterized by increased permeability of vessels adjacent to the wound site; neutrophil, monocytes, and macrophage infiltration at the injury; and the presence of growth factors. The proliferation phase is exemplified by granulation tissue containing fibroblasts, collagen and growth factors. The remodeling phase is a modification of collagen and wound contraction is the final phase when tissue continuity is generally restored (Healing). Looking at these phases in more detail is essential in order to correctly evaluate the acquired pictures at a cellular level.

Inflammation:

Inflammation is the most familiar of the healing phases as its physical manifestations are quite obvious and include the immediate response of the body: redness, warmth, pain, swelling and altered function (Wiley *et al.* 2008). However, the corresponding cellular indications are less widely known, but they are imperative to consider while evaluating the research at hand.

Healing, by definition, requires inflammation and so is an important nonspecific defense (Wiley *et al.* 2008, Silen & Ito 1985). As previously mentioned, inflammation is the initial stage which vascularizes the wound site allowing immune components to clear the area of foreign material and dying tissue to combat early stages of infection, while simultaneously allowing a rapid change in the levels of several proteins which sets the stage for enabling healing and regeneration (Kindt *et al.* 2007, Stadelmann *et al.* 1998). The two major components that make up the inflammatory response are the change in vasodilatation and vasopermeability as well as a leukocyte infiltrate.

The vasomotor-vasopermeability response allows for and increase in blood volume in the injured area as well as leakage from blood vessels to initiate edema (Kindt *et al.* 2007, Stadelmann *et al.* 1998). Bradykinin binds to capillary walls to open spaces for leukocytes to infiltrate. Bradykinin increases vasopermeability and is also responsible for vasodilatation, inducing pain, and triggering contraction of smooth muscle (Kindt *et al.* 2007). Bradykinin also causes the release of prostaglandins which are released from the polymorphonuclear leucocytes during phagocytosis. The prostaglandins bind to free nerve endings, making them fire and start a pain impulse (Wiley *et al.* 2008).

Vasopermeability leads to the leukocyte infiltration. Simultaneously it also binds to mast cells in the connective tissue associated with most small blood vessels. This activates the mast cells causing and influx of calcium ions, which leads to degranulation and release of preformed mediators such as histamine which is a key chemical mediator in vasodilatation and vasopermeablility (Wiley *et al.* 2008, Stadelmann *et al.* 1998). Histamine also widens the junctions between endothelial cells so that more fluid, leukocytes, kallikrein, and bradykinin move out, causing more swelling (Wiley *et al.* 2008). Accompanying the increased vascular

permeability, there is an influx of cell populations including polymorphonuclear leukocytes and mononuclear leukocytes. The increased permeability also allows for neutrophils which have been released by activated bone marrow to infiltrate (Kindt *et al.* 2007, Wiley *et al.* 2008). Neutrophil infiltration into the tissue peaks within the first six hours of the inflammation response and is a complex process (Kindt *et al.* 2007).

The following series of concomitant events is complicated and begins with the signals released from the injured cells and is made possible by increased vascularization and vasopermeability. Chemokines are a major group of signaling molecules released by injured cells that make it possible for leukocytes to reach the site of infection by activating the endothelium of nearby capillaries (Wiley *et al.* 2008). C3a, C5a, and C5b67 act together to induce monocytes and neutrophils to adhere to vascular endothelial cells. This is the beginning of extravasation, the event that allows for leukocytes to infiltrate the tissue at the activated epithelium site (Kindt *et al.* 2007). Neutrophil extravasation can be divided into 4 steps: rolling, activation by chemoattractant stimulus, arrest and adhesion, and transendothelial migration. During rolling, the chemokines bind to the surface of leukocytes, specifically neutophils in the case of early inflammation (Kindt *et al.* 2007). The binding between the chemokines and the receptors cause a conformational change in the surface receptor integrins which increase neutrophil affinity for intercellular adhesion molecules located on the endothelium of the various blood vessels at the injury (Kindt *et al.* 2007). These neutrophils roll along the endothelium during chemoatrractant stimulus step until they encounter selectins, another cell adhesion molecule, which slows the neutrophil and eventually stops it completely. Then the adhesion step begins (Wiley *et al.* 2008). The binding neutrophils then migrate through the endothelium into the tissue where they are attracted to the site of wound by chemoattractants (Kindt *et al.* 2007).

This is just beginning of the leukocyte response which is one component of a cascade of events originating from the inflammatory mediators and chemotactic signals sent out by injured tissue cells (Kindt *et al.* 2007, Wiley *et al.* 2008). Once in the tissue, these leukocytes phagocitize impurities to prevent the possibility of infection. As mentioned, neutrophils and other leukocytes are attracted to the infection site by chemotaxins. The chemotaxins include substances released by bacteria, endothelial cells, mast cells, and tissue breakdown products. The chain reaction leads to more chemicals calling more cells to the site of injury. Local signals released from the tissue cells include antimicrobial peptides such as defensin, cathelicidins and interferons (Kindt *et al.* 2007). In response, the innate immune system uses many molecules and receptors as effectors. Cytokines are small proteins released by cells that effect other cells. They are also released at the wound site and include the interleukins, lymphokines and cell signal molecules, such as tumor necrosis factor and the interferons. These all also contribute to the healing process.

The use of signals is necessary first to recruit leukocyte populations into the area to further clean it, and is soon followed by many other cells. Other types of leukocytes may follow the neutrophils depending on the severity and nature of tissue damage (Wiley *et al.* 2008). Typically the early stages of the wound will include large populations of leukocytes including monocytes present at the wound site (Ross *et al.* 1970). Monocytes are one type of leukocyte that will invade the inflamed tissue since they must be present for healing to proceed (Stadelmann *et al.* 1998). They are responsible for dividing into macrophages and dendritic cells to aid in the healing process. Macrophages have dense bodies in their cytoplasm which may indicate previous phagocytosis of foreign material (Ross *et al.* 1970).

Other responses:

Working at the same time as the influx of immune cells, there are four activated interconnected mediator and inflammation-producing systems that further contribute to the surge of infiltrating cells and molecules into the wound area. The kinin system, the clotting system, the fibrinolytic system and the complement system are activated by the immune cells and contribute to the inflammatory process. The clotting system is rapidly triggered after injury in order to prevent bleeding and limit the spread of infection (Kindt *et al.* 2007).

When the endothelium is damaged, in this case by mechanical means, collagen and von Willebrand factor, a cell adhesion ligand in the walls of the endothelium, becomes exposed and the endothelium can no longer produce platelet inhibitors. The platelets become activated by coming into contact with the collagen and cluster by fibrinogen. A clot is formed by the action of thrombin which is released by platelets at the wound site. Thrombin acts on soluble fibrinogen in tissue fluid or plasma and produces insoluble strands of fibrin. The insoluble fibrin strands crisscross one another to form a clot, which serves as a barrier to the spread of infection (Kindt *et al.* 2007). Platelet degranulation also initiates the compliment cascade with the formation potent anaphylatoxins promoting the release of histamine from basophils and mastcells (Stadelmann *et al.* 1998). Platelets are store houses for a myriad of growth factors and vaso-active substances: platelet-derived growth factor (PDGF), transforming growth factor beta (TGF- beta), fibroblast growth factor (FGF), epidermal growth factor (EGF), beta-thromboglobulin, platelet factor 4 (PF4), platelet derived angiogenesis factor (PDAF), serotonin, bradykinin, prostaglandins, prostacyclins, thromboxane, and histamine (Stadelmann *et al.* 1998). And so platelets also contribute to the initial inflammation response.

Effects of translocated cells/ECM breakdown:

The translocation of the immune cells results in a degradation of the extracellular matrix (ECM) and thus the breakdown of the surrounding tissue scaffolding. The exponential release of numerous mediators by the recruited immune cells causes this breakdown, which also helps to recruit immune cells. The communications between the cells and the ECM by expression and engagement with adhesive ligands that mediate cell-cell and cell-matrix interactions are important and cause further changes in the composition of the wound site (Vaday & Lider 2000). The relationship that exists between the ECM and immune cells is important and delicate.

Central to inflammation, activated neutrophils and leukocytes release free oxygen radicals and lysosomal enzymes including neutral proteases, collagenases, and elastases which help fight infection and clean the wound. However, these enzymes and mediators also cause damage to the tissue (Stadelmann *et al.* 1998, Rieder *et al.* 2007). Migratory leukocytes themselves also participate in governing ECM modifications by secreting cytokines, chemokines, and degradative enzymes (Vaday & Lider 2000). They make these complexes as they travel through the ECM, they are not stored. This is demonstrated by remarkable changes that occurred in leukocyte behavior as they adhered to and migrated through the ECM (Vaday & Lider 2000). Macrophages also secrete large amounts of reactive oxygen radicals contributing to obligatory mucosal and submucosal destruction. They also release tissue-degrading enzymes soon followed by the release of pro-inflammatory cytokines as well as chemotactic and cell-activating peptides previously bound to the matrix (Rieder *et al.* 2007).

These ECM-modifying and ECM-degrading enzymes permit further infiltration of both immune and non-immune cells into the inflamed area by allowing for more space and ultimately

allowing for myofibroblast migration (Rieder *et al.* 2007). The action of these enzymes, whether proteinases, heparanases, or other subgroups, creates a path for cells to follow as well as an assortment of cytokine and ECM substrate fragments possessing distinct properties that may affect the outcome and length of the inflammation response (Vaday & Lider 2000). Serine proteases and matrix metalloproteinases (MMPs) are the two major groups of proteinases that are secreted by the immune cells. These enzymes are capable of degrading elastin, fibronectin, laminin, collagen and proteoglycans, which are major ECM components (Rieder *et al.* 2007).

MMPs:

Metalloproteins (MMPs) aid leukocytes in their extravasation from blood vessels and are known for their ability to destroy ECM, creating paths for leukocytes to penetrate into inflamed tissue. MMPs are an enzyme capable of degrading every ECM substrate due to a common structural domain shared by ECM constituents. Through enzymatic protocol they bind to that common domain and disassemble the substrate. MMPs are synthesized by leukocytes and secreted in response to inflammatory cytokines, growth factors and eicosanoids. TIMPs, or tissue inhibitors of metalloproteinases, are responsible for keeping the proteolysis under control via a non-covalent bonding relationship (Vaday & Lider 2000). Regulation is key since levels of MMPs and their inhibitors direct the progress of inflammation (Rieder *et al.* 2007). There may be more than one way of regulating the balance between MMPs and their inhibitors including: activation and homing to specific tissues, adhesion to endothelial cells, and adhesion to extracellular matrices (Vaday & Lider 2000). This balance contributes to maintenance of the ECM, however the dynamic properties of the ECM is also important to healing.

ECM:

As the immune cells release enzymes that break down the extracellular matrix, the composition of the matrix changes and allows for inflammation to ensue. The ECM is the medium in which all cells exist. It also provides a substance through which cells can communicate via chemical signals. The ECM has a specialized role in providing intrinsic signals for coordinating actions of specific immune cells (Vaday & Lider 2000). The crosstalk that occurs between immune cells and their surroundings profoundly influences cells to adapt and respond accordingly (Vaday & Lider 2000). The ECM serves as a reservoir of factors that promote cell proliferation, differentiation, activation and migration.

The ECM often undergoes compositional changes from its latent form to its activated form. This dynamically alters its signals and the corresponding cellular responses. The enzymatic removal of certain ECM bound molecules may increase or decrease inflammation by interfering with cell behavior (Vaday & Lider 2000). As the ECM changes in this case, it acts as a signaling system to immune cells, directing them throughout the inflammatory response.

What is interesting about the ECM interaction is that since it is self renewing, as the ECM is degraded and activates immune cells, it is able to contain the inflammation response to a specific area. ECM macromolecules and cytokines appear to act synergistically: the ECM provides a substrate for cytokines to bind, while the bound cytokines consequently enhance cell-matrix interactions thus creating positive feedback (Vaday & Lider 2000). The inflammatory response is often more organized than it appears.

In addition, cytokine interactions with integrins are responsible for the control of inflammation and ultimately its arrest (Vaday & Lider 2000). Integrins are adhesion molecules that promote stable interactions between cells and their environment. They also act as cellular

sensor and signaling molecules. The integrins are activated by chemokines as well as cytokines, and chemical agents which are active during inflammation. When activated, the integrins transition from low affinity to high affinity ligand binding states allowing for cells to adhere to the ECM during cell migration (Vaday & Lider 2000).

The degradation of matrix components such as fibronectin, is a major determinant in ECM remodeling evoking cellular responses and signaling the direction of the inflammatory response. Fibronectin is produced primarily in the first 24-48 hours after injury and deposition creates a scaffolding on which fibroblasts can migrate into the wound (Stadelmann *et al.* 1998). Chemotactic fibronectin fragments among other distinct biological activities may selectively promote the recruitment of monocytes and other leukocytes into inflamed tissues (Vaday & Lider 2000). This is especially important in the foreign body response which will be discussed later.

It is possible that the sequence of signals created in the ECM microenvironment to recruit, activate and regulate immune cells varies during each inflammatory episode. Each medley of ECM constituents, cytokines and enzymes provides a context of information for migrating leukocytes, promoting reciprocal cell responses to ultimately resolve inflammation and so with differing signals, there are different cell populations each time (Vaday & Lider 2000).

The ECM changes that occur in the latter portions of inflammation involve the action of fibroblasts. The fibroblast is the main cell type implicated in the production of the ECM during the complex repair process (Schmitt-Graff *et al.* 1993). The fibroblasts must begin to repair the ECM and then prepare it for the next steps of healing. The natural dynamics of ECM remodeling during inflammation and wound repair involve changes in the balance of components, as well as the deposition of adhesive molecules not usually present in the ECM in abundant quantities.

However, cells other than fibroblast are often involved in the remodeling of the ECM. Surrounding endothelial, epithelial, and stromal cells may subsequently provide additional enzymes that are necessary to remodel ECM as inflammation resolves (Vaday & Lider 2000). Sometimes the changes that occur in these surrounding cells in order to produce ECM components cause fibrotic complications (Kedinger *et al.* 1998). This makes sense as an imbalance in the cells may cause a plethora of products to be released from the cell causing an irregular ECM leading to over production of fibroblasts and ultimately fibrosis.

Chronic Inflammation:

Inflammation in its chaotic entirety can be acute or chronic. Acute wounds heal by passing through all phases of healing, whereas chronic inflammation includes the formation of new connective tissue (Wiley *et al.* 2008, Stadelmann *et al.* 1998). Chronic inflammation is an imbalance at the cellular level that can lead to further pathological consequences and ultimately permanent tissue damage (Kindt *et al.* 2007). It is often characterized by dense infiltration of lymphocytes, plasma cells, and macrophages, as the inflammatory stage heavily depends on these cell types (Wiley *et al.* 2008). There is tissue destruction due to the infiltration of these cells and so that is yet another characteristic of chronic inflammation. There is also the remodeling of extracellular matrix components in the subepithelial region which is resultant from the invading immune cells (Otte *et al.* 2003). The remodeling involves fibroblasts and further production of connective tissue. Angiogenesis is also a characteristic of chronic inflammation and serves as a way for immune cells to reach the area. So ultimately the fibroblasts are unable to restore the ECM balance that would normally allow for healing.

Interestingly enough, in an examination of healthy individuals 12% of the subjects exhibited chronic inflammation without noticeable signs (Kreuning *et al.* 1978). Even severe duodenitis can exist without noticeable symptoms. This condition may be made possible by the constant mechanical stress that the duodenum is under combined with the bacterial contents in the lumen. Chronic inflammation is necessary for the foreign body response to occur, which will be discussed later.

In this research, chronic inflammation may be apparent by definition due to the morphology of the wound site. Chronic inflammation involves simultaneous acute inflammation, tissue destruction and attempts at healing. It also causes the continuous activation of the immune response as well as infections and possible movement of a foreign body. By one definition chronic inflammation occurs with the formation of abundant granulation tissue and excessive fibrosis leading to scar contraction and loss of function (Stadelmann *et al.* 1998). Since the barbs are continuously aggravating the tissue, chronic inflammation seems quite plausible. The amount of granulation tissue seen on slides as well as the cell populations observed also leads to that conclusion. However, the fibrosis seen in the tissue takes a specific collagenous form, and will be discussed as the foreign body response.

Restitution:

Restitution is the other end of the spectrum in which injury heals without inflammation. This is the response that is elicited by minor wounds that do not penetrate below the mucosal surface (Silen & Ito 1985). Restitution can be attributed to the constant turnover of intestinal epithelial cells. This makes it possible for epithelia integrity to endure injury before the inflammatory response occurs. The proliferating stem cells located in the gastric glands are

responsible for this behavior in the stomach and so it can be hypothesized that the proliferating cells in the crypts are responsible for analogous behavior in the duodenum. Epithelial cells make restitution possible, rapidly migrating from adjacent areas without differentiation or replication (Silen & Ito 1985). Restitution does not seem like a plausible explanation for the response elicited in the obtained tissue samples. Although restitution is possible in the duodenum, the wounds are much too deep to include restitution.

Fibrosis:

Fibrosis is the excessive buildup of fibrotic tissue that serves as the response to inflammation and the destruction of mucosa (Rieder *et al.* 2007). It is a transition between the inflammation stage and the fibroplasia stage. The inflamed tissue is transformed into a matrix of mainly fibroblasts and the normal intestinal structural design is restored by post-transcriptioal and post-translational mechanisms that prevent the net accumulation of ECM and fibrogenic cells. It also includes myofibroblasts that are continuously activated and thus produce collagen. This overabundance of collagen is important in the foreign body response. It is an important balance that must be kept in check otherwise.

Although the generation of fibrous tissue is necessary for healing to continue, if there is an imbalance, fibrosis becomes an unwanted result of healing that includes overproduction of ECM proteins such as collagen and fibroblast proliferation (Fries *et al.* 1994). The ECM is not degraded at appropriate levels and the fibrogenic cells proliferate uncontrolled (Rieder *et al.* 2007). Mesenchymal cells (fibroblasts, myofibroblasts, and smooth muscle cells) are the main producers of ECM components during fibrosis and may be regarded as the effectors of intestinal fibrosis (Rieder *et al.* 2007). Fibrosis leads to a dense conglomeration at the wound site and loss

of original tissue function in some cases. In the foreign body response fibrosis is important as a barrier function.

Granulation Tissue:

Continuing with the phases of healing, inflammation begins to subside with the infiltration of fibroblasts into the area and the beginning of fibroplasia. The lymphocytes assist the transition by secreting cytokines that are mitogens and chemoattractants for fibroblasts. They also clear the wound of old neutrophils (Stadelmann *et al.* 1998). It is the fibroblast population that will now become the dominant cell type in the healing wound as granulation tissue formation signifies the end of the inflammatory stage. Granulation tissue is characteristic of wound healing (Stadelmann *et al.* 1998, Schmitt-Graff *et al.* 1993). Granulation tissue is fibrous and contains many capillaries. It consists of fibroblastic cells separated by a collagenous matrix which contains capillary buds and remaining inflammatory cells as well as endothelial cells, pericytes and myofibroblasts (Stadelmann *et al.* 1998, Schmitt-Graff *et al.* 1993). Macrophages are the predominant white blood cell type in the wound and appear to play a central regulatory function in fibroblast chemotaxis, proliferation and the subsequent collagen synthesis and degradation. Neovascularization takes place in the granulation tissue via vascular endothelial growth factor (VEGF) and fibroblast growth factor-2 (FGF2). It is the conversion from highly vascularized granulation tissue to a relatively avascular and acellular collagen matrix that takes place during healing that is noticeable within the slides acquired during this research (Stadelmann *et al.* 1998).

An important event in granulation tissue is the transition of fibroblasts into myofibroblasts. In the underlying granulation tissue, fibroblastic cells exhibited the characteristic

cytoplasmic features of myofibroblasts. The fibroblasts are interconnected at several points by distinct gap junctions and contain a prominent microfilamentous cytoplasmic apparatus which signifies the transition to myofibroblasts (Gabbani *et al.* 1978). These microfilaments appear "de novo" in epidermal cells during the healing of an open wound and in myofibroblasts of the underlying granulation tissue (Gabbani *et al.* 1978). This transition and acquisition of microfilaments will be discussed later.

At the end of inflammation the wound begins to return to normal functioning tissue if healing has proceeded correctly. The process of tissue damage caused by the inflammation comes to an end and is followed by healing. If the basement membrane underlying the injured epithelium is intact, residual epithelial cells at the edge of the wound become motile and will meet adjacent epithelial cells to form new tight junctions (Rieder *et al.* 2007). In the case of the tissue collected, there are no final stages of healing. There is granulation and fibrosis present at all time periods collected.

Growth Factors:

Growth factors are capable of angiogenesis, stimulating cell growth, proliferation and differentiation and are major players in all steps of the healing response. Growth factors are expressed by both injured cells as well as immune cells in order to advance the healing process by many different actions. They are crucial in moving from one stage of healing to the next.

Some growth factors stimulate cells by acting as chemotactics contributing to the degradation of the ECM while other growth factors act on one particular cell type. For example PDGF, TGF beta, ILs and TNF alpha are produced by macrophages and activate fibroblasts to begin fibroplasia (Stadelmann *et al.* 1998). PDGF is responsible for increasing granulation

tissue, and increasing fibronectin and collagenase in fibroblasts thus contributing to the latter stages of fibroplasia and the early stages of remodeling. Factors like PDGF can be very potent. For example PDGF can increase granulation tissue by 200% in seven days after just one application (Schmitt-Graff *et al.* 1993). FGF is also aimed at fibroblasts and causes an increase in granulation tissue as well as collagen accumulation. Furthermore, FGF promotes angiogenesis contributing to connective tissue repair (Schmitt-Graff *et al.* 1993).

TNF-alpha

Tumor necrosis factor (TNF)-alpha is a growth factor that is used to boost the immune response by inducing fibroblasts to proliferate. It is produced mainly by macrophages and functions principally as a cytokine. TNF alpha binds avidly to the major ECM constituents, fibronectin and laminin. The association between the TNF-alpha-and fibronectin increases the adhesion of activated CD4+ T cells (Vaday & Lider 2000). This contributes to the immune response so that any foreign and harmful substance can be remembered and removed effieciently. TNF alpha is also partially responsible for the binding of neutrophils to endothelial cells during extravasation, the beginning of the inflammation cascade.

The interaction between TNF-alpha and T cells is especially important. T cells are an important part of immunity. Since TNF-alpha and interleukin are both present in the intestinal muscle layers during inflammation and it is proposed that they may serve to modulate the activation of T cells. Specifically, TNF-alpha and interleukin are hypothesized to contribute to the ability of non-professional antigen-presenting cells to activate T cells (Hogaboam *et al.* 1997). This means that non-immune cells are able to aid the immune system in recognizing self from non-self. This occurs by the action of TNF on cells, such as intestinal smooth muscle cells,

causing them to express major histo-compatability complexes (MHCs) on their surfaces. These MHCs present antigens to the T cells activating them and contributing to further immunity of the organism (Hogaboam *et al.* 1997).

TGF Beta

Transforming growth factor (TGF)-beta is similar to TNF-alpha in that it promotes proliferation and differentiation in many cell types. TGF beta is a multifunctional peptide growth factor that possesses a wide range of potential effects on growth, differentiation, extracellular matrix deposition and the immune response (Wells 2000). TGF beta is especially important in limiting the duration of the inflammation response. Macrophage-like mononuclear cells with TGF beta were found scattered throughout the mucosa and submucosa as well as in areas of subserosal granulomatous inflammation supporting the idea that they are closely related with the macrophages which in turn are responsible for much of the inflammatory response (Van Tol *et al.* 1999).

And like TNF-alpha, TGF beta also promotes fibroblast infiltration of the wound site (Kindt *et al.* 2007). Neutralization of TGFbeta1 reduces inflammation and matrix deposition, however, in uncontrolled situations it may also contribute to pathogenic fibrosis (Rieder *et al.* 2007, Schmitt-Graff *et al.* 1993, Van Tol *et al.* 1999). TGF beta may also be involved in the re-epithelialization of injured tissue by increasing laminin-collagen receptors on the mucosa and enhancing migration of epithelial cells to the site. In response to TGF beta, myofibroblasts in basal conditions express twice as much laminin than the latter (Fritsch *et al.* 1997). The TGF beta family of proteins is most involved in the control of mesenchymal cell differentiation and of

epithelial cell proliferation (Kedinger *et al.* 1998). As mentioned, the increase in laminin increases the migration of epithelial cells to the site inevitably increasing the rate of healing.

TGF beta also promotes the development of an ECM that can support the rest of the steps of the healing process. TGF beta alters the normal balance between ECM synthesis and degradation, inducing an increase in synthesis of matrix components and a parallel decrease in overall ECM proteolytic activity (Wells 2000). TGF beta is one of, if not the most powerful and widely circulated pro-fibrogenic moderator in the body (Rieder *et al.* 2007). TGF beta upregulates the fibrillar and nonfibrillar collagens, as well as other matrix components including fibronectin and tenascin, the basement membrane components laminin and entactin, and membrane proteoglycans including perlecan and biglycan. These components contribute to the changing matrix. TGF beta also alters the number and variety of integrins expressed on some cells, potentially enhancing their adhesion to the ECM and thus changing the binding properties of the ECM (Wells 2000).

TGF beta regulates many matrix degrading proteins but one of the ways TGF is able to regulate ECM consistency is by interacting with MMPs, a major class of ECM degraders that were mentioned previously (Wells 2000). TGF upregulates TIMPs, the inhibitors of the MMPs and thus stops these proteases from degrading the ECM. TGF beta also induces expression of plasminogen activator inhibitor (PAI)-1. This results in decreased conversion of plasminogen to plasmin, a protease that directly degrades matrix components and activates MMPs and this also contributes to keeping the ECM in tact (Wells 2000). TGF beta alters the balance between ECM synthesis and degradation, and mediates an increase in the synthesis of matrix components and a decrease in ECM proteolytic activity (Rieder *et al.* 2007). This inevitably changes in the ECM composition and allows for healing to continue while reducing inflammation.

Interestingly enough, TGF beta may regulate itself by modifying the ECM. Connective tissue growth factor is a mitogenic peptide induced by TGF beta that stimulates the synthesis of collagen I and fibronectin. This may mediate some of the downstream effects of TGF beta (Wells 2000)

Through many pathways TGF beta enhances subsequent healing and is capable of reducing the inflammation response. TGF beta may also reduce scarring; suggesting that it also plays a role in wound retraction and scar formation. TGF beta is able to induce the expression of alpha-SM actin in granulation tissue myofibroblasts (Schmitt-Graff *et al.* 1993). This actin is necessary in contracting the myofibroblasts and contributes largely to reduce scarring.

Fibroblasts:

Fibroblasts are the main cell type in the fibroplasia phase of wound healing. Originally, they were thought of as mere framework for other cell types, but they have been found to play key roles in the immunological response of the body to injury (Fries *et al.* 1994). Fibroblasts have great plasticity allowing them the potential to differentiate along a number of pathways (Fritsch *et al.* 1997). Remaining relatively undifferentiated, fibroblasts can assume a particular phenotype according to the physiological needs and/or the microenvironmental stimuli (Schmitt-Graff *et al.* 1993). The ECM serves as one stimulus for fibroblast differentiation. Among ECM components, different types of collagen, glycoproteins and proteoglycans are involved in fibroblastic differentiation (Schmitt-Graff *et al.* 1993). Fibroblast differentiation can also be modulated by contact with epithelial cells as well as other effecter molecules, including those involved in inflammation (Fritsch *et al.* 1997). This versatility makes them a perfect candidate for restoring tissue continuity.

Morphologically fibroblasts are flat elongated cells with an oval nucleus, physiologically they posses the ability to synthesize ECM components (Fries *et al.* 1994). Thus, they contain an abundance of endoplasmic reticulum and ribosomes allowing them to produce ample amounts of protein. In addition, the aggregates of ribosomes attached to the ER membranes of the fibroblasts are large and contain as many as 20-30 ribosomes which is yet another defining morphogenic characteristic (Ross *et al.* 1970).

There also may exist subpopulations which contain unique phenotypes that may be recruited by cytokines at the inflammation site depending on their properties. Subsets of fibroblasts are regulated in their response to cytokine released during inflammation. For example, the recruitment of fibroblasts that are capable of producing higher than normal amounts of collagen is useful at wound sites. The existence of different subtypes at the same anatomical site has made it possible for characterization of these subtypes. These subpopulations may be based on collagen production, morphology, and glycogen pools among other things such as size by sedimentation, lipid droplets, response to and synthesis of prostaglandin (Fries *et al.* 1994).

Fibroblasts appear to have a plastic phenotype that is capable of fulfilling distinct functions in normal pathological situations as well as in different locations. The modulation of fibroblastic cells involves several apparently independent biological activities including contractile activity and collagen synthesis: a co-ordinate activation of several genes (Schmitt-Graff *et al.* 1993). Differentiation states of fibroblasts are a debatable topic. They are defined morphologically and immunologically mainly by the expression of cytoskeletal proteins. Among these specific features are prominent cytoplasmic actin microfilaments, or stress fibers, and the formation of intercellular connections via adherens and gap junctions (Rieder *et al.* 2007)

Wound fibroblasts do not arise from hematogenous precursors and therefore must arise from adjacent connective tissue cells. The wound fibroblasts are derived from cells in the connective tissue adjacent to the wound (Ross *et al.* 1970). The inflammatory responses which typically precede fibrotic induction may be controlled by a subset of resident fibroblasts. Another subset may be important for the fibroblast hyperplasia. This is a condition in which there is an increase in the number of normal cells in a tissue or organ. There may also be extensive extracellular matrix production which is a hallmark of fibrosis (Fries *et al.* 1994).

Fibroblasts are the first non-immune cell to proliferate and migrate into the defect within the first 2-3 days when activity is principally confined to cellular replication and migration rather than collagen synthesis. Adhesion, proliferation and migration of fibroblasts are modulated by the changing ECM components as previously mentioned (Schmitt-Graff *et al.* 1993). Eventually, fibroblasts dominate the cell population within the first 7 days (Stadelmann *et al.* 1998, Chkravortty & Kumar 1997). The early ECM consists of fibronectin and hyaluronate which serves as essential scaffolding upon which fibroblasts can migrate and adhere (Stadelmann *et al.* 1998).

Concerning their role in fibroplasia, masses of fibroblast cells begin to synthesize and emit measurable amounts of extracellular collagen by day 2 or 3 (Stadelmann *et al.* 1998). The attachment of fibroblasts to each other has been reported to rapidly increase expression of pro alpha1(I) collagen messages (Juliano Haskill 1993). It is important that the number of fibroblasts producing collagen is controlled. This may involve the theory of subsets of fibroblasts that were previously mentioned. In the subset theory, some fibroblasts would produce collagen in vast quantities while others would be rather dormant. However, the concept of "tissue-specific

fibroblasts" differing in morphology, proliferative rates, and synthesis of cytokines and ECM components is still debatable (Fries *et al.* 1994).

The fibroblasts also secrete plethora of growth factors and substances essential to wound repair, including glycosaminoglycans and ECM molecules in addition to collagen (Stadelmann *et al.* 1998, Chkravortty & Kumar 1997).

Fibronectin, a glycoprotein, attaches fibroblasts to their surrounding stromal collagen (Eddy *et al.* 1988). The fibroblasts then begin to set up the ECM environment for contraction. Fibroblast traction can rearrange and re-pack collagen into patterns, even beginning from a totally random network (Harris *et al.* 1981). This aids in repairing the wound site by using all of the collagen being produced in the ECM. After the wound has been repopulated and the chemotactic gradient of mediators secreted by inflammatory cells has decreased, fibroblast migration will normally cease and the fibroplasia stage will wane (Rieder *et al.* 2007).

Collagen:

Collagen is one of the major ECM molecules synthesized by fibroblasts and is responsible for mediating cell attachment, and modulating signal transduction, cell proliferation and cell differentiation (Chkravortty & Kumar 1997). Collagen also plays an important role in the formation of tissues and organs and is involved in various functional expressions of cells. Its high tensile strength makes it relatively stable; a good trait for the primary structural protein in the body (Lee *et al.* 2001). Collagen is a very important component of connective tissue as well as the duodenum. In 1887, Halsted discovered that the submucosa provides the gastrointestinal tract with the majority of its tensile strength. Ultimately, submucosal collagen provides the intestinal wall with strength to withstand intraluminal pressure (Hesp *et al.* 1984). The bulk of

collagen in the duodenum is contained within the submucosa. Type I collagen predominates (68%) followed by type III collagen (20%) and type V collagen (12%). The collagen content in this layer increases significantly in response to chronic obstruction and wounding (Healing).

The basic unit of collagen is tropocollagen which is three polypeptide chains arranged in a left-handed helix. These molecules aggregate to form fibrils and collagen fibrils aggregate to form collagen fibers (Stadelmann *et al.* 1998). Each chain of collagen has an individual twist in the opposite direction contributing to the strength (Lee *et al.* 2001). Collagen synthesis begins as an intracellular process starting as a monomer which is actively secreted into the extracellular wound environment where polymerization into collagen fibers occurs. Collagen fibers are then covalently crosslinked to increase tensile strength and stability through its self-aggregation (Stadelmann *et al.* 1998, Lee *et al.* 2001). Collagen synthesis a seminal feature of the fibroplasia stage of healing (Stadelmann *et al.* 1998).

The increasing content of collagen in the wound during the fibroblastic phase correlates with the increasing wound tensile strength (Stadelmann *et al.* 1998, Hesp *et al.* 1984). Normal collagen synthesis takes place intracellularly and organization of the newly formed collagen continues in the extracellular space (Stadelmann *et al.* 1998). Collagen is produced as soon as fibroblasts reach the wound and receive appropriate signals. The signal that stimulates the production of collagen appears to be a combination of growth factors concurrently stimulated by hypoxia and by-products of anaerobic metabolism. One week following wounding, collagen synthesis reaches its maximum rate and collagen fibers become histologically apparent in the wound (Stadelmann *et al.* 1998). Collagen levels rise continually for approximately three weeks until collagen homeostasis is achieved. This is when the rate of collagen degradation by collagenase equals that of collagen synthesis by the fibroblasts. The net collagen content of a

wound at any given time is controlled by the delicate balance between collagen production and collagen degradation. Collagenase activity is thought to be controlled by a number of factors, including parathyroid hormone, adrenocorticoid steroids and colchicine (Stadelmann *et al.* 1998).

At least nineteen types of collagen have been reported. Collagen types I, II, and III as well as types V and XI are fibril forming collagens. These fibril forming collagens are responsible for most of the tensile strength in the body (Stadelmann *et al.* 1998, Lee *et al.* 2001). Many species share some homologous sequences in these types of collagens (Lee *et al.* 2001). As previously mentioned, collagen types I, III, and V are important in maintaining the tensile strength of the bowel wall.

As a wound heals the levels of collagen change and the ratio of different types of collagen also change. Normal soft tissue contains 80% type I collagen and 20% type II collagen. Acute wound granulation tissue contains approximately 30-40% type III collagen which will be converted into types I and II later in the healing process (Healing).

Myofibroblasts:

Morphologically, myofibroblasts are between fibroblasts and smooth muscle cells. Myofibroblasts normally exist below the epithelia in the duodenum in healthy tissue and are important in the healing process. The resident myofibroblasts remain present immediately below the basement membrane, close to the basal surface of epithelial cells (Otte *et al.* 2003). They have been identified as an important source of pro-inflammatory cytokines including IL-1, TNF alpha, growth and differentiation factors such as TGF beta TGF alpha, platelet derived growth factor, stem cell factor, hepatocyte growth factor, and keratinocyte growth factor as well as

chemotactic factors such as IL-8 or macrophage inflammatory protein-1alpha (Otte *et al.* 2003) Myofibroblasts play a role in the maintenance of the intestinal mucosa via secretion of cytokines and metabolites of arachidonic acid. These paracrine effects are important in mucosal immunophysiology as well as the regulation of a number of epithelial functions. Myofibroblasts are capable of producing postaglandins, which may regulate gastrointestinal barrier function, inflammation, and intestinal electrolyte transport and motility (Otte *et al.* 2003).

If tissue damage is severe, the myofibroblasts migrate to the wound site. They are also affected by chronic inflammation and will remain in the tissue if inflammation remains. Migration of myofibroblasts into and through the ECM seems to be a fundamental event during wound healing,although myofibroblasts may also be derived from surrounding fibroblasts. (Rieder *et al.* 2007). Myofibroblasts are resident to tissue however, some do arise from differentiating fibroblasts. Myofibroblasts may arise in granulation tissue from fibroblasts and are involved in healing by aiding in the contraction phase. Soon after wound contraction the converted myofibroblasts disappear (Schmitt-Graff *et al.* 1993).

The myofibroblasts are also capable of attracting additional myofibroblasts. Fibronectin can be synthesized by myofibroblasts in large quantities is essential and responsible for the migration of additional intestinal myofibroblasts. They may also call additional myofibroblasts by the use of autocrine and paracrine factors. Myofibroblasts can use PDGFA, PDGFB, TGF beta1, IGFI and epidermal growth factor which also influence intestinal myofibroblast migration. This migratory function along with the ability to contract the wound area makes intestinal myofibroblast cells important in the physiological situation (Rieder *et al.* 2007).

In granulation tissue, myofibroblasts are found in a basal-lamina-like material sometimes organized into nodular structures (Schmitt-Graff *et al.* 1993, Eddy *et al.* 1988). However, it is not

basal lamina since it lacks laminin. They are generally flat and elongated non-muscle cells with indented nuclei and prominent rough endoplasmic reticulum and golgi apparatus (Eddy *et al.* 1988). The presence of the developed RER indicates synthetic activities that must take place for myofibroblasts to contribute to healing. Myofibroblasts contain stress fibers like fibroblasts and are surrounded by fibronectin (13, Eddy *et al.* 1988). They have abundant cytoplasmic actin microfilaments, and dense bodies (Schmitt-Graff *et al.* 1993). The bundles of microfilaments resemble the myofibrils of smooth muscle and play a role in contraction (Eddy *et al.* 1988).

Fibroblasts respond to TGF beta and other cell signals and begin to differentiate into myofibroblasts. The transformation of fibroblasts into myofibroblasts is initiated by signals such as TGFbeta as well as mechanical stimuli generated from forces resisting wound contraction (Stadelmann *et al.* 1998). This transformation is an example of cellular adaptation (Schmitt-Graff *et al.* 1993). One week after wounding a portion of the wound fibroblasts undergo a transformation into specialized myofibroblast cells that contain alpha SM-actin (Stadelmann *et al.* 1998). The SM actin is characteristic of myofibroblasts. Activated cells with a myofibroblast phenotype typified by expression of alpha SM-actin are important mediators of increased collagen synthesis and deposition in chronically inflamed tissue (Van Tol *et al.* 1999). Alpha SM-actin expressing myofibroblasts persist in hypertrophic scars and in fibrotic lesions of many organs in ECM accumulation. Granulation tissue fibroblasts, or myofibroblasts, also develop several ultrastructural and biochemical features of smooth muscle cells, including the presence of microfilament bundles in addition to the expression of alpha-SM actin (Schmitt-Graff *et al.* 1993).

Myofibroblasts are numerous at wound sites because they are able to form secure intracellular attachments via desmosomes and maculae adherens (Stadelmann *et al.* 1998). The

myofibroblasts are interconnected by gap junctions and are affixed to the ECM via intercellular microfilaments that are continuous with the fibronectin fibers about their perimeter (Schmitt-Graff *et al.* 1993). These stable organized contacts allow for the myofibroblasts to take part in contraction, an important part of wound healing (Fritsch *et al.* 1997). Myofibroblasts have structural and biological properties intermediate between those of resident fibroblasts and those of smooth muscle cells suggesting that they produce the force of wound contraction. When contraction stops and the wound is fully epithelialized, myofibroblasts containing alpha-SM actin disappear, most likely through apoptosis. The scar becomes less cellular and composed of typical fibroblasts with well developed ER but with no more microfilaments (Schmitt-Graff *et al.* 1993).

Wound Contraction:

Contraction is a myofibroblast-dependent process that occurs after fibroplasia and remodeling. Fibroblasts rearrange the ECM which allows for the generation of strong force and prepares it for contraction by myofibrobalsts (Harris *et al.* 1981). As previously mentioned, some fibroblasts transform into myofibroblasts about one week after wounding. There may also be migration of resident myofibrobalsts. The important characteristic of the myofibroblats is alpha SM-actin which allows for contractile forces to be generated by the myofibroblasts (Stadelmann *et al.* 1998, Eddy *et al.* 1988).

Myofibroblast contraction is different because it is strictly non-muscle. In smooth muscle cells, which are closely related to myofibroblasts morphogenically, there is a type of myosin that is distinct from the myosin found in non-muscle cells (Eddy *et al.* 1988). The contraction that is generated is transmitted across the ECM which is rich in collagen and fibronectin after fibroplasia. The myofibroblasts cells produce an extensive contractile apparatus prior to

contraction (Gabbani *et al.* 1978). The previously mentioned gap junctions that exist between the myofibroblasts are essential to the orchestrated contraction process that takes place. The gap junctions allow for synchronized contraction and often increase at the same time that contractile elements do. These gap junctions are often increased after stressful changes in the environment such as the infiltration of immune cells (Gabbani *et al.* 1978).

The actual action of contraction is induced by serotonin, angiotensin, vasopressin, bradykinin, epinephrine and prostaglandin F1 alpha (Schmitt-Graff *et al.* 1993). The entire granulation bed of myofibroblasts contract simultaneously and collagen is deposited and then crosslinked. This crosslinking creates a scaffolding holding the contracted wound stable (Stadelmann *et al.* 1998). It creates a stable environment that allows for the slow contraction of the wound.

Foreign Body Response:

There is a more complex healing reaction occurring in the tissue that was obtained from the porcine duodenum. Since the tissue was mechanically injured over a period of time, the foreign body reaction was elicited. The foreign body reaction occurs after the inflammation and wound healing stages (Ward 2006). It is a primary reaction of the non-specific immune system and occurs to create a barrier between the body and the foreign object (Luttikhuizen *et al.* 2006). The foreign body response occurs instead of resorption and reconstruction of the wound site. It is characterized by chronic inflammation, macrophages and foreign body giant cells, which are agglomerations of macrophages (Anderson *et al.* 2008). The formation of foreign body giant cells often occurs when the body is implanted with foreign materials (such as the barbs used in this experiment) if the surface allows for macrophage attachment (Anderson *et al.* 2008). The giant cells exist to phagocytose the implanted material as one collective mass. Since it usually

cannot, inflammation continues and the tissue encapsulates the foreign body. The collected tissue was in contact with metal barbs and so in compliance with the foreign body response, the tissue encapsulated the barb site with collagen through a specific process.

There are some differences between the FBR and normal wound healing. The first is that soon after the foreign body is in place, there is deposition of fibrinogen onto it (Ward 2006). As with the normal wound response, the blood vessels become more permeable and coagulation occurs. The fibrinogen, which is released by the liver to clot, collects on the surface of the foreign body. Complement and antibodies also tend to play a role because they adhere to the foreign body surface as well. The fibrinogen serves as a binding site for platelets as well as macrophages. The platelets will bind to the fibrinogen and a fibrin clot will form (Luttikhuizen *et al.* 2006).

The macrophages bind to the fibrinogen and the healing response continues. The macrophages, as previously mentioned, produce many substances including TGF-beta which causes the fibroblasts to produce pro-collagen.

Inflammation in the case of the foreign body response is chronic. Chronic inflammation will often include plasma cells as seen in the picture obtained. This chronic inflammatory response is usually confined to the site of the foreign body and is generally short in time (Anderson *et al.* 2008). The granulation tissue begins to form and is characterized by the same set of cells (fibroblasts and macrophages). The granulation tissue in this case will become the fibrous capsule formation (Anderson *et al.* 2008). The procollagen, fibronectin, and proteoglycans surround the implant and are produced by the fibroblasts and myofibroblasts (Luttikhuizen *et al.* 2006). The collagen becomes cross-linked as the wound healing continues. This collagen then creates a capsule around the foreign body inhibiting communication between

the foreign object and the bloodstream to prevent further responses (Ward 2006). The capsule thickness depends on many factors one of which is motion. With the intestines moving constantly through peristalsis, the collagenous capsule is rather thick. The fact that there is a sharp point also makes it more likely for a thicker capsule to form.

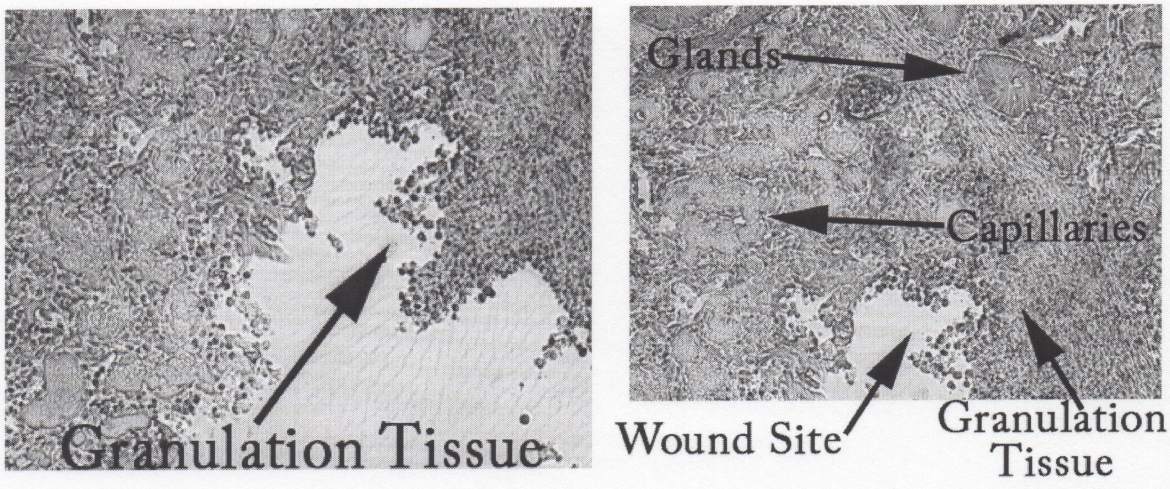
Another type of cell that is characteristic of the foreign body response is the mast cell which is responsible for releasing histamine (Zdolsek *et al.* 2007). Histamine is a vasodilator and an activator of nerve endings (causes pain sensation). This is very important to the inflammatory response to implanted materials. The histamine recruits macrophages and also increases the permeability of capillaries. This is important to the medical device industry because if histamine receptor antagonists were given to a patient, the inflammatory response may be lessened (Zdolsek *et al.* 2007).

The foreign body response appears to be the culprit in the collected samples. The tissue eventually encapsulates the barbs after they have irritated the area for over three days, as seen in the fourteen and ninety-four day samples.

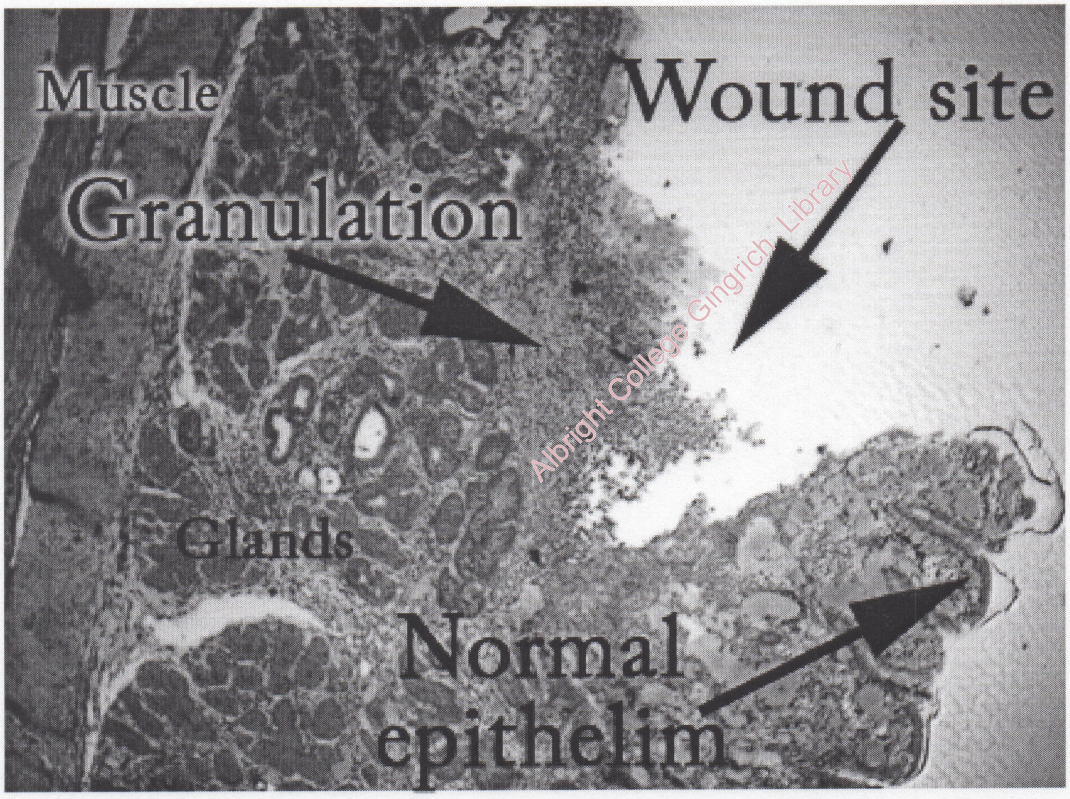
Results:

3 Day Sample

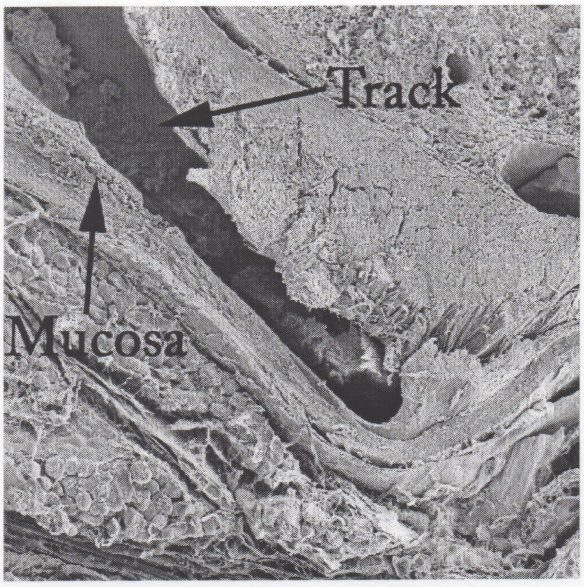
Light Microscope Hematoxylin & Eosin (100 x)



Light Microscope Hematoxylin & Eosin (40 x)

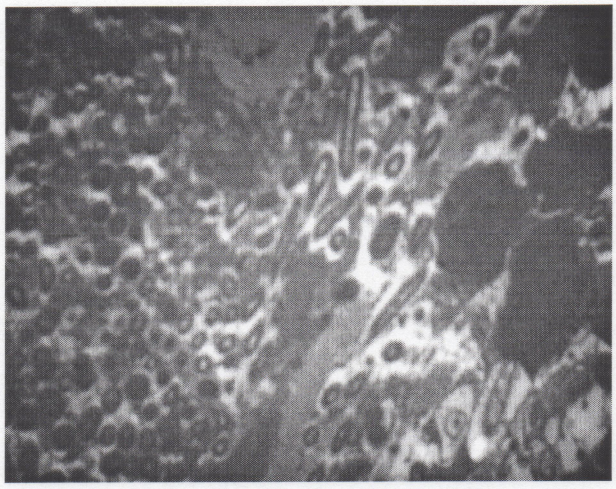


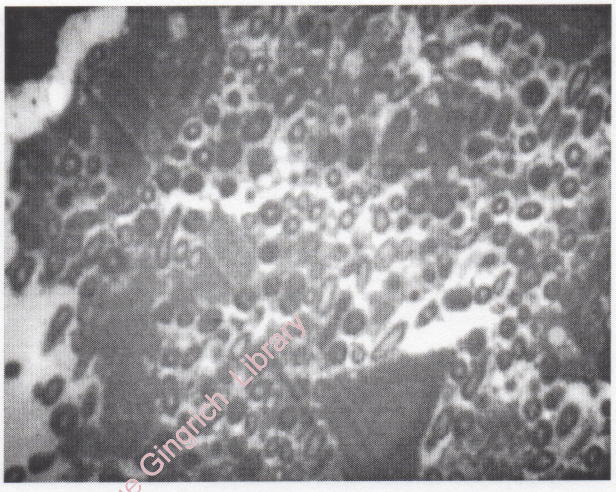
SEM (40 x)



SEM (100 x)

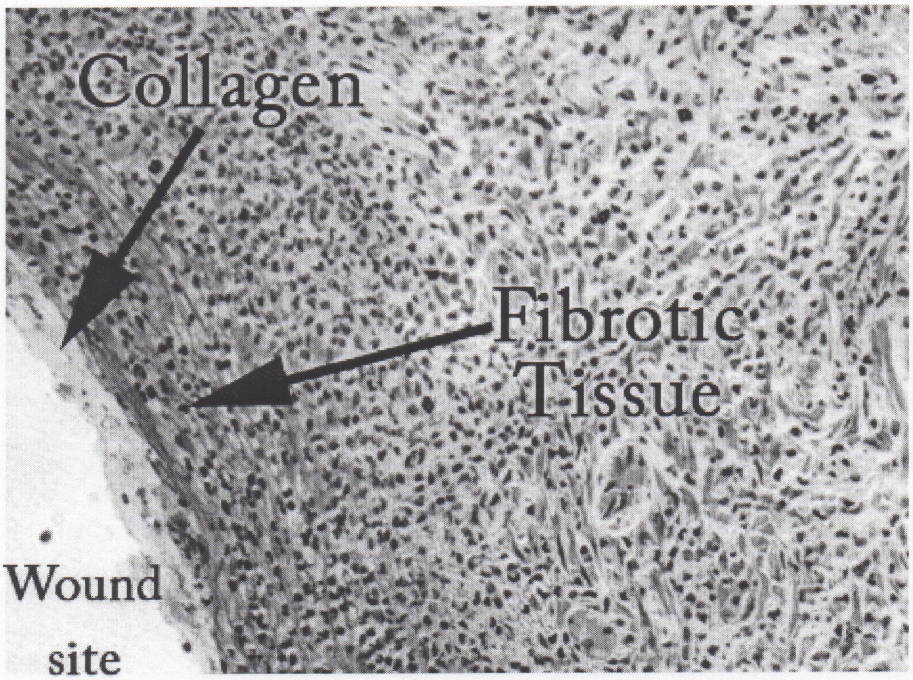
TEM (Uranyl acetate & lead citrate)



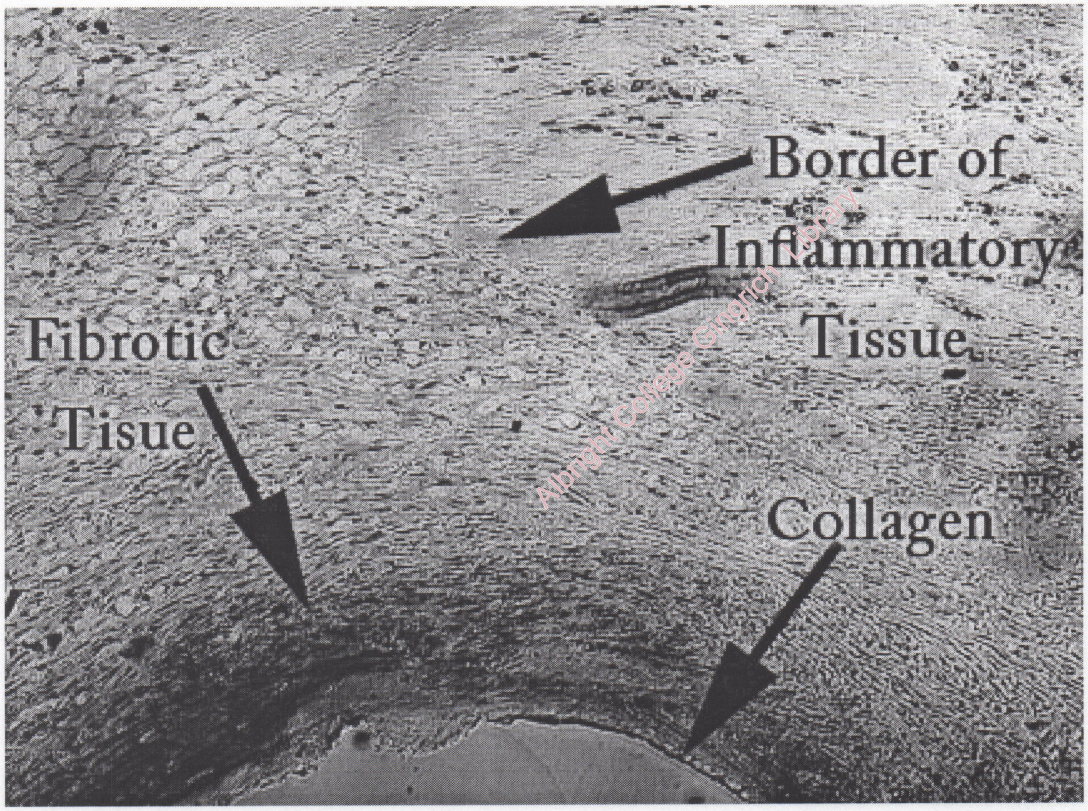


14 Day Sample

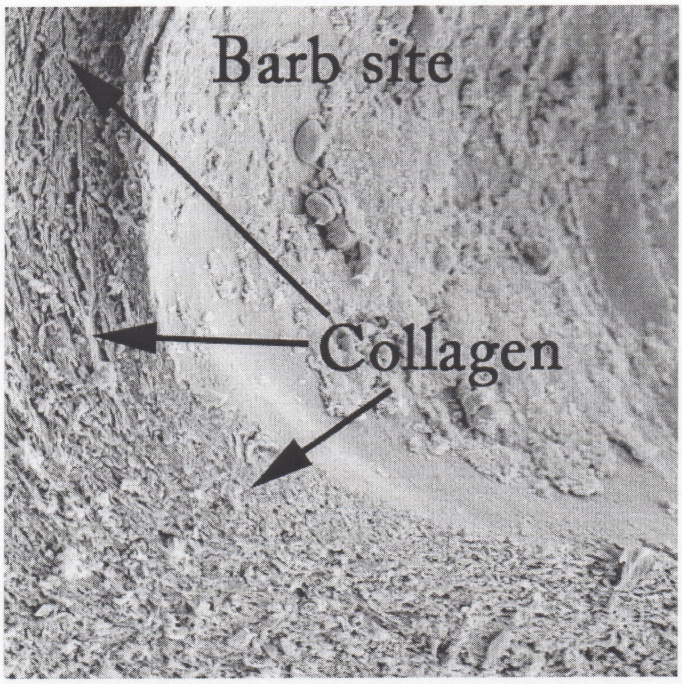
Light Microscope Hematoxylin & Eosin (200 x)



Light Microscope Massson's Trichrome Stain (200 x)

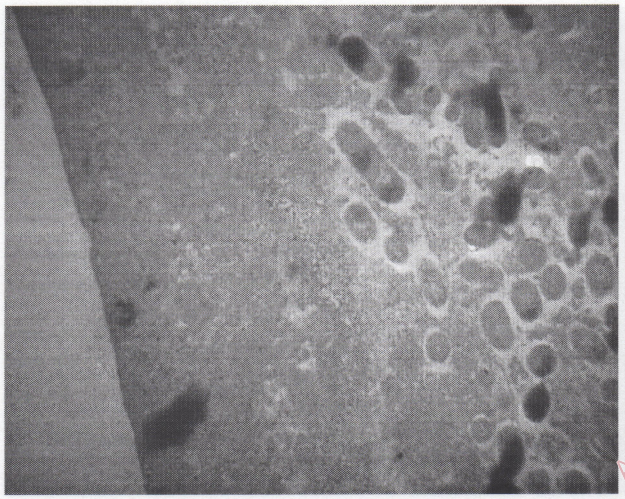


SEM (552 x)

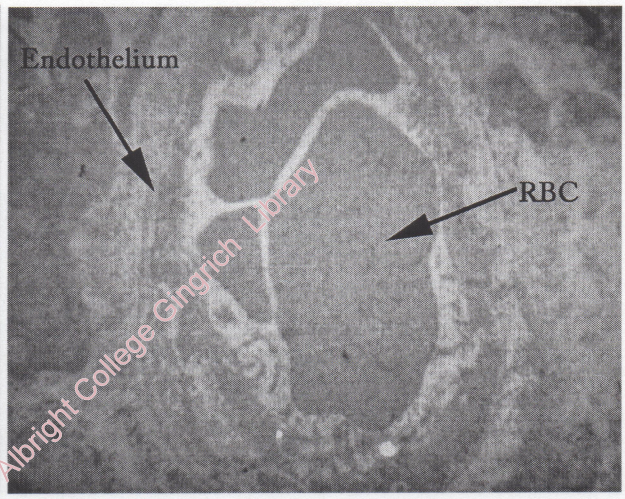


TEM (Uranyl acetate & lead citrate)

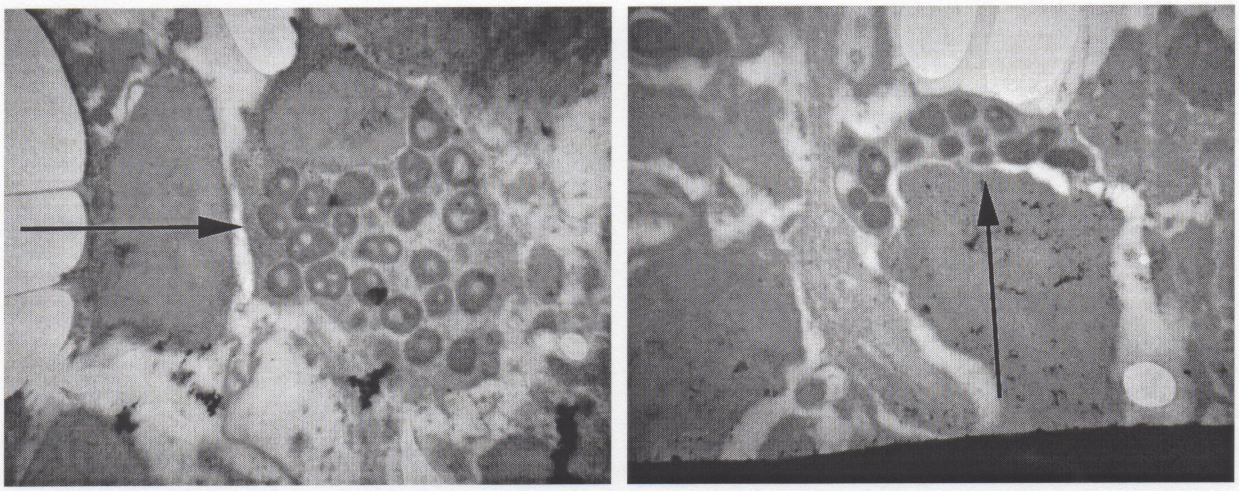
Border of barb site



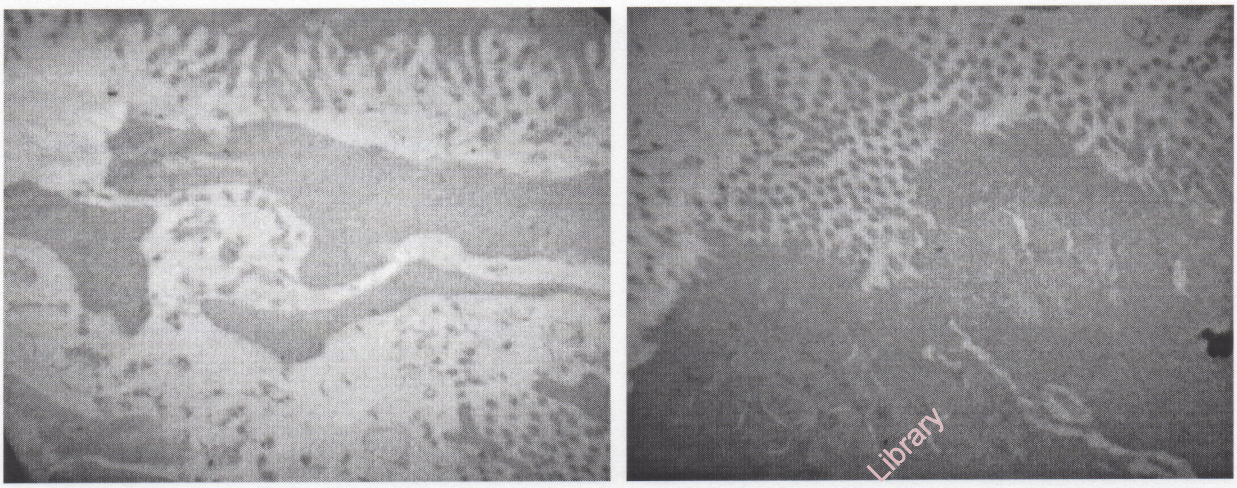
Capillary



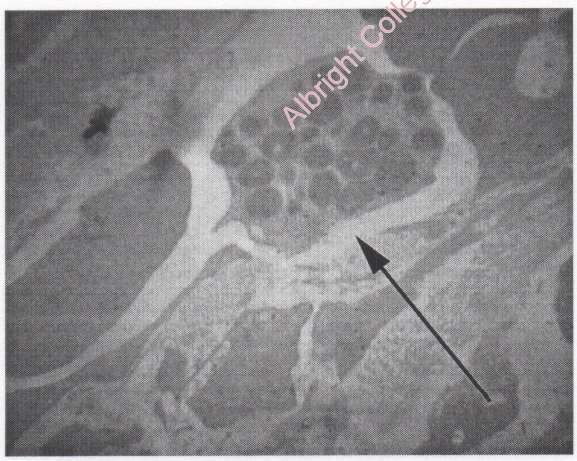
Macrophages



Microvilli

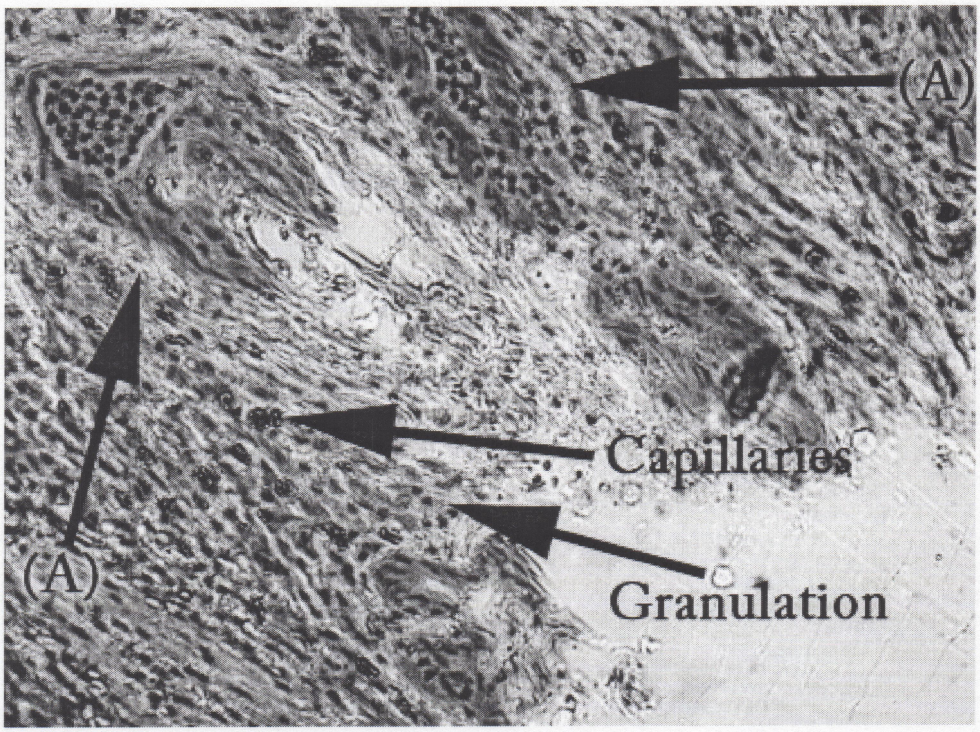


Basophil

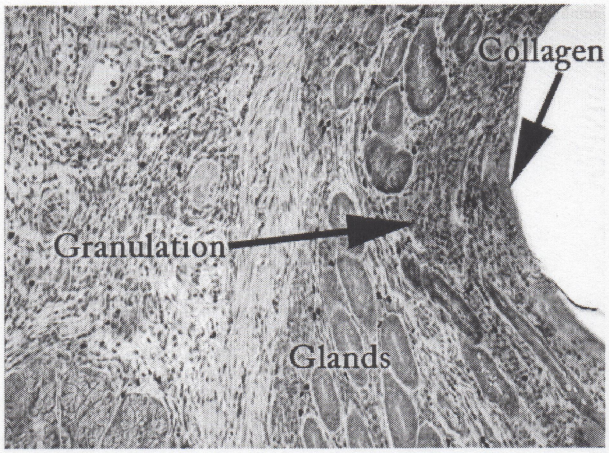


94 Day Samples

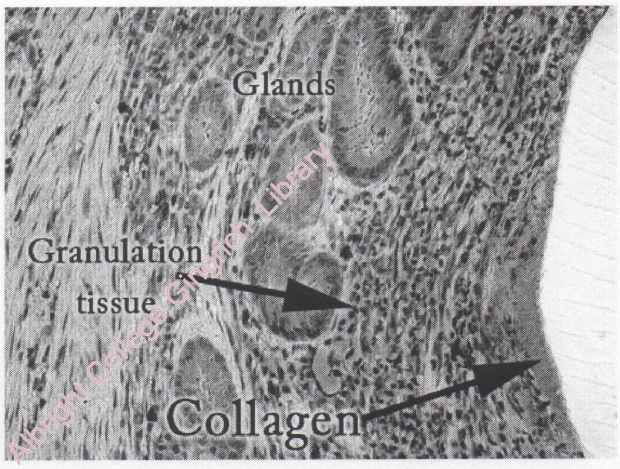
Light Microscope Hematoxylin & Eosin (200 x)



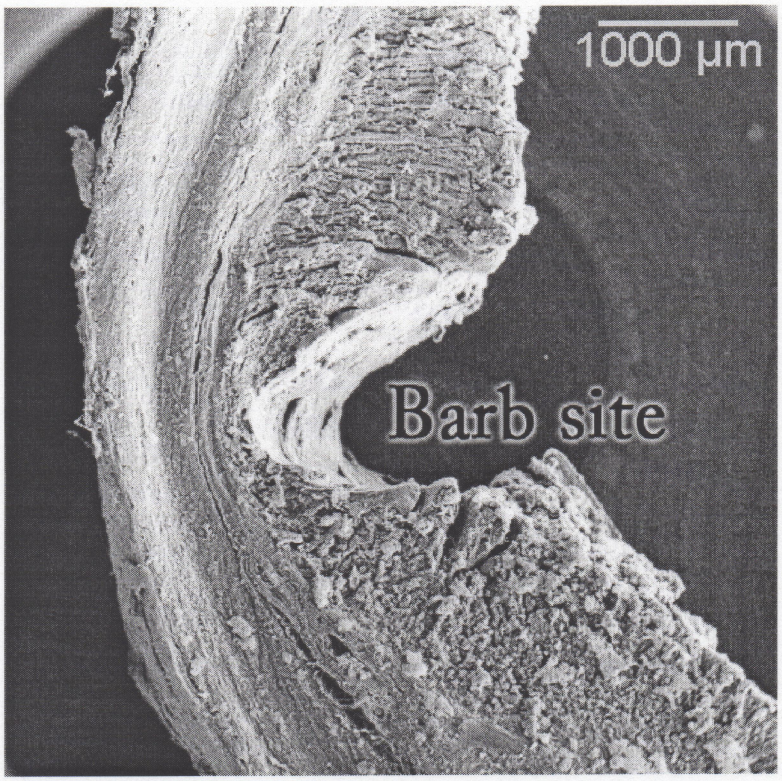
LM H&E (40 x)



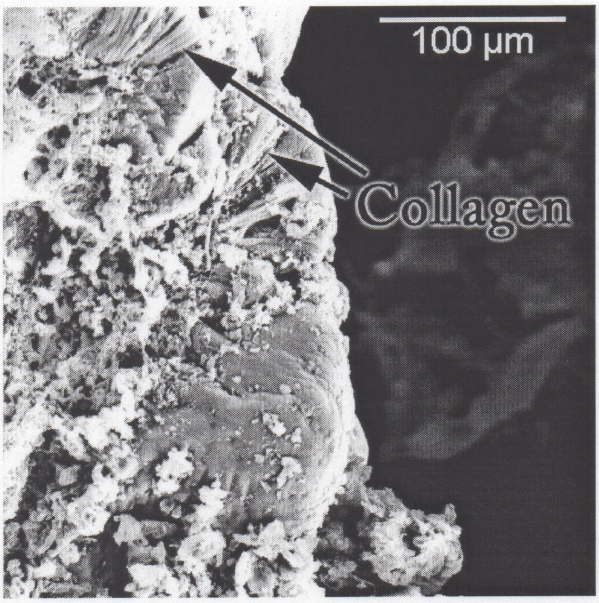
LM H&E (100 x)



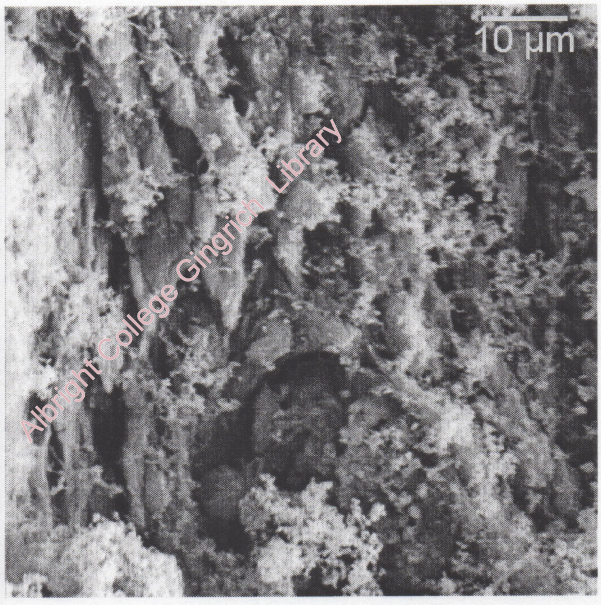
SEM (29 x)



SEM (502 x)

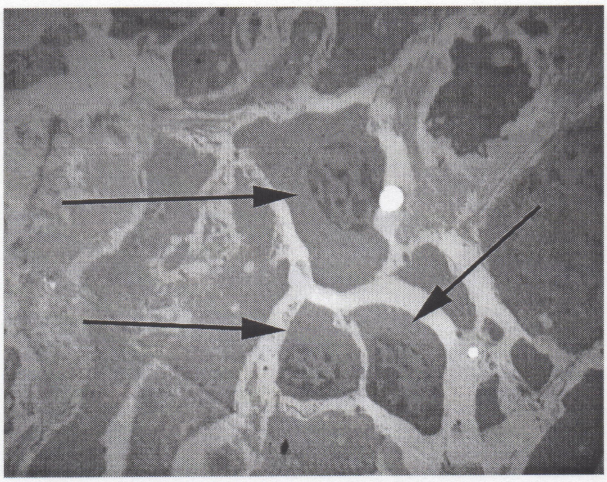


SEM (2330 x)

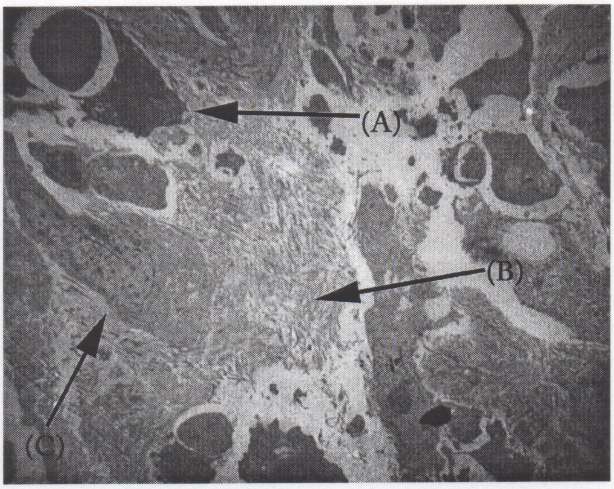


TEM (Uranyl acetate & lead citrate)

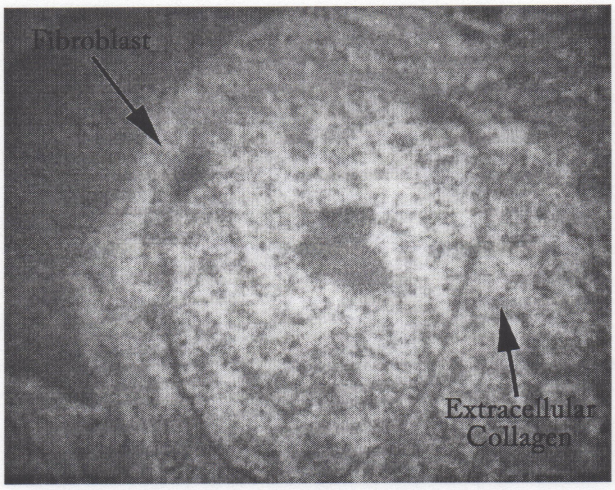
Plasma Cells



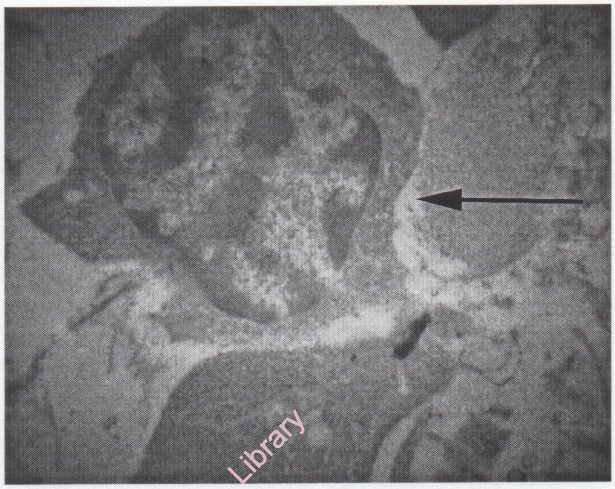
ECM



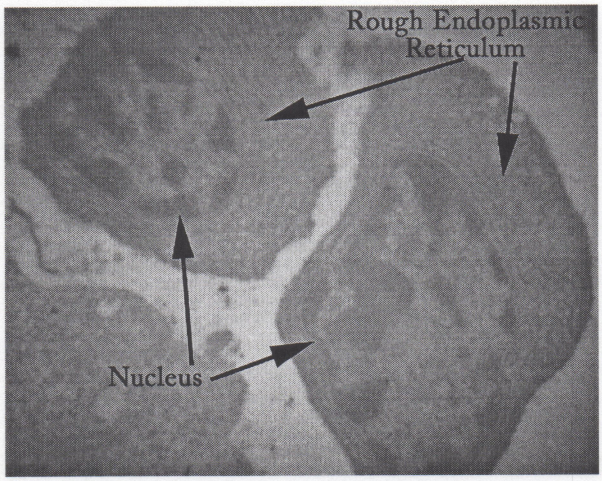
Fibroblast



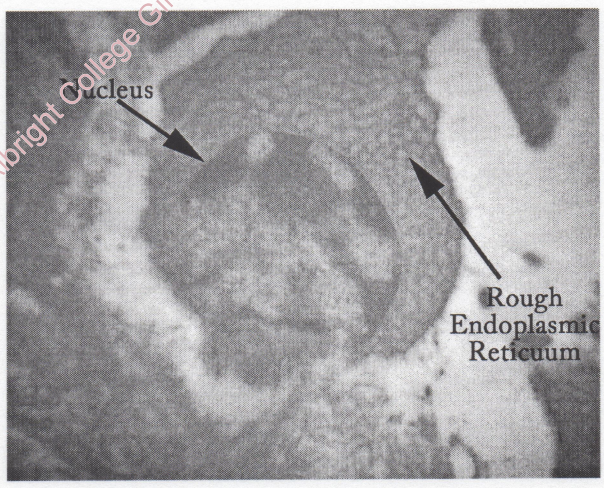
Neutrophil



Plasma cells



Plasma cell



Discussion:

3 Day Sample

Light microscope, SEM, and TEM slides of the three day sample depict characteristics of the early stages of healing; especially inflammation. There are certain activities that take place in the early response of the body and evidence of those processes can be seen in these slides. There is not a great representation of the later stages of healing and so it cannot be determined from the three day sample whether or not normal healing, fibrotic disorder, or foreign body response is acting. Through closer interpretation of all the slides, one can materialize a larger picture.

The light microscope pictures stained with hematoxylin and eosin show that there is an area of granulation tissue at the site of the barb. It is much different from the normal epithelium of the mucosa. The normal epithelia has villi and microvilli, both of which have been disrupted in the barb area. In the three day sample, the barb has not penetrated much past the mucosa and seems to have only disrupted the mucosal layer.

The granulation tissue is seen as the small, roundish cells that are clustered in no particular arrangement at the barb site. This granulation tissue is loose tissue that is often fibrotic due to the migration of fibroblasts into the area. There are also many capillaries seen in the granulation tissue which are present in the light microscope slides. The capillaries as previously mentioned make it possible for various immune cells to invade the area.

The SEM pictures show the layers of the duodenum that have been disrupted by the barb. As seen in the light microscope pictures, only the mucosa is disturbed while the muscularis mucosa and submucosa appear only to be distorted.

The TEM pictures from the three day samples were quite interesting. There are many small round and rod-like objects on the slide. After much research, the only frequently occurring

and feasible explanation is the presence of *Helicobacter pylori* which often resides in the stomach and duodenum of organisms. It is a rod-shaped flagellated bacterium which fits the TEM. There are both round and oblong shapes about similar sizes that are very small compared to body cells. The round shapes are cross sections of the rods. See Appendix A for comparison. In the United States, thirty percent of adults are affected by *Helicobacter pylori* and many are asymptomatic.

14 Day Sample

The 14 day sample showed a more mature wound site complete with the formation of collagen at the barb site. The collagen seemed to mimic the collagen encapsulation seen in a foreign body response. The collagen that is being deposited is most likely collagen type III which will eventually be replaced by collagen type I (Stadelmann *et. al* 1998). The tissue directly below the collagen appeared to be fibrotic and thus may be due to chronic inflammation resulting in fibrosis. The fibrosis is a normal response to mucosal damage but in the case of a barb being implanted for fourteen days, the response may be chronic inflammation. This also leads to the foreign body response which also makes sense since the barb is a foreign body in the mucosa of the duodenum. The presence of collagen at the barb site also supports this theory.

The Masson's Trichrome stained slide further typifies the tissue seen. The collagen fibers are seen in the slide as the dark areas. The stain did not come out as protocol as the collagen fibers are supposed to be blue, but the slides may have not been rinsed thoroughly during the procedure. The Masson's Trichrome slide does however depict a distinct line of where the inflammation begins. As discussed previously, it is the work of many cytokines that keep the inflammation in check. This border serves as a place where the organized healthy tissue on the

right meets the inflamed tissue on the left. The inflamed tissue appears differently due to the alterations that have occurred in the ECM. The infiltration of cells and the vascularization changes the tissue. Since the tissue does appear to be still inflamed, it serves as more evidence towards chronic inflammation.

The SEM pictures of the fourteen day sample show signs of collagen formation as well. This is supported by the appearance of collagen in Appendix B. Collagen is fibrous and stringy in appearance and can also appear to be branched or networked. This is visible at the edge of the barb site in the SEM pictures. This further supports the foreign body response in which collagen cross-links into a capsule around the foreign body.

The TEM slides of the fourteen day sample shows many of the characteristics of healing tissue that would be expected. There are capillaries which are a characteristic of inflammation (chronic). The capillaries are present to allow more immune cells to infiltrate the area. There are also macrophages that will consume foreign particles and fight disease. They will also send out the necessary signals for other cells to continue in the healing process. Basophils are also white blood cells involved in keeping the area clean of infection.

The microvilli that are present do show that some mucosa is in tact. This intact mucosa could be the area immediately outside of the barb affected area or it could be a healed wound that was more superficial. The microvilli belong to the epithelial layer and may be at the periphery of the wound site (Gabbiani et. *al* 1978).

94 Day Sample

The light microscope slides from the 94 samples infiltrated very well and provided more information about the healing taking place. There is still granulation tissue that holds some

capillary beds supporting the idea that chronic inflammation is at work here. There are also two structures (labeled A) in the light microscope images. At first glance, white blood cells came to mind, but their massive size leads to the suggestion that they may be the foreign body giant cells seen in the foreign body reaction. These giant multinucleated cells are masses of macrophages that join together to fight off the foreign body, in this case the barb. Reference pictures are seen in Appendix C. It would be expected that the barb would elicit a foreign body response and so these giant cells may very well be present, especially 94 days after the wounding occurred.

The other LM pictures show a full cross section of the affected area. There appears to be a build-up of collagen at the barb site which is seen as the dark purple homologous area. Directly below there appears to be fibrosis and the inflammatory response or foreign body response continuing. The foreign body response includes chronic inflammation as seen in the slide. Beyond the submucosa, the tissue appears normal.

The SEM slide shows that there is no damage beyond the submucosa. It also shows the presence of collagen at the barb site. The collagen appears as fibrous substance and sometimes forms networks. This collagen is important in the foreign body response to encapsulate the foreign body so that it no longer elicits and immune response.

The TEM pictures support the foreign body response theory since there are specific cells associated with it. There are numerous plasma cells at the barb site in the 94 day tissue. They release cytokines and continue the immune response which supports the idea that this is chronic inflammation. There are also collagen fibers seen in the ECM (B) they are most likely being secreted by the fibroblast (C). There may also be a myofibroblast to the right of the collagen. The cell has a spindle shape and prominent nucleus as well as what appears to be an RER at the

caudal end. The presence of myofibroblasts would allude that contraction would begin occurring by the movement of the microfilaments and their interaction with the ECM.

There is another fibroblast seen in the TEM pictures which leads to the fibrotic healing phase. The fact that fibroblasts are producing the collagen still means that the fibrotic stage is happening.

Neutrophils are also on site. They are the most abundant type of white blood cell in the body. In accordance with (Stadelmann *et. al* 1998), these types of cells are seen at day 6 in normal healing and so seeing them this late also supports the idea of chronic inflammation.

Conclusion:

By examining the tissue samples at three time intervals, it can be concluded that there is significant evidence that these barbs implanted in the duodenum over time cause chronic inflammation followed by the foreign body response. The techniques used were effective in displaying the effect on the tissue at a superficial level as well as a cellular level. After obtaining an understanding in the healing process and the different outcomes of a wound, it can be concluded that there is support for the inflammation process followed by fibrosis and finally the foreign body response.

The appearance of the tissue in the LM pictures shows definite granulation tissue at the site of the wound as well as the possibility of giant cells in the later time interval. The later intervals also show the possibility of collagen capsule formation. The SEM pictures also point to a collagen capsule formation.

The TEM pictures provide the most support for the healing process since the types of cells that are present communicate exactly what is happening in the tissue. The appearance of

fibroblasts especially confirms the fibrotic stage of healing. The various white blood cells confirm the inflammatory stage continuing in the tissue. The appearance of the ECM is chaotic, and much different than healthy tissue, which also supports the idea that inflammation is occurring. The appearance of capillaries also shows that inflammation is still occurring. The microvilli that are present are compliant with the periphery cells in most wounds. The appearance of collagen fibers in the TEM pictures further supports the information gathered from the SEM and LM pictures.

Appendix A

Helicobacter pylori (TEM)

(http://www.scielo.br/img/revistas/jbpml/v41n2/a09fig02.gif)

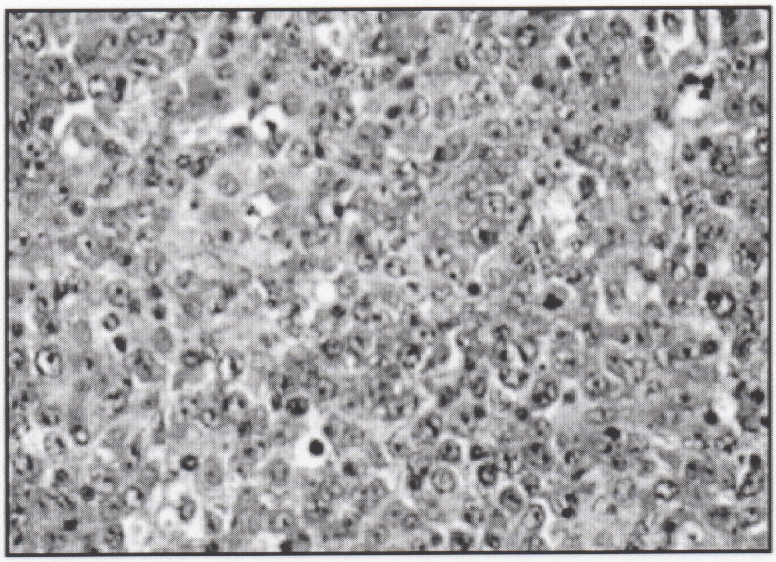
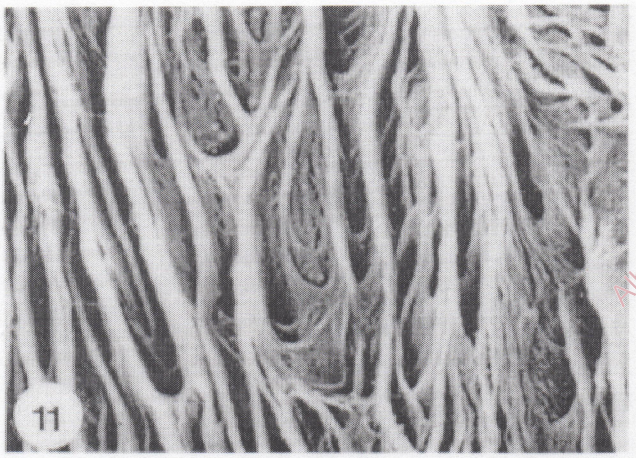


Figura 2 – *Linfoma difuso de grandes células B (alto grau). Células predominantemente não-clivadas, com diferenciação centroblástica (HE 400x)*

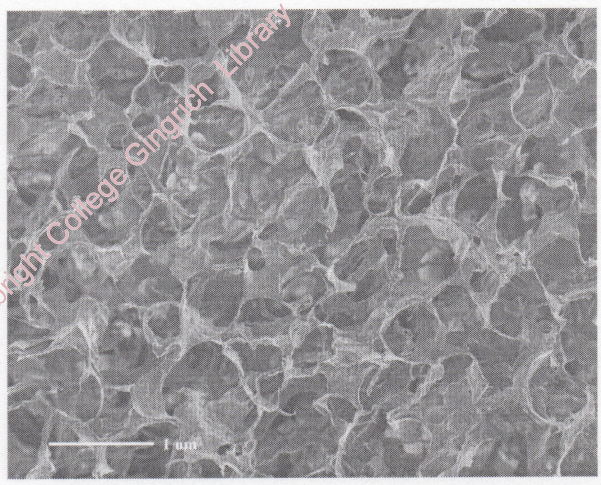
Appendix B

Collagen (SEM)

(http://www.scielo.cl/fbpe/img/rca/v15n1/Image5.jpg)



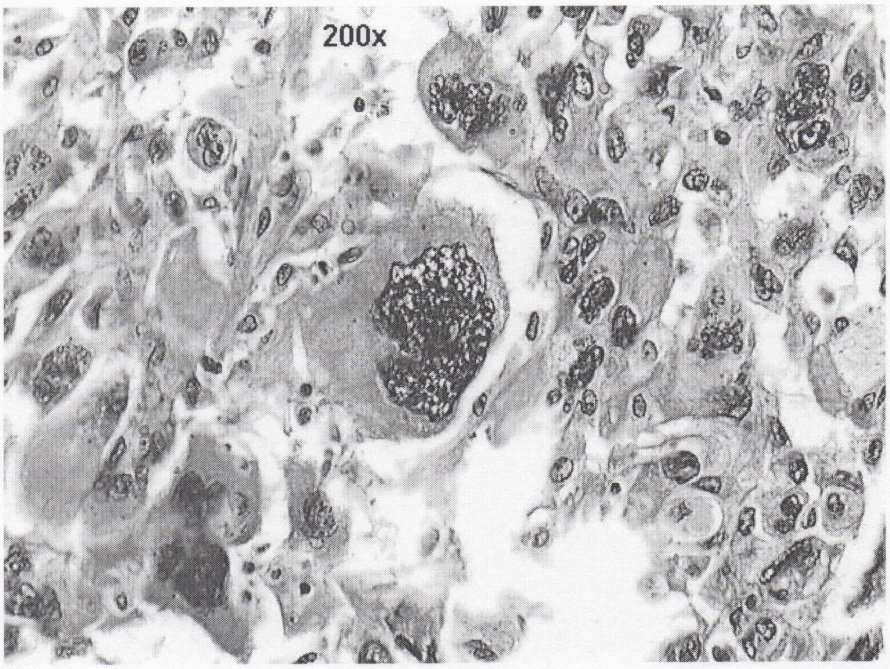
(http://www.seas.upenn.edu/nanotechfacility/images/collagen.jpg)



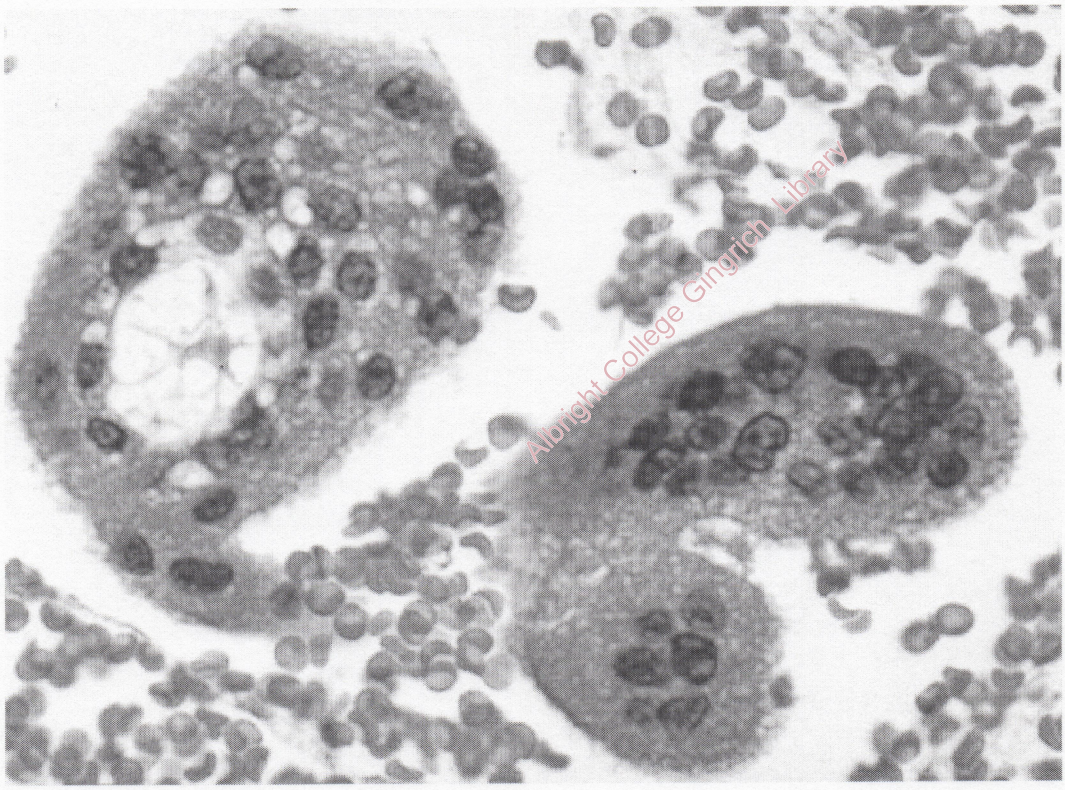
Appendix C

Foreign Body Giant Cells (light microscope)

(http://www.geocities.com/jcprolla/86024e_HE_200x.jpg)



(http://granuloma.homestead.com/files/asteroid85.jpg)



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