ANALYSIS OF IMMUNOLOGICAL RESPONSES OF MURINE MACROPHAGES TO HYDROXYAPATITE

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ABBREVIATIONS

ALCAP	Aluminium calcium phosphate
ADP	Adenosine diphosphate
ACP	Acid phosphatase
BSA	Bovine serum albumin
CaP	Calcium phosphate
CB	Cytochalasin B
CL	Chemiluminescence
COX-2	Cyclooxygenase- 2
CGMP	Cyclic guanosine 3′:5′-monophosphate
CMF	Cell movement factors
CR	Complement receptors
DNA	Deoxyribonucleic acid
DBM	Demineralized bone matrix
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl Sulfoxide
eNOS	Endothelial nitric oxide synthase
ECW	Extracellular matrix
FCS	Fetal calf serum

g	grams
HA	Hydroxyapatite
HAC	Hydroxyapatite crystals
IgG	Immunoglobulin G
iNOS	Inducible NOS
IL	Interleukin
INFγ	Interferon gamma
ICE	IL-1β converting enzyme
KDa	Kilodalton
LDH	Lactate dehydrogenase
LSP	Large size polyethylene
LTCP	Large particle size tricalcium phosphate
LPS	Lipopolysaccharide
L-NIL	L-N^6-(1-iminoethyl)lysine hydrochloride)
M	Molar
µm	micrometer
mg	milligram
µg	microgram
µl	microliter

ml	milliliter
µM	micromolar
mM	millimolar
mRNA	Messenger ribonucleic acid
MDA	Malondialdehyde
MSUM	Monosodium urate monohydrate
MMP-1	Metalloproteases-1
NOS	Nitric oxide synthase
NMA	N^G-methyl-L-arginine
NO	Nitric oxide
nNOS	Neural NOS
nm	nanometer
nM	nanomolar
ng	nanogram
NCP	Nitrocellulose membrane
OD	Optical density
PBS	Phosphate buffered saline
pg	picogram
PSG	Pollen starch granules

PGE2	Prostaglandin E2
PLC	Phospholipase C
PKC	Protein Kinase C
PI	Phagocytic index
PMN	Polymorphonuclear leukocytes
RAW	Murine macrophage cell line
SDS	Sodium dodecyl sulfate
SEM	Scanning electron microscopy
SPE	Submicron polyethylene
STCP	Small particle size tricalcium phosphate
SiC	Silicon Carbide
Ti	Titanium
Tris-Hcl	Tris(hydroxymethyl)aminomethane-hydrochloride
TCP	Tricalcium phosphate
TEM	Transmission electron microscopy
TNF	Tumor necrosis factor
TGF-β1	Transforming growth factor beta 1
TLRs	Toll like receptors
VD3	Vitamin D3

Abstrak

Analisis gerak balas imunologi makrofaj murin terhadap hidroksiapatit

Tujuan kajian ini adalah untuk menganalisis tindak balas immunologi hidroksiapatit (HA) ke atas sel RAW264.7 dengan menentukan fagositosis terangsang HA. Parameter imunologi yang dikaji termasuk peranan polimerisasi mikrotubul dan aktin, protein kinase-C (PKC), nitrik oksida (NO), interleukin-1β (IL-1β) dan faktor nekrosis tumor-α (TNF- α).

Keputusan menunjukkan bahawa HA difagositosis oleh sel warisan makrofaj (sel RAW264.7) pada tempoh eraman yang berbeza. Peningkatan adalah selaras dengan peningkatan PI di dalam sel yang telah dirangsang dengan HA tempoh eraman. Analisis TEM telah menunjukkan bahawa partihel HA telah ditelan oleh sel dan berada di dalam vakuol sel berkenaan. Cytochalasin B atau colchicine didapati secara signifikan merencat aktiviti fagositosis sel ke atas kedua-dua partikel HA dan manik lateks dalam bentuk bergantung dos. Sel yang dirangsang pula menghasilkan enzim PKC seawal awal fagositosis iaitu pada masa 7 minit. Sel yang telah dirangsang dengan Bisindolylmaleimide didapati labih sedikit partikel dalam bentuk menelan bergantung dos. Penghasilan NO daripada

sel-sel yang dirangsang oleh HA adalah lebih rendah berbanding sel yang dirangsang oleh manik lateks. Ekspresi iNOS di dalam kedua-dua sel yang dirangsang oleh manik lateks dan juga HA telah dikesan pada 7, 15, 30 dan 60 minit tempoh eraman. L-arginin meningkatkan kedua-dua fagositosis dan dan penghasilan NO oleh sel yang dirangsang oleh HA tetapi sebaliknya L-NIL merencat aktiviti tersebut. HA merangsang sel-sel untuk merembes kedua-dua IL-1β dan TNF- α dalam bentuk bergantung masa. Dengan kehadiran antibodi anti-murine IL-1β dan TNF- α aktiviti fagositosis oleh sel RAW264.7 yang dirangsang oleh HA didapati dikurangkan dengan signifikan.Olehitu, keputusan kajian ini mencadangkan bahawa partikel HA boleh merangsang aktiviti fagositosis makrofaj murine (RAW264.7 cells) melalui mekanisme yang berkait dengan polimerisasi aktin dan miktotubul, yang mungkin berperantarakan enzim PKC. NO juga mungkin memainkan peranan penting dalam fagositosis terangsang HA oleh sel-sel RAW264.7 yang mungkin juga bergantung kepada IL-1β dan TNF- α.

Abstract

The aim of the present study was to analyze the immunological response of hydroxyapatite (HA) to RAW264.7 cells by determining HA-induced phagocytosis. Immunological parameters included in this study were the role of polymerization of actin and microtubule, protein kinase-C (PKC), nitric oxide (NO), interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α).

The results showed that HA were phagocytosed by murine macrophage cell line (RAW264.7 cells) at different incubation time. Increased PI of HA-treated cells were paralleled with increased period of incubation. TEM (Transmission electron microscopy) analysis showed that HA particles were engulfed by the cells and located within cell vacuoles. Cytochalasin B or/and colchicine significantly inhibited phagocytic activity of the cells to both HA particles and latex bead in a dose-dependent fashion. Stimulated cells produced PKC enzyme right at the early stage of phagocytosis at 7 minutes. Pre-treated cells with Bisindolylmaleimide ingested fewer particles in a dose dependent fashion. NO production was less by HA stimulated cells than by latex bead-stimulated cells. Inducible nitric oxide synthase (iNOS) expression in both latex bead- and HA-stimulated cells was observed at 7, 15, 30 and 60 minutes of

incubation time. L-arginine enhanced but L-NIL inhibited both phagocytosis and NO production by HA-stimulated cells. HA stimulated the cells to release both IL-1 β and TNF-α in a time-dependent fashion. In the presence of anti-murine IL-1 β and TNF-α, HA-induced phagocytic activity by RAW264.7 cells was significantly reduced. Therefore, the results of the present study suggest that HA particles may induce phagocytic activity of murine macrophages (RAW264.7 cells) in an actin and microtubule polymerization dependent mechanism, which may be mediated through PKC enzyme. NO may play a crucial role in HA-induced phagocytosis by RAW264.7 cells which may also depend on IL-1 β and TNF-α.

CHAPTER 1

INTRODUCTION

1.1 Bone Replacement Materials

Effective repair of bone defects of the skull and facial bones secondary to traumatic, inflammatory, neoplastic, or iatrogenic lesions had always been a challenging problem to surgeons. The first attempt to use bone for cranial reconstruction was made as far back as 1670 by Van Meekren (Van Meekren, 1682). In 1821, Von Walther performed the first autologous bone graft (Von Walther, 1821), and in 1867 Ollier emphasized the role of periosteum in bone regeneration (Ollier, 1867).

Bone grafting is intended to bridge bone defects there by establishing native bone architecture. One of the most effective methods of bridging bone defects is by the use of autograft. However several limiting factors such as operative time, blood loss, postoperative pain, length of hospital stay, and cost are the factors, which are not in favor of autografts. On the other hand fresh or frozen allografts are available in different forms with strong mechanical properties. However, these have the disadvantage of inducing bacterial and viral

infection in the recipient due to the infected graft, which may lead to the possibility of graft rejection. All these facts forced the researches to indulge in developing the synthetic materials for bone replacements (Ilan and Ladd, 2002).

Demineralized Bone Matrix (DBM) is an allograft, which is used commonly nowadays and is available as dry, moldable or injectable forms. It is in the form of powder, paste, or putty, using a carrier that renders it suitable for placement. However the demineralization leads to denaturation of the protein matrix in the bone because of chemical and radiation treatment resulting in weakening of the bone and the bone formation potential. Growth factors might survive processing, although their actual presence is not very clear (Ilan and Ladd, 2002).

Endobon is a bovine-derived ceramic consisting of cancellous bone, sintered and processed to eliminate all but the mineral components (Werber *et al.*, 2000). This material lacks in immunogenicity and has a poor remolding capacity. It is currently used in the treatment of craniofacial fractures and bone defects. Coralline HA (Pro Osteon, Interpore Cross International, Irvine, CA) is coral that is thermo chemically treated with ammonium phosphate, which demonstrates

porosity similar to bone and finds its application in the treatment of fractures (Wolfe *et al.*, 1999).

Alloplastic materials are synthetic materials that are used in reconstructive dental and orthopedic surgery. The source from which these materials are derived were of non-human, non-animal, and hence, non-organic. Since these materials are of non-organic source they are easily available for reconstructive work and have an added advantage of avoiding donor scar and infection in the recipient as a result of contaminated graft from the donors. However for the alloplastic material to be clinically successful, it must be biocompatible, implying an acceptable interaction between the host and the implant material. These alloplastic materials such as solid silicone, polytetrafluoroethylene, polyethylene and acrylic when implanted into defected bone, they are generally encapsulated by a fibrous tissue, which is initiated by a host inflammatory response (Anderson and Miller, 1984). As a result of this response, the implant materials do not adhere to bone and this is a critical problem in their use in bone repair.

Bioactive ceramics that spontaneously bind to and integrate with bone in the living body have been investigated (Kokubo *et al.*, 2003). Various types of bioactive ceramics have been developed over the last three decades. Among these the main

bioactive ceramics used clinically are hydroxyapatite (HA) (Jarcho *et al.*, 1977), HA micro crystals (Fukuchi *et al.*, 1995), Calcium phosphate (CaP) ceramic (Benahmed *et al.*, 1996b) etc.

Macrophage actively phagocytosed the HA micro crystals. However, no damage was observed in macrophages exposed to HA micro crystals by transmission electron microscopy (TEM). Macrophage in the presence of HA micro crystals showed less acid phosphatase (ACP) and lactate dehydrogenase (LDH) activity and higher intracellular calcium content than those in the presence of calcined HA and alumina. HA micro crystals as well sintered HA were reported to have excellent biocompatibility to macrophages (Fukuchi *et al.*, 1995).

Studies have been conducted on an ultrastructural scale to determine the specific behavior of human monocytes with regard to CaP ceramic (Benahmed *et al.*, 1996b). Phagocytosis of CaP coincided with autophagy and accumulation of residual bodies in the cells. Addition of HILDA/LIF leukemia inhibitory factor to these cultures induced a very marked decrease in phagocytotic activity on CaP crystals. Autophagy was reduced, and residual bodies were absent

Studies on the evaluation of the importance of particle characteristics on cytokine production by human monocytes *in vitro* demonstrated that needle shaped particles induced larger production of tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6) and interleukin-10 (IL-10) by cells as compared to spherical and indifferent shaped particles (Laquerriere *et al.*, 2003b). To a less extent, the smallest particles induced an increase in the expression and production of the cytokines.

In view of these limitations in using autogenous and allogenic bones as replacement materials, alloplastic implant materials offer an excellent substitute in soft and hard tissue replacement and repair (Eppley, 1999). The ranges of materials included are dimethylsiloxane (Silicone), polytetrafluoroethylene, polyethylene, polyseters, polyamides, acrylic, metals, cynoacrylate adhesives and calcium phosphates.

Ilan and Ladd (2002) described several minerals/ ceramics such as HA, corraline HA granules or blocks, calcium sulfate pellets, β-tricalcium phosphate, bioactive glass, polymer implant with bioactive glass were osteoconductive solid formulations that were used as bone implant material.

1.2 Hydroxyapatite (HA)

HA ceramic was found to have chemical compositions closely resembling that of mineral phase of natural bone (Liu, 1998). Implants composed of CaP have been available as bone replacement/augmentation materials for more than 20 years (Jarcho, 1981). CaP materials are not osteoinductive by themselves, but they do provide a physical substrate on to which new bone from adjacent surfaces may be deposited and guided into area occupied by the material (Alexander, 1987).

Currently available CaP materials were manufactured as HA with a chemical formula of $Ca10(PO_4)_6(OH)_2$. It can be manufactured either as ceramic or as non ceramic apatites and can be formed into a wide variety of physical configurations (Yuen *et al.*, 1994). Ceramic HA were made from crystals that are sintered at high temperatures into a hard nonresorbable solid. They were available as dense granules or blocks in the early 1980s and were used in maxillofacial reconstruction, particularly alveolar ridge augmentation (Frame *et al.*, 1987). The dense form of the granules was prone for migration before significant fibro-osseous in growth, and the dense blocks were difficult to

shape and were prone for extrusion. Therefore, dense HA were replaced by a different physical structure.

Porous HA were based on the structure of marine corals (calcium carbonate skeleton), which have interconnecting porosity of a size varying from 50 to 200 μm that permitted fibrovascular and osseous growth with the potential for cell-mediated resorption and osseous replacement (Holmes and Hagler, 1988). Of the available porous HA forms, the granules have achieved the greatest current use as an augmentation material for the craniofacial skeleton (Byrd *et al.*, 1993). The block forms were used primarily as an interpositional graft material in facial skeletal osteotomies (Salyer and Hall, 1989).

In dentistry, HA is used as a bone replacement material in surgical work. Study undertaken to evaluate the histological effect of HA–collagen on twenty-four proximal periodontal defects in mongrel dogs revealed that the implanted biomaterial promoted cementogenesis of the demineralized root surfaces and established a strong interdigitation between the root surface and the gingival connective tissue fibers (Minabe *et al.*, 1988). Similar investigations on the histological response of HA on infrabony periodontal pockets in humans by Galgut *et.al* (1990) showed the healing response that vary between specimens and between sites within the same

specimen at 22, 40 and 60 weeks after placement of implant. HA finds its application as a bone implant material in clinical dentistry particularly in the fields of periodontology, oral surgery and endodontic work.

Although HA is well suited as biomaterial to replace autogenous bone in skeletal reconstruction it has been observed that this biomaterial when come in intimate contact with the tissues of the body, might initiate several complicated biological reactions such as the release of chemotactic mediators and growth factors that might elicit and sustain inflammatory responses at the implant site (David *et al.*, 1964, Bloom and Bennett, 1966, Nathan *et al.*, 1971).

It had been found that neutrophils, monocytes and macrophages invaded the tissues surrounding the implant. This was controlled and directed by chemotactic or chemokinetic agents that included complement factors, lymphokines, platelet factors, leukotrienes and bacterial fragments (Remes and Williams, 1990). Studies have shown that in the case of ceramic implants, different calcium phosphate powder stimulated the activation of complement involving phagocytes, which were attracted to the inflammatory site (Klein *et al.*, 1983). It was known that biomaterials such as metallic and ceramic implants could

cause sensitivity during exposure to the tissues, with attraction and activation of macrophages (Bosetti *et al.*, 1999). Previous studies indicated that a wide variety of particles could prime the macrophages to give a marked increase in their oxidative response (Myrvik *et al.*, 1993).

The effects of HA particulate debris on the production of cytokines like Interleukin-1β (IL1β), IL-6, TNF-α on human fibroblasts demonstrated that HA and HA/TCP particles were capable of stimulating the expression and secretion of cytokines and proteases that enhanced bone resorption, suggesting that particulate debris from implants using these coating also might increase osteolysis and loosening of the implant (Ninomiya *et al.*, 2001). During pro-inflammation, macrophages release pro-inflammatory cytokines like IL1β and TNF-α. These Cytokines have been identified in the preprosthetic tissues of patients with implants (Goodman *et al.*, 1998, Catelas *et al.*, 2003). It has also been observed that HA activated human monocytes *in vitro* stimulated the production of IL-1β and TNF-α (Laquerriere *et al.*, 2003a). However it is not clear whether the released cytokines would act in an autocrine fashion to regulate HA induced macrophage activity.

Studies have demonstrated that nitric oxide (NO) has negative regulatory role in cytoskeletal assembly, pseudopodia formation, phagocytosis and adherence of murine macrophages in association with the ADP– ribosylation of actin on a laminin substratum. NO, depending on the extent and duration of its production, resulted in reversible inhibition of macrophage function, thereby protecting the tissue microenvironment from harmful effect of activated macrophages (Jun *et al.*, 1996). Studies by Ke *et.al* (2001) have postulated that cyclic guanosine' 5'–monophosphate (cGMP) and subsequent ca^{2+}/calmodulin might be key regulators of actin reorganization in NO – stimulated RAW 264.7 cells.

Macrophages, when involved in phagocytosis, release NO but it is still unclear weather this NO will upregulate or downregulate HA-induced macrophage functions. *In vivo,* macrophages migrate to and phagocyte the implanted HA (Rahbek *et al.*, 2005). It is assumed that macrophages would release NO when phagocytosed by HA. It is also possible that this gaseous molecule would also regulate the levels of HA induced macrophage phagocytic activity.

Protein kinase C (PKC) is required at the earliest stage of particle internalization since inhibition of PKC blocks the

formation of actine filaments beneath the site of particle binding (Allen and Aderem, 1995). PKCs are key participants in numerous signaling pathways to the actin cytoskeleton and the nucleus, including signals stimulated by hormones, cytokines, and adhesion, suggesting multiple level of regulation of phagocytic efficiency (Kikkawa *et al.*, 1989, Zhu *et al.*, 2001). However there was no data to show that PKC might play a role in HA induced macrophage phagocytic activity.

1.2.1 HA as Bone Substitute or Replacement Material

CaP biomaterials are used as an augmentation material in reconstructive surgery to bridge surgical bone defects (LeGeros, 2002). Commercially available CaP biomaterials differ in origin (natural or synthetic), composition (HA, beta-tricalcium phosphate and biphasic CaP) and physical forms (particulates, blocks, cements, coating on metal implants, composites with polymers) and in its physicochemical properties.

The properties which are in favor of CaP biomaterials include the similarity in composition to bone mineral, bioactivity (ability to form bone apatite like material or carbonate HA on their surface), ability to promote cellular function and expression leading to formation of a uniquely strong bone

CaP biomaterial interface and osteoconductivity (ability to provide the appropriate scaffold or template for bone formation) (LeGeros, 2002).

It had been observed that CaP biomaterials in its three-dimensional geometry was able to bind and concentrate endogenous bone morphogenetic proteins in circulation, and might become osteoinductive (capable of osteogenesis), and could be the effective carriers of bone cell seeds (LeGeros, 2002).

Other advantages of HA over autografts and allografts are unlimited supply, easy sterilization and storage (Bucholz, 2002). In view of the above advantages, CaP biomaterials find their potential use in tissue engineering for regeneration of hard tissues.

1.2.2 *In Vivo* Studies with HA

Various researches have investigated HA, when this material is implanted into the tissue monocytes/ macrophages, are attracted to the implant site (Heymann *et al.*, 1999). Analysis of retrieved tissues from animal models (Rahbek *et al.*, 2005) and in humans have confirmed phagocytosis of HA particles (Bloebaum *et al.*, 1994).

The *in vivo* effect of the phagocytosis of pure HA particles and HA/dichloromethylene bisphosphonate (clodranate) suspension were investigated by TEM and standard chemiluminescence (CL) assays following intra peritoneumal injection in rats (Hyvonen and Kowolik, 1992). Macrophages were harvested at 12, 24, 48 and 96 hours. HA was completely phagocytosed by 24 hours and HA reacted with clodronate was completely phagocytosed by 48 hours. From 48 hours onwards HA dissolution was observed in the phagosomes of the cell in the two groups. Clodronate seemed to exhibit an inhibitory effect on the phagocytic activity and an enhancement of the chemiluminescence production by the cells in this model, indicating that it was modifying the inflammatory cell response

In a similar study, HA-induced macrophage interactions were investigated after implantation into spongy bone of the distal femur of rabbits (Muller-Mai *et al.*, 1990). The specimen was examined under TEM and scanning electron microscopy (SEM) following transverse fracture in the interface. It was observed that the implants displayed considerable changes in the surface morphology caused by leaching, corrosion and active resorption by osteoclast-like cells. Macrophages were also involved in cleaning the surface via phagocytosis of loose

implant particles. Subsequent mineralizations of these areas were also observed. *In vivo* mechanisms of HA ceramic degradation by osteoclast cells were investigated by TEM (Wenisch *et al.*, 2003). The results revealed that the osteoclasts mediated the degradation of HA ceramic implanted into the sheep bone by simultaneous resorption and phagocytosis.

In vivo animal studies were done to see the effect of phagocytosable particles of HA on bone in-growth in the bilaterally implanted harvest chamber in the proximal tibial metaphyses of 13 mature rabbits (Wang *et al.*, 1994). The results of this study showed that after 6 weeks, HA particles were incorporated within the matrix of ingrown new bone and there was no evidence of granuloma formation or inflammation. Thus, HA particles, which were small enough to be phagocytosed by macrophages, had no adverse effect on bone in-growth. Balla *et.al* (1991) also studied the effect of HA in relation to the histology of furcation perforations created in the mandibular and maxillary premolars and molars of six rhesus monkeys. They observed that after 6 months HA treated tooth revealed that perforated defect was filled with ill-oriented fibrous connective tissue and globules of HA with minimal inflammation and bone resorption.

Van Blitterswijk *et.al* (1985) have studied the events at the HA implant material/tissue interface in the middle ear of the rat. The results suggested that resorption of the implant material occurred by mono and multinuclear phagocyte activity. Resorption decreased 6 months after the operation, possibly due to the decreasing number of phagocytes at the interface and the increasing amount of bone in the micropores.

Studies were carried out on the effect of HA in human periodontal tissues, dental pulp, and following tooth implantations. The biocompatibility of HA crystals implanted into infrabony periodontal defects in human was also studied (Benque *et al.*, 1985). They demonstrated that the biocompatibility was accompanied by a normal fibrogenesis and apparent osteogenesis after 6 years. Filled extraction sockets in miniature swine treated in a similar manner with apatite were also studied.

Noguchi (1989) demonstrated osteodentin bridging in 6 months following direct pulp capping with HA on experimental exposure of pulp in teeth, which were to be extracted for orthodontic reasons. It was observed that in the deeper region of the dentin bridge, tubular dentin was formed newly. Both osteodentin and tubular dentin fused tightly without any organelles in the border between them.

The importance of crystal size of bioceramic on bone formation in human periodontal lesions was studied (Frank *et al.*, 1991). The results suggested that micro sized HA generated significant amount of peripheral bone formation in 6 months when implanted into human periodontal lesions

Yu *et.al* (2003) also reported 15-months follow-up after treating a case of combined endodontic-peridontic lesion on a mandibular first molar by intentional replantation and application of HA. They found that tooth was clinically and radiographically healthy and functioned well at the end of the follow-up period.

1.2.3 *In vitro* Studies on HA

Various researchers have investigated HA *in vitro* which help us to understand the response of the materials better in various experimental designs.

Interaction of human monocyte and monocyte derived macrophages to HA, tricalcium phosphate (TCP), and aluminum calcium phosphate (ALCAP) were investigated (Ross *et al.*, 1996). The data from these experiments suggested that monocytes and macrophages were capable of adhering to the surface of HA, TCP and ALCAP in an *in vitro* environment for over a 7 day period. However, long-term incubation of the ceramic capsules with macrophages

revealed that the cells experienced a gradual disassociation phenomenon. Similar studies on the interrelationship of various biomaterials such as HA, titanium (Ti), large size polyethylene (LSP), submicron polyethylene (SPE), large particle size tricalcium phosphate (LTCP) and small particle size tricalcium phosphate (STCP) towards human monocytes/macrophages suggested that regardless of the biomaterial used, all experimental groups experienced remarkable phagocytosis in the first two phases (24, 48 hours) (Carr *et al.*, 1999).

Human monocytes placed on the surface of HA and biphasic calcium phosphate (BCP) tablets in the presence of vitamin D3 (VD3) and interferon gamma (INF gamma) showed monocytes being influenced by soluble factors (vitamins, cytokines) in initiating the degradation process on biomaterial (Benahmed *et al.*, 1996a).

Bosetti *et.al* (1999) investigated on the biological reaction of the macrophages to natural apatite (heat treated bovine bone), synthetic apatite (HA), and three types of alumina as control. Ultrastructural observation and electron microscopic analysis showed that the macrophages grown in the presence of natural and synthetic apatite were seen with features of healthy cells while macrophages grown in the presence of

alumina seemed to be negatively affected. Biocompatibility of biomaterials (titanium, mixed particle size polyethylene (MPE), ultra high molecular weight polyethylene, mixed particle size of tricalcium phosphate, and hydroxyapatite) in response to RAW macrophages was evaluated at 24, 48 and 72 hours using biochemical markers (Johnston *et al.*, 1999). At 24 hours there was an increase in catalase levels and no initial cell membrane damage was observed by malondialdehyde (MDA) assay, but in 48 and 72 hours, cellular injury occurred in all treatment groups as evidenced by lactate dehydrogenase (LDH) levels.

HA-induced neutrophil interactions were studied under TEM and CL by Dowsett *et.al* (1997). The TEM results confirmed the functional integrity of the neutrophils, particularly those phagocytosing HA particles up to 24 hours. Based on these results it was demonstrated that human peripheral blood neutrophils could be maintained in a fully functional state with respect to the respiratory bust and morphology at least for 24 hours.

In an other identical report the interaction between human neutrophils and pure HA particles were assessed *in vitro* by TEM and CL (Hyvonen and Kowolik, 1991). Neutrophils convincingly phagocytosed HA particles within 15 minutes and

a high CL response was elicited by the zymosan-stimulated CL reaction. Clodronate (dichloromethylene bisphosphonate) alone appeared to have little effect on the cell morphology or CL. When HA was combined with clodronate, phagocytosis was more rapid, and the zymosan-stimulated CL was 50% of that of the HA group suggesting the anti-inflammatory role of clodronate.

Various researchers studied the *in vitro* response of fibroblast cells to HA. Gregoire *et.al* (1987) studied the *in vitro* effect of synthetic granular HA on cultured fibroblastic cells (L929, human bone and gingival cells). Phagocytosis of synthetic granular HA particles resulted in morphological cell changes which were demonstrated by microscopic examinations.

In a similar study human fibroblasts were incubated in the presence of hydroxyapatite or calcium hydroxide ($Ca(OH)_2$) (Alliot-Licht *et al.*, 1994). With $Ca(OH)_2$, the cells exhibited alteration in morphology, DNA synthesis, alkaline phosphatase activity and protein synthesis, in accordance with the necrosis observed when $Ca(OH)_2$ was used as a pulp-capping agent. However with HA, phagocytic activity of pulpal fibroblast towards HA was seen. As a consequence, DNA synthesis was affected with inhibitory effect on alkaline phosphatase activity, which correlated with clinical

observation where reparative dentin bridge was observed directly on the pulp tissues when HA was used as a pulp-capping agent.

Further reports on fibroblast interactions with finely ground powder of synthetic HA by Evans (1991) suggested a reduction in total growth rate and mitotic rate of the cells and increase in the number of pycnotic cells. The effect was dose related and any occurred with small particles. The small particles appeared to either adhere to the cells or phagocytosed by them. The toxic effect was assumed to be physical rather than chemical.

The ability of monosodium urate monohydrate (MSUM), HA and diamond crystals to stimulate phagocytosis, degranulation and secretion of cell movement factors (CMF) from polymorphonuclear leukocytes (PMN) were also assessed (Swan *et al.*, 1990). The ability of each crystal to absorb PMN derived enzymes and CMF was also compared. MSUM crystals stimulated greater enzyme release and generation of CMF than HA; in contrast, HA crystals exhibited greater absorption of PMN products. Diamond crystals clearly interacted with PMN, but they did not stimulate degranulation or CMF production.

The influence of HA particle size (0.5-3.0, 37-63, 177-250 and 420-841 micron) on osteoblasts was also investigated *in-vitro* at 1 hour, 3 hours, 1day, 3 days and 7 days (Sun *et al.*, 1998). It was observed that adding HA to the culture reduced the osteoblast cell count. Transforming growth factor-beta1 (TGF-β1) concentrations in culture decreased significantly with the addition of HA particles. Prostaglandin E2 (PGE2) concentration in the medium increased significantly. The changes in TGF-β1 and PGE2 concentration were more significant and persisted longer in smaller-particle groups.

Thus, the above *in-vitro* studies highlighted the HA interactions with various cells such as human monocytes, macrophage, neutrophils, fibroblast, polymorphonuclear leukocytes, and osteoblast.

1.3 Complexity in Phagocytosis

The term phagocytosis means engulfing or ingesting particulate materials and microorganisms by phagocytic cells which form an essential component of the innate immune system (Stossel, 1999).

The phagocyte-microbe interaction had been widely investigated which was accompanied by intracellular signals that triggered various cellular processes such as cytoskeletal rearrangement, alteration in membrane trafficking, activation

of microbial killing mechanisms, production of pro-and anti-inflammatory cytokines and chemokines, activation of apoptosis, and production of molecules required for efficient antigen presentation of the adaptive immune system (Aderem and Underhill, 1999). Even the fundamental processes of internalizing particles were to be proceeded through a variety of distinct molecular and morphological processes.

This complex phagocytic mechanism involves many underlying principles such as the involvement of many receptors and many signaling molecules have been described as key signaling molecules in phagocytic responses. In one recent study, Garin *et.al* (2001) purified macrophage phagosomes containing latex beads and identified more than 140 proteins associated with the phagosome by two-dimensional electrophoresis and mass spectrometry. These investigators identified many proteins which were not previously known to be associated with phagosomes, as well as many novel proteins.

Phagocytosis occurs in four stages (Underhill and Ozinsky, 2002). First, many different receptors recognize microbes and phagocytosis is usually mediated simultaneously by multiple receptors. Second, different microbe-recognition receptors induce different signaling pathways and their signals interact

cooperatively (and sometime destructively) to mediate ultimate response to particles. Third, microbe recognition is coupled (either directly through phagocytic receptors or indirectly through co-receptors) to inflammatory responses that in turn affect the efficiency of particle internalization by phagocyte or neighboring phagocytes. Fourth, many pathogenic microbes actively attempt to regulate the mechanisms of phagocytosis to evade destruction.

Phagocytosis is an inherently complex process that requires coordinated activation of signaling leading to events as diverse as actin remodeling, alterations on membrane trafficking, particles engulfment, microbial killing and production of appropriate inflammatory mediators that direct the adaptive immune response (Underhill and Ozinsky, 2002). The consequences of phagocytosis vary and they depend on the identity of the microbial target and many factors that modulate the activation state of the phagocyte. Many proteins have been identified that play important role during phagocytosis. It is important to integrate these molecules into pathways that account for the diversity of phagocytic responses.

1.3.1 Receptors for Phagocytosis

Phagocytes express a broad spectrum of receptors that participate in particle recognition and internalization (Underhill and Ozinsky, 2002). Some of these receptors are capable of transmitting intercellular signals that trigger phagocytosis while other receptors appear primarily to participate in binding or to increase the efficiency of internalization. The main classes of phagocytic receptors that participate in phagocytosis of microbes include Fc-receptors, complement receptors, various integrins, scavenger receptors, and mannose receptor.

1.3.2 Role of Signaling Pathways during Engulfing

Particle internalization is accompanied by activation of many signaling pathways that together coordinate rearrangement of the actin cytoskeleton, extension of the plasma membrane, and engulfment. Number of signaling molecules including actin binding proteins, membrane traffic regulators, ion channels, kinases and lipases are activated during phagocytosis of complex particles (such as opsonized bacteria) and may contribute for the efficient internalization (Underhill and Ozinsky, 2002). However, certain signaling molecules stand out as participants both in phagocytosis and in many other signaling pathways. Phosphoinositide 3-kinase