Nanobacteria: Fact or Fiction? Characteristics, Detection, and Medical Importance of Novel Self-Replicating, Calcifying Nanoparticles

Neva Ciftcioglu, David S. McKay, Grace Mathew, and E. Olavi Kajander

Key words: nanobacteria, nanoparticles, calcification, biomineralization

What Do We Know about Biomineralization/ Calcification?

Including humans, many multicellular organisms produce similar hard tissues, such as bones, teeth, shells, skeletal units, and spicules. These hard tissues are biocomposites and incorporate both structural macromolecules (lipids, proteins, and polysaccharides) and inorganic minerals.[1] We do not fully understand the control mechanism of biomineralization in primitive or in developed organisms. The mineral phase of hard tissue is sometimes called biologic apatite, that is, a nonstoichiometric hydroxylapatite. Pure hydroxyapatite has the formula $Ca_{10}(PO_4)_6(OH)_2$. In contrast, a biologic apatite (like in bone) is nonstoichiometric and contains several other ions, mainly carbonate and other elements in traces such as Mg^{2+}, Na^{+}, Fe^{2+}, HPO_4^{2-}, F^{-}, and Cl^{-}. Consequently, a more appropriate structural formula for the composition of bone is $(Ca,X)_{10}(PO_4,CO_3,Y)_6(OH,Z)_2$, with X substituting cations and Y and Z substituting anions (with the indices 10, 6, and 2 changing according to stoichiometry).[2]

There is a paradox in medicine. Whereas some researchers have been discussing the cytotoxic effect of apatite in vitro,[3,4] others have been announcing the safety of in vivo apatite applications.[5–8] Although these disagreements have not been completely resolved, both biogenic and nonbiogenic apatite materials have been continuously used in drug delivery and transplantation.[6,9] We know that when apatite is found in soft tissue, it is considered to be pathologic calcification.[10] The causes of apatite-deposit formations in soft tissue have been discussed for decades but still remain speculative. For example, calcification in the coronary arteries has been widely regarded as an uncommon, end-stage, insignificant, passive, degenerative process of aging—a notion that has paralyzed research in this area for decades.[11] Interestingly, these same terms were once used to describe atherosclerosis.[11] Today, we know that coronary artery calcification occurs, almost exclusively, at sites of atherosclerotic lesions.[12] Calcification in the development of these plaques is a complicated, actively regulated process of mineralization that is similar to bone formation and remodeling.[13,14] Mineralogists explain that all that is needed for crystal formation/biomineralization to start is nidi (nucleus) and an environment of available dissolved components at or near saturation concentrations, along with the absence of inhibitors for crystal formation.[15] Bacteria or other agents producing such nidi, if present in blood and in urine, are very likely candidates to launch and accelerate pathologic calcification in vivo.[16,17] This is clinically important because blood contains phosphate near its saturation level.[18]

"Nanobacteria": Potential Nidi for Calcification, a Good Model for Studying Calcification Mechanism

What is known about microbial infections is based on the study of well-known microbes. A poorly known bloodborne agent (discovered and tentatively

From Nanobac Pharmaceuticals Inc. (N.C., G.M.), Johnson Space Center, Houston, TX; 2NASA Johnson Space Center (D.S.M.), Houston, TX; Nanobac Life Sciences (E.O.K.), FIN 70211 Kuopio, Finland.

Presented at Experimental Biology 2006, San Francisco, CA, April 1–5, 2006.

Address correspondence to: Dr. Neva Ciftcioglu, NASA Johnson Space Center, 2101 Nasa Parkway, Mail Code KA, Houston, TX 77058; e-mail: Neva.Ciftcioglu-1@nasa.gov.

Journal of Investigative Medicine 2006; 54:385–394.

DOI 10.2310/6650.2006.06018

termed ''nanobacteria'' [NB] by our team)[19] exists that behaves as a microbe and appears to show a correlation with such diverse calcification-related health problems as arterial heart disease,[20–22] Alzheimer's disease,[23] kidney stone formation,[24–28] polycystic kidney disease (PKD),[29,30] gallstones and gallbladder inflammation,[31] prostatitis,[32,33] calciphylaxis,[34,35] and cancer.[36,37] NB are also called Calcifying Nano Particles or CNP. This agent has unique properties, including an extremely small size (0.1–0.5 μm), as seen in Figure 1. Although the biologic characterization of NB is yet to be fully understood, the precipitation and growth of calcium phosphate readily occur in systems containing trace amounts of NB but not in identical control systems lacking NB.[19] The exact mechanism(s) by which apatite is nucleated and formed around NB is unknown. When the serum concentration in the medium is reduced (≤ 5%) in the NB culture conditions, NB start to mineralize and grow larger in size owing to calcium and phosphate deposition on their surface (Figure 2). Differential interference contrast microscopy revealed several micrometer–thick age ring–like mineral layers forming around NB in

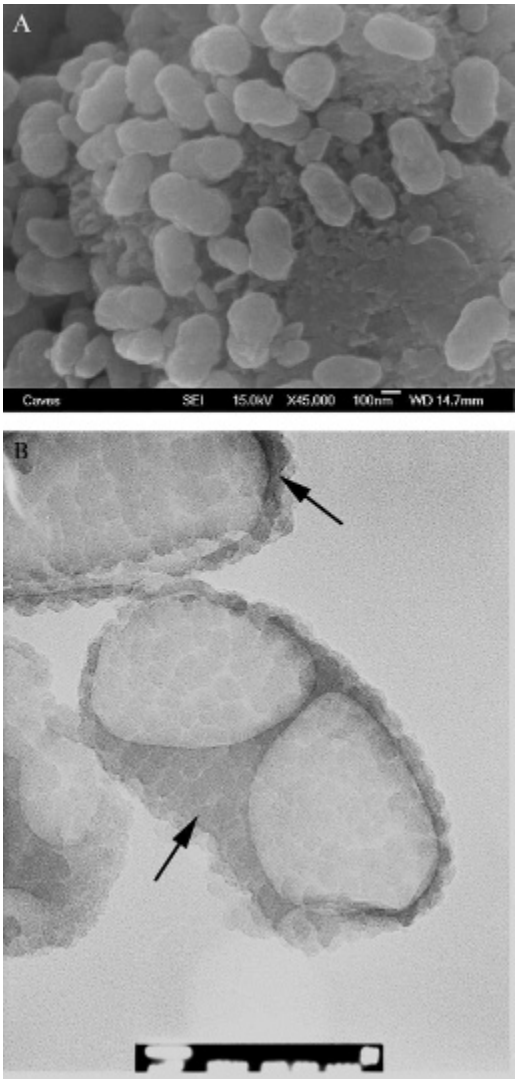

Figure 1 Scanning electron microscopic (*A*), and transmission electron microscopic (*B*) images of cultured nanobacteria (NB). *Arrows* on (*B*) show an apatite envelope of apparently multiplying NB. Bars = 100 nm left, 50 nm right.

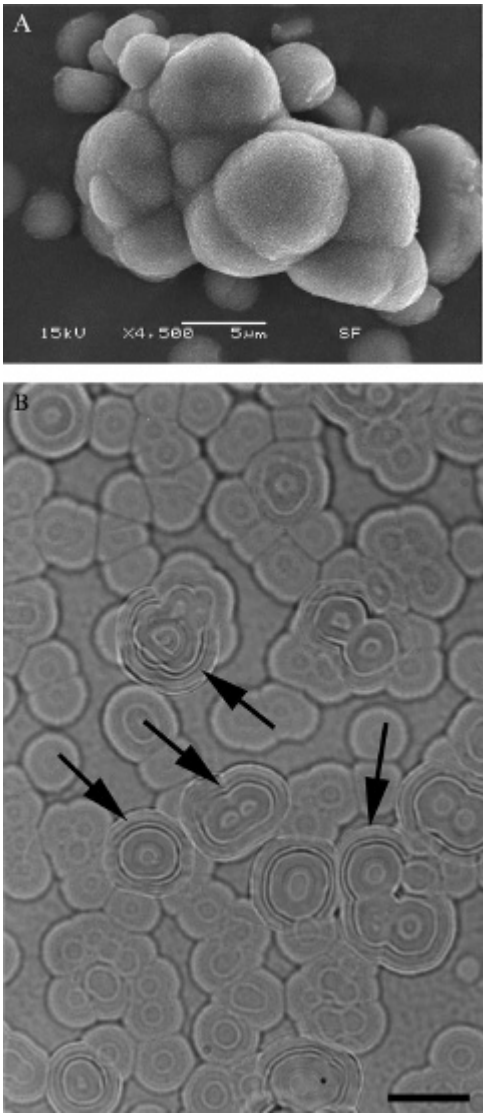

Figure 2 Scanning electron microscopic (*A*) and differential interference contrast microscopic (*B*) images of apatite clusters formed by nanobacteria under serum-free culture conditions. *Arrows* on (*B*) show age ring–like apatite layers surrounding the nanobacteria ''colonies.'' Bars = 5 μm.

these cultures (see Figure 2B). Chemical analysis using energy-dispersive x-ray (EDX) microanalysis of these mineral layers shows Ca and P peaks.[19] The effectiveness of NB biomineralization is remarkable: apatite formation in vitro stopped only when the calcium level decreased by 50% from 1.8 to 0.9 mM and the phosphate levels fell to near zero.[24] Our results indicate that the NB calcium phosphate phase can be formed at pH 7.4, consistent with human physiologic phosphate and calcium concentrations.[19,24,38,39] This can also be monitored by ^{85}Sr incorporation and provides a unique model for in vitro studies on calcification.[24] NB-induced apatitic biofilm formation is dependent on the presence of oxygen[19,40] and can be prevented with several antibiotics and antimetabolites and by high gamma irradiation at sterilizing doses.[39,41] The apatite produced by NB is biogenic because it is formed in a carbon-containing biomatrix, forms small spherical units of apatite in nanoscale crystal size (that are very resistant to acid hydrolysis), and can be formed at nonsaturating concentrations of calcium and phosphate.[19] Such spherical units were identified in most of the human kidney stones examined (Figure 3).[24,42,43] Nonbiogenic apatite has larger crystals that are easily dissolved in acidic solution. Fourier transform infrared spectroscopy (FTIR) of NB revealed the mineral as almost identical to bone mineral (Figure 4). Models for bone formation, which use metastable concentrations of calcium and phosphate, involve gels that include nidi, such as matrix vesicles, apoptotic vesicles, or collagen, but exclude the known proteinaceous inhibitors for crystal formation.[44] Such systems have not been tested with NB. Vali and colleagues showed that nanoforms contain apatite-protein complexes and immunoelectron microscopy reveals protein antigens in proximity to apatite, suggesting a novel form of protein-associated mineralization.[45]

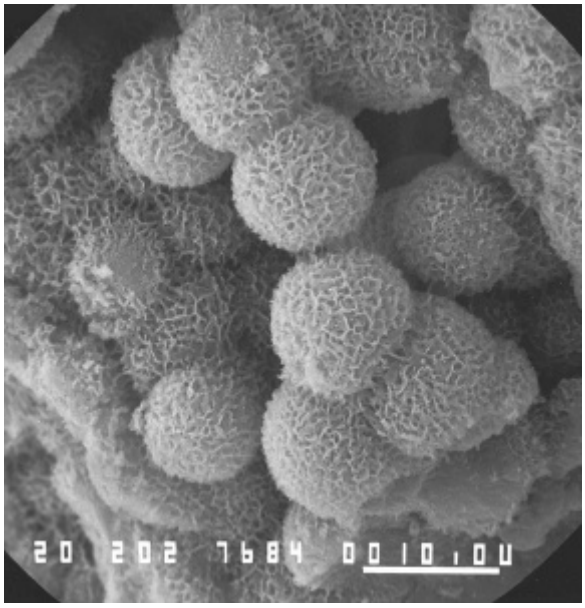

Figure 3 Scanning electron microscopic image of spherical apatite formations in an apatite kidney stone. Bar = 10 μm.

In our earlier studies, we examined NB cultures in high-aspect rotating vessels (HARVs) designed at NASA's Johnson Space Center, which are designed to simulate some aspects of microgravity.[25] NB cultured in HARVs multiplied 4.6 times faster than under stationary conditions and 3.2 times faster than in shaker flask incubation. Interestingly, the results demonstrated that the degree of apatite crystal formation on NB (biomineralization) and the properties of the apatite are strongly affected by the gravity and other specific culture conditions used.[25] Although some researchers believe that microgravity does not affect crystal formation and biomineralization,[46] it has been shown that long periods in a microgravity environment do cause loss of bone and enhance kidney stone formation–like biomineralization disorders in astronauts.[47–49]

In summary, NB is a perfect model for studying biogenic mineralization/calcification because NB (1) are self-replicating particles and have less complicated metabolic pathways, (2) accumulate calcium and phosphate under physiologic conditions, (3) produce a calcium phosphate mineral similar to bone, and (4) exist in physical conditions (eg, pH, gravity, temperature) that are easy to manipulate and that can be replicated for the physiologic model.

Controversy

The first debate about NB revolved around whether these minute particles are alive. To date, critics argue that a particle just 50 to 200 nm in diameter cannot possibly harbor the components necessary to sustain life. Maniloff's work suggests that to contain the deoxyribonucleic acid (DNA) and proteins needed to function, a cell must be at least 140 nm across.[50] However, recently, it has been shown that a genome constructed to encode 387 protein-coding and 43 structural ribonucleic acid (RNA) genes could sustain a viable synthetic cell, a *Mycoplasma laboratorium* that can shrink its size below that limitation.[51] NB are also incredibly resistant to heat and other methods that would normally kill bacteria, which makes some scientists wonder if they might be an unusual form of crystal rather than organisms. Cisar and colleagues presented an alternative theory for the experimental

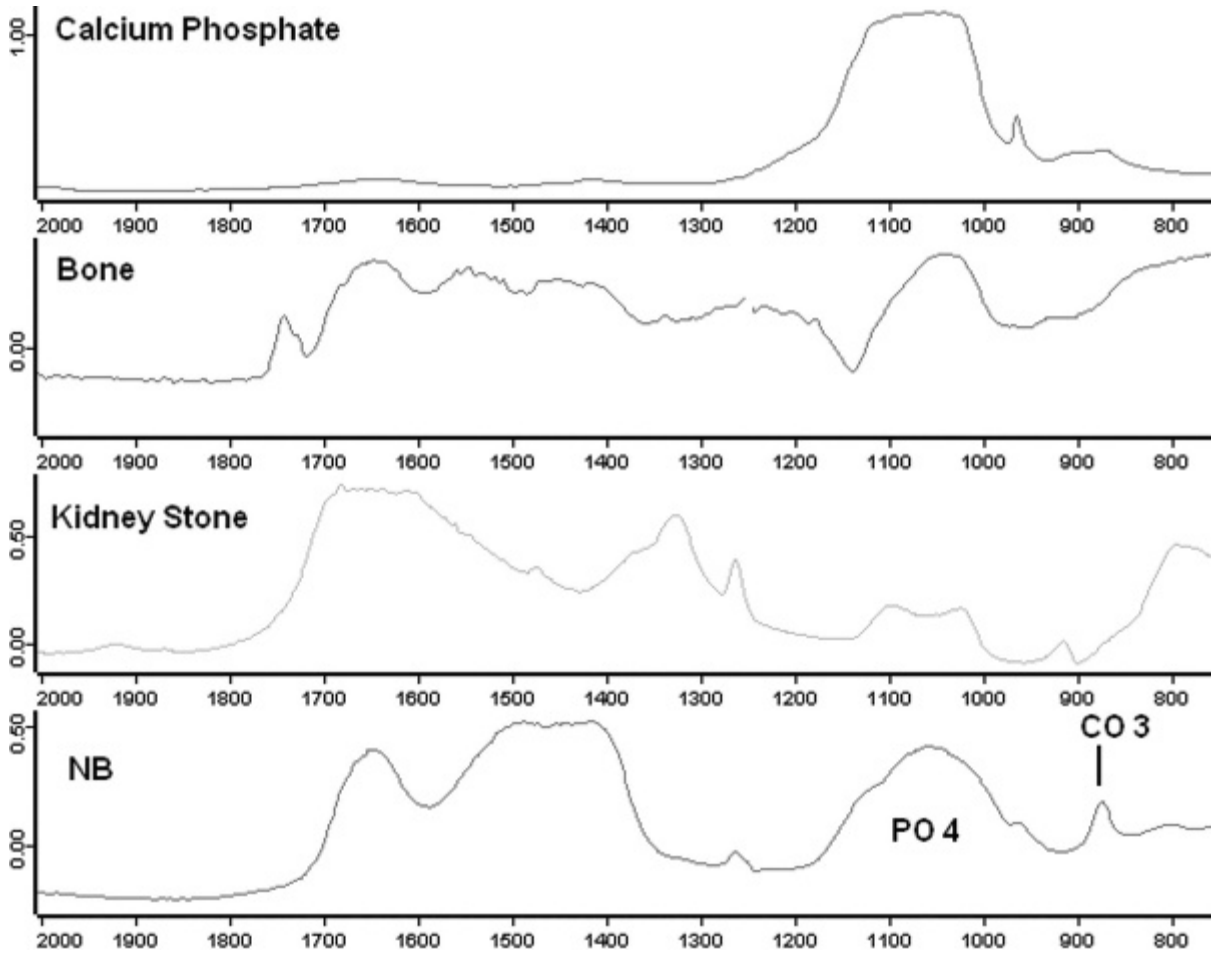

Figure 4 Fourier transform infrared spectroscopy showing the distinct phosphate absorption signature at 1,000 to 1,200 cm^{-1} seen in all four spectra, with inorganic apatite, bone, kidney stones, and nanobacteria (NB).

findings of NB scientists. They stated that biomineralization previously attributed to NB may be initiated by nonliving macromolecules and transferred on "subculture" by self-propagating microcrystalline apatite.[52]

Detection Methods for NB

Methods to diagnose NB in biologics, cells, tissues, blood, and urine include immunodetection with NB-specific monoclonal antibodies, electron microscopy, and culture techniques.[39,53] Because NB pass through 0.22 μm–pore size filters, which exclude most common microbes, filtration is often used to clean up fluid specimens before culture for NB.[53] Replication can be measured by particle counting and optical density at 650 nm.[41] It has also been shown that the growth of the NB could be detected by specific methods, such as enzyme-linked immunosorbent assay, turbidity, sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), or methionine and uridine incorporation.[19,20] Susceptibility tests can be used to test the effects of antibiotics and other chemotherapeutics.[19] Growth can be prevented with tetracycline, high doses of aminoglycoside antibiotics, ethylenediaminetetraacetic acid (EDTA), cytosine arabinoside, 5-fluorouracil, and gamma irradiation.[19]

Are NB a Living Entity?

Isolation of any kind of nucleic acid from NB has been difficult, in part because of the mineral surface that they produced during their culture period. They could not be lysed with lysozyme, proteinase K, several other proteinases, lipases, amylases, alkali, ultrasonography, X-press, detergents, or solvents.[40] We needed to use acid or EDTA-like chelators before the analysis, which were the factors for structural change in nucleic

acid. It has been our experience that NB actually inhibit the amplification of added exogenous classic bacterial DNA by polymerase chain reaction (PCR) methods.

In preliminary research, NB cultures incorporated increasing amounts of radiolabeled ^{35}S into macromolecules separable by gel electrophoresis during the 18-day incubation period. Protein pattern detected with Coomassie's stain of SDS-PAGE transferred to an Immobilon membrane (Millipore, Billerica, MA) and autoradiography showed methionine incorporation into several proteins (Figure 5). Miller and colleagues showed that autoradiographs of isolated NB nucleic acid indicated incorporation of uridine into several ''nucleic acid'' bands.[20] The negative control, media containing gamma-irradiated serum (ie, inactivated NB), did not yield radioactive bands after exposure to these radiolabeled DNA/RNA and protein precursors. To define the mechanism of incorporation and systematic location of NB, further molecular research is needed.

Are NB Cytotoxic?

Calcified and noncalcified forms of NB have been observed as free, cell-attached, and internalized particles in mammalian cell cultures in vitro.[19,53] In our experiments, we chose six different fibroblast lines as experimental models because they are the most ubiquitous cells in the animal body and might be most accessible in wound tissue for invading pathogens, with the exception of professional phagocytic leukocytes.[53] We showed that NB were bound as clusters on the cell surfaces within 15 minutes. It was concluded that NB are internalized either by receptor-mediated endocytosis or by a closely related pathway within 12 hours.[53] Internalization seems to be necessary for cytotoxicity. We showed that cytotoxicity was dependent on NB concentration and exposure time. Dying cells always contained numerous ingested NB.[53] Hybridomas and many lymphocytes were found to be affected by NB, but considerably higher doses were needed.[40,53] The findings that NB are cytotoxic are of interest to nanoparticle toxicity mechanisms in general; similar mechanisms could be used by other nanoparticles for entering cells and causing cytotoxicity.[54]

In Vivo Effects of NB

Åkerman and colleagues reported that radiolabeled (^{99m}Tc) viable NB accumulated in the kidney and appeared in urine after 15 minutes of their intravenous injection into rabbits.[55] This could be due to the fact that kidneys are the preferred sides for this agent, unlike other known nanoparticles, and the presence of injured epithelium or a nucleus in the kidney or

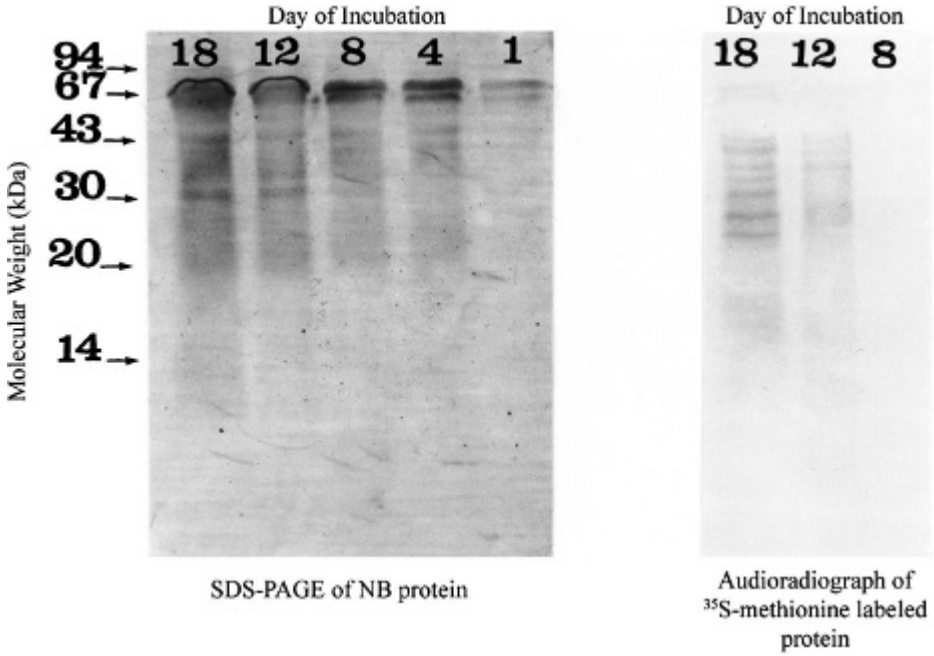

Figure 5 Metabolic labeling of nanobacteria (NB). Images show incorporation of radiolabeled precursor molecules into macromolecules following 1 to 18 days of incubation of NB cultures with the label. SDS-PAGE = sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

urinary tract provides a preferable niche for NB to adhere and grow, resulting in biocrystallization. A control study in rabbits was performed in which a similar dose of ^{99m}Tc-labeled albumin cross-linked particles or tin-technetium nanoparticles as nanocolloids was administered. Nanocolloids were not targeted to kidneys as NB were.[55] Shiekh and colleagues also observed that NB, when injected intravenously into rats, were localized in kidneys.[28] In their research, they observed regions of chronic inflammation infiltrated in the cortex and medulla, which could be due to damage induced by NB. The research team has also shown NB adhering to the surface of the epithelium and their penetration into epithelial cells.

NB and Connections to Calcification-Related Diseases

Calcium is the most important intracellular regulator of physiologic responses, is a messenger for hormonal actions, and regulates cell death and multiplication. Additionally, calcium regulates inflammation, blood clotting, immunologic responses, and neural transmission and muscle contraction. In these actions, calcium-binding proteins are the main players. Recently, a large number of calcium-binding proteins have been mapped to be bound on NB.[56] NB may thus participate in activation-inhibition processes regulating a large number of responses inside and outside cells. Thus, NB could have multiple pathologic actions in the body.

The lifelong prevalence of kidney stones appears to have increased throughout the whole twentieth century and occurs in up to 15% of the population.[57] Treatment of kidney stones is now estimated to cost over $3,000 per patient per year. The incidence of new cases and recurrences may continue to rise. Thus, new approaches in treatment and prevention could have a huge economic effect apart from benefits in terms of reduced morbidity. Electron microscopic observations show that apatite units produced in serum-free NB cultures are very similar to human apatitic kidney stones (see Figures 2 and 3). Both grow as layers of mineral (see Figure 2) and matrix. Chemical analysis and EDX microanalysis revealed that the composition of this solid mineral formation was similar to that of most extraskeletal tissue calcification and stones.[19] The crystalline components of kidney stones are calcium oxalate, calcium phosphate, struvite, purines, or cystine.[58] Fermentor model studies have shown that in kidney stones, calcium phosphate nidi are always formed initially and may subsequently become coated with oxalate or other components.[59,60] Apparently, apatite may play a key role in the formation of all kidney stones. Our hypothesis underlines the role of active nidi: even supersaturated urine needs nidi for crystallization to appear, and active nidi can make the process more thermodynamically favored so that it could happen apparently outside equilibrium. We have proposed that NB may be active nidi that attach to, invade, and damage the urinary epithelium of collecting ducts and papilla, forming a calcium phosphate center(s).[19,24–27,30] We found NB in 70 of 72 human kidney stones studied in Finland.[24] Khullar and colleagues demonstrated 62% culture positivity of NB in stones collected from an Indian population.[61] Additionally, they showed DNA and distinct protein bands of NB. However, one group has been unsuccessful at replicating NB culture from stones, although they have observed nanoparticles that are morphologically similar to NB in scanning electron microscopy.[62]

Another urinary disease, PKD, is the most common autosomal dominant lethal disease in humans. There are reports of endotoxin, NB, and fungal antigens and antibodies in human kidney cyst fluids. Interestingly, a higher prevalence of kidney calcifications is observed in PKD than in the normal population.[63] It has been proposed that the currently known cellular toxicities, tissue distribution, and pharmacology of NB are plausibly related to the known pathology and pharmacology of PKD.[29] Hjelle and colleagues evaluated 13 PKD cyst fluids and detected NB antigen positivity in each sample as well as in liver cystic fluid from affected individuals.[29]

Biopsies, urine, and prostatic secretion cultures fail to demonstrate bacterial pathogens more frequently than in asymptomatic controls, yet inflammation or at least inflammatory markers are often detected in chronic prostatitis.[64] Likewise, the presence of prostatic calculi in younger men is associated with both inflammation and symptoms of chronic pelvic pain syndrome.[65,66] The core of prostatic calculi is typically calcium apatite,[67] which is the hallmark of NB action. Wood and Shoskes proposed that there is a potential role of NB in chronic prostatitis.[32] Recent clinical research targeting these agents has proven effective in treating some patients with refractory category III prostatitis (chronic pelvic pain syndrome).[33] In that research, NB antigen or antibody was found in 60% of serum and 40% of urine samples. In 10 patients who underwent transrectal ultrasonography after therapy, prostatic stones were decreased in size or resolved in 50%.[33]

Mechanisms mediating vascular calcification remain incompletely understood. Some have hypothe-

sized the potential role of NB in arterial calcification.[20,21,35] Miller and colleagues cultured nanosized particles from calcified but not from noncalcified aneurysms.[20] These particles were stained with NB-specific monoclonal antibodies, recognized by a DNA-specific dye and incorporated radiolabeled uridine, and, after decalcification, they appeared via electron microscopy to contain cell walls.[20] Therefore, nanometer-scale particles similar to those described as NB isolated from geologic specimens and human kidney stones can be visualized in and cultured from calcified human cardiovascular tissue.[20] Puskas and colleagues propagated NB-like spherical particles from 26 of 42 sclerotic aorta and carotid samples and confirmed their nature by dot immunoblot by using NB-specific monoclonal antibodies, light microscopy, and transmission electron microscopy.[21] [^{3}H]L-Aspartic acid was incorporated into high-molecular-weight compounds of demineralized particles. PCR amplification of 16S ribosomal DNA sequences from the particles was unsuccessful using traditional protocols.[21] Identification of NB-like particles at the lesion supports but does not by itself prove the hypothesis that these agents contribute to the pathogenesis of atherosclerosis, especially vascular calcifications. Specific therapies targeting these particles have demonstrated reduced plaque formation, regression of plaques, and improved lipid profiles.[68] The potential of anti-NB treatments is controversial and awaits larger clinical trials. Epidemiologic studies have implicated antibodies made by the body against NB to be a strong independent risk factor for coronary artery calcification. The risk appears to be comparable to that of diabetes in the two preliminary studies.[69,70] The importance of this is that coronary artery calcification is an excellent predictor of future coronary events and death.

Originally, Sedivy and Battistutti reported that NB promoted crystallization of psammoma bodies in ovarian cancer.[71] Hudelist and colleagues soon verified the 100% concordance between the expression of NB and the presence of psammoma bodies in malignant ovarian tumors. In their research, several lines of evidence suggest the involvement of these organisms in the process of biomineralization. Therefore, they have concluded that NB infection of malignant ovarian tissue contributes to mechanisms leading to the formation of calcified deposits known as psammoma bodies.[36]

Additionally, NB have been shown to be detected at higher rates in the serum of patients with gallstone disease[31] and mitral valve calcification.[34] Others have suggested that NB may contribute to the development of peripheral neuropathy in human immunodeficiency virus (HIV)-positive patients,[72,73] periodontal problems,[22,74] and even osteoporosis.[75] All of these hypothetical approaches require further investigation.

Koch's Postulates

In a small study, García-Cuerpo and colleagues found that translumbar, percutaneous, intrarenal injection of NB (isolated from kidney stones) into rats resulted in kidney stone formation.[76] Additionally, Shiekh and colleagues examined NB's role in biocrystallization and the in vivo effects on kidney pathology. A calcium oxalate monohydrate assay (COM) was carried out in the presence of NB to study biocrystallization. Wistar rats were given an intravenous injection of NB, and the kidneys were examined for pathologic changes. The COM assay showed accelerated biocrystallization of ^{14}C-oxalate in the presence of NB, indicating them to be efficient candidates for biomineralization. Histopathologic studies revealed bacteria-induced renal tubular calcifications and various manifestations of infection.[28] Their studies confirm that NB may be involved in the pathogenesis of renal tubular calcification. Such findings are required to prove Koch's postulates linking NB to other pathologic calcification-related diseases.

Conclusion

Whether NB themselves serve as the nucleus for crystal formation or whether the NB are simply able to lower the activation energy barrier and thus allow precipitation and growth of crystals under much lower supersaturation conditions is yet to be determined. However, it is immaterial whether they are bacteria, viruses, or other living or nonliving forms; their properties of promoting ready crystallization and growth of Ca minerals are well established. These self-replicating particles may induce calcification and stone formation in vivo because NB (1) have been detected in human blood, (2) are transported from blood into urine and bile as living organisms, (3) are renotropic, (4) cause apoptotic cell death, (5) are present in human stone isolates and tissues with calcification, and (6) cause kidney stone formation in rats within 1 month when injected in an intrarenal route.

Since NB have been detected in blood and blood products, they should be of interest to the biopharmaceutical industry. For example, recently, NB have been isolated and cultured from the cultured nasopharyngeal carcinoma epithelia HNE1 cell supernatant.[77] The safety of vaccines produced in cell culture by using (bovine) serum or serum-derived materials in the culture of the cells, sterilized by filtration, is an

issue needing thorough risk analysis and method validation.[78,79]

Only continued research will reveal the nature of NB and their impact on health and disease. NB are a good model system to use in developing drugs to alter the likely diverse pathways involved in tissue calcification. Although the controversy of whether they are living or nonliving entities will continue until new definitive data are collected, this controversy should not overshadow the critical medical importance of understanding the already demonstrated effects of NB on pathologic calcification in the human body and on research into countermeasures to reverse or eliminate these effects.

References

1. Sarikaya M. Biomimetics: materials fabrication through biology. PNAS 1999;96:14183–5.
2. Tadic D, Peters F, Epple M. Continuous synthesis of amorphous carbonated apatites. Biomaterials 2002;23:2553–9.
3. Inayat-Hussain SH, Rajab NF, Roslie H, et al. Cell death induced by hydroxyapatite on L929 fibroblast cells. Med J Malaysia 2004;59:176–7.
4. Barroug A, Kuhn LT, Gerstenfeld LC, Glimcher MJ. Interactions of cisplatin with calcium phosphate nanoparticles: in vitro controlled adsorption and release. J Orthop Res 2004;22:703–8.
5. Rauschmann MA, Wichelhaus TA, Stirnal V, et al. Nanocrystalline hydroxyapatite and calcium sulphate as biodegradable composite carrier material for local delivery of antibiotics in bone infections. Biomaterials 2005;26: 2677–84.
6. Tamai N, Myoui A, Tomita T et al. Novel hydroxyapatite ceramics with an interconnective porous structure exhibit superior osteoconduction *in vivo*. J Biomed Mater Res 2002;59:110–17.
7. Albiar E, Holds JB. Hydroxyapatite orbital implants: indications for use and nursing considerations. J Ophthalmic Nurs Technol 1992;11:71–6.
8. Mizushima Y, Ikoma T, Tanaka J, et al. Injectable porous hydroxyapatite microparticles as a new carrier for protein and lipophilic drugs. J Control Release 2006;110:260–5.
9. Guo D, Xu K, Zhao X, et al. Development of a strontium-containing hydroxyapatite bone cement. Biomaterials 2005; 26:4073–83.
10. Giachelli CM. Inducers and inhibitors of biomineralization: lessons from pathological calcification. Orthod Craniofac Res 2005;8:229–31.
11. Parhami F, Bostrom K, Watson K, et al. Role of molecular regulation in vascular calcification. J Atherioscler Thromb 1996;3:90–4.
12. Schoenhagen P, Tuzcu EM. Coronary artery calcification and end-stage renal disease: vascular biology and clinical implications. Cleve Clin J Med 2002;69:12–20.
13. Demer LL. A skeleton in the atherosclerosis closet. Circulation 1995;92:2029–32.
14. Wexler L, Brundage B, Crouse J, et al. Coronary artery calcification; pathophysiology, epidemiology, imaging methods, and clinical implications. Circulation 1996;94: 1175–92.
15. Jono S, Shioi A, Ikari Y, et al. Vascular calcification in chronic kidney disease. J Bone Miner Metab 2006;24:176–81.
16. Blair B, Fabrizio M. Pharmacology for renal calculi. Exp Opin Pharmacother 2000;3:435–41.
17. Sohshang HL, Singh MA, Singh NG, et al. Biochemical and bacteriological study of urinary calculi. J Commun Dis 2000;32:216–21.
18. Driessens FC, Verbeeck RM, van Dijk JW. Plasma calcium difference between man and vertebrates. Comp Biochem Physiol A 1989;93:651–4.
19. Kajander EO, Ciftcioglu N. Nanobacteria: an alternative mechanism for pathogenic intra- and extracellular calcification and stone formation. Proc Natl Acad Sci U S A 1998; 95:8274–9.
20. Miller VM, Rodgers G, Charlesworth JA, et al. Evidence of nanobacterial-like structures in calcified human arteries and cardiac valves. Am J Physiol Heart Circ Physiol 2004;287: 1115–24.
21. Puskas LG, Tiszlavicz L, Razga Z, et al. Detection of nanobacteria-like particles in human atherosclerotic plaques. Acta Biol Hung 2005;56:233–45.
22. Ciftcioglu N, McKay DS, Kajander EO. Nanobacteria might be one of the potential agents in oral flora triggering peripheral arterial diseases. Circulation 2003;108:58–9.
23. Kajander EO, Liesi P, Ciftcioglu N. Do autonomously replicating sterile-filterable particles have an association with amyloid accumulation? Viruses and virus-like agents in disease [abstract]. In: 2nd Karger Symposium, Basel, Switzerland. 1993. p. 41.
24. Ciftcioglu N, Björklund M, Willman K, et al. Nanobacteria: an infectious cause for kidney stone formation. Kidney Int 1999;56:1893–8.
25. Ciftcioglu N, Haddad RS, Golden DC, et al. A potential cause for kidney stone formation during space flights: enhanced growth of nanobacteria in microgravity. Kidney Int 2005;67:483–91.
26. Ciftcioglu N. Kidney stone formation: an infectious disease? Jpn J Urol Sur 2002;15:228–32.
27. Kajander EO, Ciftcioglu N, Aho K, et al. Characteristics of nanobacteria and their possible role in stone formation. Urol Res 2003;31:47–54.
28. Shiekh FA, Khullar M, Singh SK. Lithogenesis: induction of renal calcifications by nanobacteria. Urol Res 2006;20:1–5.
29. Hjelle JT, Miller-Hjelle MA, Poxton IR, et al. Endotoxin and nanobacteria in polycystic kidney disease. Kidney Int 2000;57:2360–74.
30. Kajander EO, Ciftcioglu N, Miller-Hjelle MA, et al. Nanobacteria: controversial pathogens in nephrolithiasis and polycystic kidney disease. Curr Opin Nephrol Hypertens 2001;10:445–52.
31. Wen Y, Li YG, Yang ZL, et al. Detection of nanobacteria in serum, bile and gallbladder mucosa of patients with cholecystolithiasis. Chin Med J 2005;118:421–4.

32. Wood HM, Shoskes DA. The role of nanobacteria in urologic disease. World J Urol 2006;24:51–4.
33. Shoskes DA, Thomas KD, Gomez E. Anti-nanobacterial therapy for men with chronic prostatitis/chronic pelvic pain syndrome and prostatic stones: preliminary experience. J Urol 2005;173:474–7.
34. Jelic TM, Malas AM, Groves SS, et al. Nanobacteria-caused mitral valve calciphylaxis in a man with diabetic renal failure. South Med J 2004;97:194–8.
35. Lopez-Brea M, Selgas R. Nanobacteria as a cause of renal diseases and vascular calcifying pathology in renal patients (''endovascular lithiasis''). Enferm Infecc Microbiol Clin 2000;18:491–2.
36. Hudelist G, Singer CF, Kubista E, et al. Presence of nanobacteria in psammoma bodies of ovarian cancer: evidence for pathogenetic role in intratumoral biomineralization. Histopathology 2004;45:633–7.
37. Wainwright M. Nanobacteria and associated 'elementary bodies' in human disease and cancer. Microbiology 1999; 145:2623–4.
38. Ciftcioglu N, Björklund M, Kajander EO. Stone formation and calcification by nanobacteria in human body. Proc SPIE 1998;3441:105–11.
39. Miller-Hjelle MA, Hjelle JT, Ciftcioglu N, et al. Nanobacteria: methods for growth and identification of this recently discovered calciferous agent. In: Olson WP, editor. Rapid analytical microbiology, The chemistry and physics of microbial identification. Surrey (UK): Davis Horwood International Publishing; 2003. p. 297–312.
40. Kajander EO, Kuronen I, Åkerman K, et al. Nanobacteria from blood, the smallest culturable autonomously replicating agent on earth. Proc SPIE 1997;3111:420–8.
41. Ciftcioglu N, Miller-Hjelle MA, Hjelle JT, et al. Inhibition of nanobacteria by antimicrobial drugs as measured by a modified microdilution method. Antimicrob Agents Chemother 2002;46:2077–86.
42. Evan AP, Bledsoe SB, Connors BA, et al. Sequential analysis of kidney stone formation in the Aprt knockout mouse. Kidney Int 2001;60:910–23.
43. Naito Y, Ohtawara Y, Kageyama S, et al. Morphological analysis of renal cell culture models of calcium phosphate stone formation. Urol Res 1997;25:59–65.
44. Riccio V, Della Ragione F, Marrone G, et al. Cultures of human embryonic osteoblasts. A new *in vitro* model for biocompatibility studies. Clin Orthop Relat Res 1994;308:73–8.
45. Vali H, McKee MD, Ciftcioglu N, et al. Nanoforms: a new type of protein-associated mineralization. Geoch Cosmoch Acta 2001;65:63–74.
46. Becker W, Marxen J, Epple M, et al. Influence of microgravity on crystal formation in biomineralization. J Appl Physiol 2000;89:1601–7.
47. Whitson PA, Pietrzyk RA, Sams CF. Urine volume and its effects on renal stone risk in astronauts. Aviat Space Environ Med 2001;72:368–72.
48. Pak CY, Hill K, Cintron NM, et al. Assessing applicants to the NASA flight program for their renal stone-forming potential. Aviat Space Environ Med 1989;60:157–61.
49. McCarthy ID. Fluid shifts due to microgravity and their effects on bone: a review of current knowledge. Ann Biomed Eng 2005;33:95–103.
50. Maniloff J. Nannobacteria: size limits and evidence. Science 1996;273:924–30.
51. Glass JI, Assad-Garcia N, Alperovich N, et al. Essential genes of a minimal bacterium. Proc Natl Acad Sci U S A 2006;103:425–30.
52. Cisar JO, Xu DQ, Thompson J, et al. An alternative interpretation of nanobacteria-induced biomineralization. Proc Natl Acad Sci U S A 2000;97:11511–5.
53. Ciftcioglu N, Kajander EO. Interaction of nanobacteria with cultured mammalian cells. Pathophysiology 1998;4: 259–70.
54. Kajander EO. Calcific nano-particles; nature's ''nanotechnology'' as a cause of human disease [abstract]. In: Nanotox 2006, January 29th-February 1st, Miami, FL.
55. Åkerman KK, Kuikka JT, Ciftcioglu N, et al. Radiolabeling and *in vivo* distribution of nanobacteria in rabbit. Proc SPIE 1997;3111:436–42.
56. Aho KM, Kajander EO, Ciftcioglu N. A novel multiplex-like ELISA test reveals that both Gla-clotting and anti-calcification proteins are present on calcifying nano-particles [abstract]. The ASCB 45th Annual Meeting, December 10-14, 2005, San Francisco.
57. Wahl C, Hess B. Kidney calculi—is nutrition a trigger or treatment? Ther Umsch 2000;57:138–45.
58. Trinchieri A, Castelnuovo C, Lizzano R, et al. Calcium stone disease: a multiform reality. Urol Res 2005;33:194–8.
59. Evan AP, Coe FL, Lingeman JE, et al. Insights on the pathology of kidney stone formation. Urol Res 2005;33: 383–9.
60. Evan AP, Coe FL, Rittling SR, et al. Apatite plaque particles in inner medulla of kidneys of calcium oxalate stone formers: osteopontin localization. Kidney Int 2005;68: 145–54.
61. Khullar M, Sharma SK, Singh SK, et al. Morphological and immunological characteristics of nanobacteria from human renal stones of a north Indian population. Urol Res 2004; 32:190–5.
62. Drancourt M, Jacomo V, Lepidi H, et al. Attempted isolation of Nanobacterium sp. microorganisms from upper urinary tract stones. J Clin Microbiol 2003;41:368–72.
63. Burton EM, Hanna JD, Mercado-Deane MG. Nephrocalcinosis in a child with autosomal dominant polycystic kidney disease and a prolapsing ectopic ureterocele. Pediatr Radiol 1995;25:462–5.
64. Anim JT, Kehinde EO, Prasad A, et al. Relationship between serum prostate specific antigen and the pattern of inflammation in both benign and malignant prostatic disease in Middle Eastern men. Int Urol Nephrol 2006;38:27–32.
65. Muezzinoglu B, Gurbuz Y. Stromal microcalcification in prostate. Malays J Pathol 2001;23:31–3.
66. Geramoutsos I, Gyftopoulos K, Perimenis P, et al. Clinical correlation of prostatic lithiasis with chronic pelvic pain syndromes in young adults. Eur Urol 2004;45:333–7.
67. Torres Ramirez C, Aguilar Ruiz J, Zuluaga Gomez A, et al. Ultrastructure of primary or endogenous prostatic calculi.

Scanning electron microscopic study. Arch Esp Urol 1981; 34:13–22.
68. Maniscalco BS, Taylor KA. Calcification in coronary artery disease can be reversed by EDTA-tetracycline long-term chemotherapy. Pathophysiology 2004;11:95–101.
69. Zhu J, Kajander EO, Katz RJ, et al. Increased serum levels of nanobacteria antibodies are associated with high coronary calcification score. Supp Circ 2004;110: 627.
70. Ertas F, Hasan T, Akan O, et al. Anti-nanobacterial antibody titer is an independent risk factor for coronary artery calcification [abstract]. American College of Cardiology 55th Annual Scientific Session Exposition. March 11-14, Atlanta.
71. Sedivy R, Battistutti WB. Nanobacteria promote crystallization of psammoma bodies in ovarian cancer. APMIS 2003;111:951–4.
72. Sommer AP, Pavlath AE. Primordial proteins and HIV. J Proteome Res 2005;4:633–6.
73. Pretorius AM, Sommer AP, Aho KM, et al. HIV and nanobacteria. HIV Med 2004;5:391–3.
74. Ciftcioglu N, Ciftcioglu V, Vali H, et al. Sedimentary rocks in our mouth: dental pulp stones made by nanobacteria. Proc SPIE 1998;3441:130–5.
75. Sommer AP. Could reduced bone mineral densities in HIV be caused by nanobacteria? J Proteome Res 2004;3:670–2.
76. García-Cuerpo E, Kajander EO, Ciftcioglu N, et al. Nanobacteria: un modelo de neo-litogenesis experimental. Arch Esp Urol 2000;53:291–303.
77. Zhou HD, Li GY, Yang YX, et al. Intracellular co-localization of SPLUNC1 protein with nanobacteria in nasopharyngeal carcinoma epithelia HNE1 cells depended on the bactericidal permeability increasing protein domain. Mol Immunol 2006;43(11):1864–71.
78. Ciftcioglu N, Kuronen I, Åkerman K, et al. A new potential threat in antigen and antibody products: nanobacteria. In: Brown F, Burton D, Doherty P, et al, editors. Vaccines 97. Cold Spring Harbor (NY): Cold Spring Harbor Laboratory Press; 1997. p. 99–103.
79. Kajander EO, Aho K, Ciftcioglu N. Detection of nanobacteria in viral vaccines. 101th General Meeting of American Society for Microbiology in May 20-24, 2001 in Orlando, USA. Paper Y-3.