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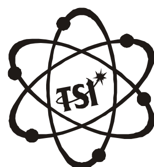

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Characteristics of nanobacteria in man and animals

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ABSTRACT

Nanobacteria are the smallest described bacteria to date, with dimensions of 0.08 to 0.50 μm. Furthermore, these organisms were found to produce a biofilm containing hydroxyl apatite or carbonate, preventing their effective staining. They were also isolated from commercial serum used in the cell culture. Nanobacteria have been detected thereafter in blood and blood products derived from horses, as well as blood from human blood donors. Some strains were isolated from kidney stones and bovine sera. To confirm the data, we searched for Nanobacteria from aseptically removed urinary tract stoned. We used scanning electron microscopy for these stones. Our objectives are identification and determination of Nanobacteria by cell co culture and apatite formation by Nanobacteria in Loeffler medium and SF medium. Also production of polyclonal antibodies, immune-fluorescence assays. Moreover, identification using molecular characterization of Nanobacteria by PCR amplification of 16S rRNA gene of selected isolates and cloning for PCR amplified products. **Microbiological characteristics:** We have reviewed recent publications regarding the microbiological characteristic and pathogenicity of a novel infectious agent, the mineral-forming , sterile-filterable, slow-growing Gram-negative Nanobacteria, detected in bovine/human blood, kidney cyst fluid, urine and kidney stones. According to their 16S rDNA structure, nanobacteria belong to the alpha-2 Proteobacteria, subgroup, which includes the Brucella and Bartonella species. Their cell diameter is 0.2-0.5 microm (the smallest known cell-walled bacteria). Their most remarkable characteristic is the formation of carbonate apatite crystals of neutral pH and at physiologic phosphate and calcium concentrations. The extracellular mineralization forms a hard protective shelter for these hardy microorganisms, and enables them to survive conditions of physical stress that would be lethal to most other bacterial species.

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INTRODUCTION

Immunological characteristics

Urine is a complex solution, and in it crystal nuclei usually form on existing surfaces. Epithelial cells, cell debris, urinary casts, other crystals and red blood cells can all act as heterogenous nuclei[11]. Biological processes can create nucleation sites[7,14,17,18,25,36,39] and stones are then formed on the preformed nuclei. It has been suggested that tiny bacteria called nanobacteria may cause kidney stones[7,14,17,18,36,39].

Nanobacterial antigen has been reported in 97% of human kidney stones[7,14,17-19,36,39,40,70]. Apparently, these bacteria surround themselves with a mineral coating and can serve as nidi for the genesis of renal calculi[7,9,14,17-19,36,39,40,70]. However, a significant contro-

versy has erupted over the existence and significance of nanobacteria[1,2,26]. India nanobacteria may be initiated by non-living macromolecules such as phospholipids and by self-propagating microcrystalline apatite[21].

Microbiological characteristics

The Olavi Kajander group (Finland) suggests that the apatite produced by nanobacteria may play a key role in the formation of all kidney stones, by providing a central calcium phosphate deposit around which other crystalline components can collect. Nanobacteria seems to be a causative agent of diseases related to biomineralization processes[72].

How small are the smallest living organisms? There seem to be many types of bacteria of 300 nm and upwards in diameter, but to many microbiologists it seems a rule of thumb that if something can get through a 0.2 μm filter (200 nm) it isn't alive. Thus the discovery of so-called "nanobacteria", with sizes between 50 nm and 200 nm, in the human blood-stream, and their putative association with a growing number of pathological conditions such as kidney stones and coronary artery disease, has been controversial. Finnish scientist Olavi Kajander, the discoverer of "nanobacteria", presents the evidence that these objects are a hitherto undiscovered form of bacterial life in a contribution to a 1999 National Academies workshop on the size limits on very small organisms. But two recent papers give strong evidence that "nanobacteria" are simply naturally formed inorganic nanoparticles[43].

In the first of these papers, Nanobacteria Are Mineralo Fetuin Complexes, in the February 2008 issue of PLoS Pathogens, Didier Raoult, Patricio Renesto and their coworkers from Marseilles report a comprehensive analysis of "nanobacteria" cultured in calf serum. Their results show that "nanobacteria" are nanoparticles, predominantly of the mineral hydroxyapatite, associated with proteins, particularly a serum protein called fetuin. Crucially, though, they failed to find definitive evidence that the "nanobacteria" contained any DNA. In the absence of DNA, these objects cannot be bacteria. Instead, these authors say they are "self-propagating mineral-fetuin complexes that we propose to call "nanons"[43].

A more recent article, in the April 8 2008 edition of PNAS, Purported nanobacteria in human blood as calcium carbonate nanoparticles (abstract, subscription required for full article), casts further doubt on the nanobacteria hypothesis. These authors, Jan Martel and John Ding-E Young, from Chang Gung University in Taiwan and Rockefeller University, claim to be able to reproduce nanoparticles indistinguishable from "nanobacteria" simply by combining chemicals which precipitate calcium carbonate – chalk – in cell culture medium. Some added human serum is needed in the medium, suggesting that blood proteins are required to produce the characteristic "nanobacteria" morphology rather than a more conventional crystal form[43].

So, it seems the case is closed… "nanobacteria" are nothing more than naturally occurring, inorganic nanoparticles, in which the precipitation and growth of simple inorganic compounds such as calcium carbonate is modified by the adsorption of biomolecules at the growing surfaces to give particles with the appearance of very small single celled organisms. These natural nanoparticles may or may not have relevance to some human diseases. This conclusion does leave a more general question in my mind, though. It's clear that the presence of nucleic acids is a powerful way of detecting hitherto unknown microorganisms, and the absence of nucleic acids here is powerful evidence that these nanoparticles are not in fact bacteria. But it's possible to imagine a system that is alive, at least by some definitions, that has a system of replication that does not depend on DNA at all. Graham Cairns-Smith's book Seven Clues to the Origin to Life offers some thought provoking possibilities for systems of this kind as precursors to life on earth, and exobiologists have contemplated the possibility of non-DNA based life on other planets. If some kind of primitive life without DNA, perhaps based on some kind of organic/inorganic hybrid system akin to Cairns-Smith's proposal, did exist on earth today, we would be quite hard-pressed to detect it. I make no claim that these "nanobacteria" represent such a system, but the long controversy over their true nature does make it clear that deciding whether a system is being living or abiotic in the absence of evidence from nucleic acids could be quite difficult[43].

One reason nanotechnology and medicine potentially make a good marriage is that the size of nano-objects is very much on the same length scale as the basic operations of cell biology; nanomedicine, there-

fore, has the potential to make direct interventions on living systems at the sub-cellular level. A paper in the current issue of Nature Nanotechnology (abstract, subscription required for full article) gives a very specific example, showing that the size of a drug-nanoparticle assembly directly affects how effective the drug works in controlling cell growth and death in tumour cells[44].

MATERIALS AND METHODS

Patients who had undergone operative procedures such as pyelolithotomy, extended pyelolithotomy and/ or nephrolithotomy for the removal of renal stones were included in the study. Surgically removed calculi from patients with renal stones were collected. The stones were analysed for their chemical composition by standard chemical analytical methods. The stone samples were processed for the culture of nanobacteria according to the method of Ciftcioglu et al.[19,40,70]. The stones were pulverized, demineralised in 1 N HCl and neutralized with 0.5 M Tris, and the solutions were centrifuged at 20,000 g for 30 min at 4°C in a Sorvall RC5B centrifuge.

The pellet was suspended in serum free RPMI 1640, sterile filtered through 0.2 lm Millipore filters and the filtrate cultured in flasks containing RPMI 1640 with 10% fetal calf serum (FCS) and kept under tissue culture conditions. As a control, RPMI was incubated with FCS but without stone filtrate. Subcultures were carried out in serum free RPMI after 4 weeks of initial inoculation and subsequently after every 15 days. The cultures were harvested by centrifugation at 20,000 g for 45 min at 4°C, washed with phosphate buffered saline (PBS, pH 7.2) and used for characterization. Gram staining was done with a commercially available kit. Urease enzyme activity was assessed using the standard method[22].

Nanobacterial cultures were assayed for antibiotic sensitivity by subculturing them in serum free RPMI in the presence of different concentrations (1, 2, 5 and 10·) of penicillin (100 IU=1·), streptomycin, gentamicin and kanamycin (100 lg/ml=1· for each) for 6 weeks.

The cultures were examined for nanobacterial growth, every week over a period of 6 weeks. Scanning electron microscopy A 30-day old bacterial culture was centrifuged at 20,000 g for 30 min at 4°C and washed with PBS. Pellets were subcultured in serum free RPMI-1640 on glass cover slips for 72 h.

The bacterial pellet was fixed with 3% glutaraldehyde overnight, followed by treatment with Os04 for 1 h. The bacteria were dehydrated in ethyl alcohol, embedded in epoxy and ultra thin sections were cut and placed on 200 mesh copper grids. The sections were stained with uranyl acetate and lead citrate and subjected to transmission electron microscopy (TEM). Nanobacterial cultures in serum free media were analysed for the presence of DNA by a fluorescence activating cell sorter (FACS) using a modified method of Ormerod, (1990). The nanobacterial pellet was dematerialized with 1 N HCl, washed in PBS and treated with 70% ethanol. The pellet was then treated with RNase (1 mg/ml) and incubated with propidium iodide (500 lg/ml).

Nanobacterial samples without demineralization were also processed by the same method. In vitro calcium oxalate monohydrate crystallization assay Calcium oxalate monohydrate (COM) seed crystals were prepared by the method of Pak et al.[54], and crystal growth was measured by the method of Nakagawa et al.[50].

The result was expressed as percent decrease in radioactivity compared to the control. Nanobacterial protein profile and immunological characterization Demineralized pellet was lysed by sonication with 10% wave intensity in the presence of 2 lm EDTA and 1 mM PMSF. Lysate was centrifuged at 10,000 g for 10 min and the supernatant subjected to SDS-PAGE under reducing conditions following the method of Laemmlli[45] and stained by Commasie blue 250.

For raising polyclonal antibodies, demineralized nanobacteria were emulsified with Freund's complete adjuvant (1:1, v/v) and injected intramuscularly into New Zealand white rabbits. Two booster doses were given with Freund's incomplete adjuvant at 14 day intervals. Serum was separated and stored at 20°C. The reactivity of the serum with isolated nanobacterial content was examined by the Ouchterlony immunodiffusion method[53]. Western blot of the nanobacterial lysate was done by the standard method of Towbin et al.[68].

Microbiological characteristics

In this work, the authors bound a drug molecule to a nanoparticle, and looked at the way the size of the

nanoparticle affected the interaction of the drug with receptors on the surface of target cells. The drug was herceptin, a protein molecule which binds to a receptor molecule called ErbB2 on the surface of cells from human breast cancer. Cancerous cells have too many of these receptors, and this affects the communications between different cells which tell cells whether to grow, or which marks cells for apoptosis – programmed cell death. What the authors found was that herceptin attached to gold nanoparticles was more effective than free herceptin at binding to the receptors; this then led to reduced growth rates for the treated tumour cells. But how well the effect works depends strongly on how big the nanoparticles are best results are found for nanoparticles 40 or 50 nm in size, with 100 nm nanoparticles being barely more effective than the free drug[44].

What the authors think is going on is connected to the process of endocytosis, by which nanoscale particles can be engulfed by the cell membrane. Very small nanoparticles typically only have one herceptin molecule attached, so they behave much like free drug – one nanoparticle binds to one receptor. 50 nm nanoparticles have a number of herceptin molecules attached, so a single nanoparticle links together a number of receptors, and the entire complex, nanoparticles and receptors, is engulfed by the cell and taken out of the cell signalling process completely. 100 nm nanoparticles are too big to be engulfed, so only that fraction of the attached drug molecules in contact with the membrane can bind to receptors. A commentary (subscription required) by Mauro Ferrari sets this achievement in context, pointing out that a nanodrug needs to do four things: successfully navigate through the bloodstream, negotiate any biological barriers preventing it from getting it where it needs to go, locate the cell that is its target, and then to modify the pathological cellular processes that underly the disease being treated. We already know that nano-particle size is hugely important for the first three of these requirements, but this work directly connects size to the sub-cellular processes that are the target of nanomedicine[44].

RESULTS AND DISCUSSION

The result showed that, Nanobacteria (NB) were discovered as a contaminating agent in cell culture over 10 years ago. Despite visible biomass present on a cell culture dish, standard microbiological methods failed to detect any known microbe[5,16,38]. Culture studies indicated that the novel agent was apparently indefinitely passagable in cell culture medium (beyond 10 years) and could adapt to growing in plain DMEM or RPMI-1640. Omitting serum supplementation resulted in larger cells[5,7,14,16-18,36,38,39] being formed inside cavities formed by thick apatite layers in old cultures, large colonies with slimy, but only slightly mineralized walls were observed indicating social behaviour. Figure also shows the release of tiny forms of NB from the colony.

Further studies with electron microscopy revealed mineralized igloos consisting mainly of carbonate apatite. Such igloos could grow in size, bud-off new ones and fuse with others to form stones visible to the naked eye. When the gamma-irradiated serum culture was inoculated with unirradiated positive serum or cultured agent, growth was restored. In this way, the source of the agent was tracked to 'sterile' FBS used as a supplement for culture medium[5,16,38]. Over 80% of tested commercial FBS batches from many different manufacturers were positive for NB[19,70]. NB were found in a significant number of humans as well, in serum and/or in urine. To date, NB have been isolated from bovine serum and from human serum, urine, kidney stones, dental stones and tissue samples[5,7,14,16-20,30,36,38-40,59,70].

Interestingly, NB show improved growth properties in artificial urine[12]. The general characteristics of NB are given. The compilation of data is based on research results obtained by the present authors on a standard "strain" (SeraLab901045). Intrastrain differences due to prolonged culture have been found in cytotoxicity[7,14,17,18,36,39] and in the kinetics of elimination into urine after intravenous injection into rats[4].

In addition to culture methods[15,23,29,30,37], several their diagnostic tools have been developed for the identification of NB. One of the most powerful methods is transmission electron microscopy (TEM). TEM sample preparation for negative staining takes only a few minutes and allows for the detection of NB as dark particles, due to their apatite content[49]. NB culture sample dried on a carbon-coated grid can be inspected either without staining, or after staining with 2% uranyl acetate. The latter reveals slimy material around the par-

ticles. The most powerful tool is a novel technique in which an unstained grid is incubated for 20 min with colloidal gold-labelled anti-NB antibody, washed, dried and inspected. The antibody reveals its target (NB surface epitope) inside of the slimy material on the NB surface.

These techniques allow imaging at high resolution without any fixation steps. NB have apatite mineral as a structural support, which makes them visible and so robust that fixation is unnecessary for TEM. This is a unique feature of NB, which allows fast and specific diagnosis using electron microscopy. Many properties of NB are rare and extreme. Relatively tiny mineral-associated microbes have been found by geologists[28,69].

Older findings link such small forms to cancer[71]. Many properties of NB support the theory that they might be primitive life forms[7,14,17,18,36,39]. The extremophilic characteristics of NB would be beneficial in surviving hostile conditions[7,15,17,23,29,30,37,,39]. Several groups are researching NB and a Nanobacteria (NB) cultured without serum in DMEM, a phase contrast microscopy image, magnification 800. The results shows a mineralized colony with two large organisms inside a mineralized igloo. b NB biofilm in DMEM. The micrograph shows a large community with relatively thin walls, see the two arrows. Small particles have been released outside the community. Magnification 800°C.

Transmission electron microscopy (TEM) micrograph of a section of NB igloo. The micrograph shows an igloo similar to that in a. A layer of apatite crystals is evident on the surface. Bar=0.2 lm. d Scanning electron microscopy (SEM) micrograph of igloos. Bar=1 lm 48 have succeeded in the detection or culture of NB or NB like forms[12,21,23,29,30,48,59,70]. However, the concept that NB are living organisms is controversial as long as their putative nucleic acid is not sequenced. How nanobacteria are involved in stone formation? The theory of NB-linked stone formation[41,42] is based on: (1) finding NB in kidney stones (2) finding similar forms of NB in kidney stones culture, (3) in vitro calcific stone formation by NB and (4) kidney stone formation after NB inoculation to rat kidneys.

In a study by Ciftcioglu et al.[19,40,70], 70 out of 72 kidney tones contained NB. The presence of NB was independent of the stone type, although apatite stones gave the highest immunopositivity. Kidney stones were crushed, treated with 1 M HCl followed by neutralization and then analyzed using culture, immunological methods and electron microscopy. Surprisingly, kidney stones contained NB that started to replicate under culture conditions and formed calcium phosphate stones in vitro. In addition, NB were able to produce stony colonies in modified Loeffler medium, cause intra- and extracellular calcium deposits and cell damage in many cultured cell lines[7,14,17,18,36,39].

Importantly, dose-dependent kidney stone formation was observed within 1 month in rats after injection of NB using the translumbar, percutaneous renal puncture method[23,29]. Despite the small number of experimental animals (n=4), the result provides evidence that kidney stone formation can be caused by introducing NB into kidney. The suggested involvement of NB in kidney stone formation[41,42] supports the observations made by Carr and Randall[13]. Carr's concretions are small shiny deposits of calcium phosphate in kidney lymphatics and collecting ducts. Calcium phosphate formed above the collecting duct might induce heterogeneous nucleation of calcium oxalate at lower levels of the renal collection system[32] and be a risk factor for Randall's plaque formation. Randall described calcium-containing plaques in the kidney papilla[60].

According to his hypothesis, the formation of kidney stone starts from these plaques due to a primary lesion in the tissue. Cell culture experiments have revealed that the adherence and internalization of calcium oxalate crystals into cultured cells is an active process potentially important for kidney stone formation.

Recent studies on NB have produced findings suggesting that NB might be calcium phosphate nidi for kidney stone formation. NB are renotrophic, as reported from rabbit experiments using radiolabelled NB[5,38]. They are eliminated from the circulation through excretion into the urine[3-5,38]. NB were found to adhere, invade and damage cells in collecting tubuli and the papillary area in the rat and rabbit models[5,38].

NB colonization could lead to the accumulation of calcium deposits on the lesions and trigger stone formation as described by Randall. Nanoscale biocrystallization by nanobacteria many kidney stones have a core composed of apatitic spheroids. NB cultured from human kidney stones formed apatitic spheroid particles in vitro with a similar architecture to that in

kidney stone; observe the broken spheroids. Tiny nanoscale crystals show a highly organized structure resembling the nanosphere structures found in pearls and other calcium carbonate formations in living bodies. This suggests a protein mediated mineral growth mechanism. Pearls are known to grow in this way.

The biomineralization process of NB takes place inside a mucus-protein matrix around the NB that is detectable with uranyl acetate staining. This suggests that nanoscale apatite crystals grow as a result of mucus-protein mediated crystal formation on the surface of NB. This theory was first introduced by Vali et al.[70]. Crystalluria appears to form at a lower urinary ionic concentration in stone formers[27], suggesting higher crystallization potency in these individuals, i.e., active nidi or weaker crystallization inhibitor activity. NB are transportable apatitic nidi from blood into kidney tissue and urine[5,38], and their active role in crystallization may explain the observation above[7,14,17-19,36,39,40-42].

Biomineralization is an effective process: 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[19,40]. NB can use dolomite[7,14,17,18,36,39] and synthetic apatite (Kajander, unpublished observation) as a calcium source. NB-induced biomineralization is dependent on the presence of oxygen[5,7,14,16-18,36,38,39]. Gamma irradiation at doses that prevented the TEM micrograph of a sample from NB culture in DMEM supplemented with 10% FBS. Negative staining technique omitting the stain. Bar=1 lm. b TEM micrograph of NB culture sample incubated for 20 min with colloidal goldconjugated anti-NB 8D10 monoclonal antibody, otherwise as in a. Three approximately 20 nm-sized gold-particles are indicated by the arrow. There is highly significant binding of the goldlabelled antibody to NB. Bar=0.12 lm. c TEM micrograph of NB subjected to negative staining with 2% uranyl acetate. Bar=1 lm. D TEM image as in c at high magnification. Mucus-protein layer surrounding NB can be seen (see arrow in c). Such a sticky layer apparently helps NB to adhere on surfaces, to grow as a social colony or biofilm, and promotes apatite crystallization. Bar=0.1 lm 50 replication of NB, abolished the biomineralization[4,5,16,20,38].

Biomineralization was abolished with several antibiotics and antimetabolites that showed a nanobactericidal effect at concentrations relevant for human therapy[4,20]. Further proof that biomineralization by NB are a biological phenomenon related to being a living entity came from recent experiments with light. Low intensity light treatment (without thermal effects) at certain wavelengths stimulated NB replication as detected by particle numbers, incorporation of uridine and electron microscopy. Concomitantly, light stimulated apatite formation as detected by 85-strontium incorporation[65]. Synthetic apatite did not respond.

Biostimulation by light treatment is a general phenomenon observed in living entities from bacteria to mammalian cells with the used light treatment[64,66]. Macromolecule-calcium phosphate mineral complexes have also been recently observed in human and animal circulation by other researchers[55-57]. Price hypothesized that these particles cause soft tissue calcification, such as atherosclerosis and kidney calcification. The source of Price's particles was an enigma. These high molecular weight complexes of calcium phosphate together with proteinaceous calcification inhibitors were circulating in rats (subjected to atherogenic treatments) after a single subcutaneous dose of etidronate[57].

The maximum concentration of complexes was observed at 6 h after the drug dose and complexes were cleared from circulation within 24 h after injection. The route of elimination was not studied. The presence of the protein-mineral complex increased total serum calcium and phosphate 1.8- and 1.6-fold, respectively, after a dose of 8 mg/100 g body weight etidronate, and even more with higher doses[57,58]. It was suggested that the complex originates due to the inhibition of bone mineralization by etidronate[57].

These findings confirm our detection of high molecular weight mineral-protein complexes containing calcium phosphate in serum (NB). We have shown that the treatment of such complexes inside calcific biofilms or stones with bisphosphonates, chelating agents and some antibiotics, resulted in the release of destroyed particles into the medium[4].

We propose an explanation for the appearance of complexes as described by Price et al.: bisphosphonate administration causes the destruction of NB into "popcorn-like" floating particulate debris General characteristics and behaviour of nanobacteria (NB) Morphology Stained Gram-negative, sterile-filterable (0.22 lm

pore-size), bacteria-like particles with varying amounts of a carbonate apatite coat Size of individual NB ranges from 80 nm to500 nm By light and electron microscopy, apatite "igloos" have a central chamber occupied by one or more NB Under low nutrient conditions (e.g., serum-free), NB tend to form microscopic colonies in liquid media surrounded by a thick coat of calcium apatite; calcified colonies can approach >1 mm in size Exhibit budding and fragmentation, social behaviour, and communities reminiscent of biofilms, but with unique characteristics consistent with that of extremophiles; withstand 90°C for 1 h, 15 kGy gamma irradiation, 5% NaCl Growth and metabolism Serum forms have a generation time of about 3 days Serum-free forms double about every 6 days Can be passaged indefinitely in DMEM with or without serum Metabolism is 10,000 times slower than in E. coli Incorporate uridine and methionine into DNA and protein, respectively.

Grow best under aerobic conditions: 5% CO2:95% air Inhibitors of nucleic acid synthesis, 5-fluorouracil and cytosine arabinoside, inhibit NB growth Tetracycline, an apatite-binding protein synthesis inhibitor, inhibits NB growth at therapeutically achievable blood levels, as do trimethoprim, sulfamethoxazole, nitrofurantoin and ampicillin; at supra-pharmacologic levels, aminoglycosides also inhibit growth Calcium chelators, such as EGTA and citrate, inhibit growth in vitro Bisphosphonates are highly nanobactericidal Structure NB biomass contains novel proteins and "tough" polysaccharides Over 30 proteins have been found by SDS-PAGE One of these proteins is a bacterial porin protein Muramic acid, a major component of bacterial peptidoglycans, was identified The 16S rDNA of NB obtained with PCR places it in the alpha-2 subgroup of proteobacteria; further proof for nucleic acids are needed, since many data indicate that nucleic acids are modified and PCR methods may not work well Detection Monoclonal antibodies to the nanobacterial porin protein and peptidoglycan recognize intact NB as shown by immunogold labeling Hoechst DNA fluorochrome stains NB Demineralization of NB enhances their endotoxin positivity in the Limulus amebocyte lysate assay Monoclonal antibodies to chlamydial lipopolysaccharide (endotoxin) react with NB Effect on cells Some, but not all isolates of NB exhibit cytotoxicity to mammalian cells in vitro NB can bind to mammalian cells in vitro and be internalized by endocytosis In human and animal tissues, transmission electron microscopy has revealed intracellular putative NB I.v. administered NB were excreted to urine in rodents.

In rats they caused apoptosis and sloughing of renal epithelium in collecting ducts and papilla 51 few hours[4,20], and these detached particles could appear in the blood until removed by the reticuloendothelial system. Bisphosphonates and chelating agents, either alone or together with antibiotics might thus be useful agents in the treatment of pathological calcification, whether in the form of atherosclerosis or stone formation. In fact, a recent summary advocates bisphosphonate treatment for stopping or preventing atherosclerosis[73], and one earlier report has shown bisphosphonate therapy to decrease the recurrence of kidney stones[8].

Larger studies are warranted, because this approach might have deep implications in the treatment of recurrent kidney stones, nephrocalcinosis and atherosclerosis. Cisar et al.[21] were able to culture NB-like apatitic particles from human saliva and dental plaques. They reported unsuccessful DNA extraction, failed PCR detection due to bacterial contamination and a negative result from protein isolation although some protein bands were obtained. Their conclusion was that the particles were self-replicating inorganic apatite. The use of positive and negative controls and methods to identify NB could have been used in their study to confirm or exclude the presence of NB, but were not performed.

Interpretation should not be based only on failed nucleic acid results. Nucleic acid research on NB has many problems, e.g., nucleic acid extraction is difficult due to apatite and extracted DNA-like material has inhibited the amplification of exogenous bacterial DNA in PCR methods. More effort should be made for the characterization of NB. Ongoing research on nanobacteria ongoing research aims at solving the mystery behind nanoscale biomineralization: What are NB? What are their survival and growth strategies? How do they mineralize and what is their role in kidney stone disease and other calcifications? Does their eradication prevent stone formation? Research is now being carried out by an increasing number of researchers, among others, in the Mayo Clinic and NASA. Effective eradication therapies may arise as a consequence of such

international research efforts. New approaches in the treatment and prevention of kidney stones could significantly reduce health care costs and increase the quality of life. Emerging knowledge on the drug sensitivity of nanobacteria/kidney stone forming units[4,20] suggests that novel treatment strategies could be based on a combination therapy using "old" drugs.

A major hindrance in adopting such new therapies can be the reluctance of drug companies to carry out the necessary but expensive clinical studies with generic drugs. Epidemiological studies are important for determining the prevalence of NB in various populations and diseases. Serum prevalence of the antigen in adult Finnish volunteers is about 5%[19,70]. Our recent collaboration with Holmberg et al. from Uppsala revealed that about 14% of healthy Swedish blood donors have antibodies to the agent[33]. Furthermore, gamma globulin preparations pooled from thousands of healthy volunteers revealed NB antibodies[3,4]. In some disease states, e.g. atherosclerosis and hemodialysis, NB markers, antibodies and antigen, can be found in the serum/urine in the majority of cases (Kajander, unpublished data).

Whether NB are bacteria, mineral autocatalytic aggregates or self-replicating biological particles, they should be regarded as an infectious agent which can be involved in the pathogenesis of pathological calcifications. Exposure to NB can cause an immune response and may result in chronic bacteremia. One accidental exposure to NB during laboratory work has been monitored. The exposure was followed by the development of antibodies against NB, antibody levels remaining high for several years after the accident (Kajander, unpublished data). This finding suggests that NB may cause chronic infection without immediate clinical symptoms. It has been estimated that the growth of a 3 mm thick layer of calcium oxalate takes approximately 2.7 years, based on crystal growth rate[67]. For this and other reasons, exposure to NB infection might have serious consequences several years after exposure. It is suspected that biopharmaceuticals might be contaminated via FBS[19,70] and some viral vaccines were found to contain NB[41,42,70]. This possibility should be kept in mind and efforts should be made to determine the role of NB in the etiology of kidney stones and pathological calcification, diseases with an apparently increasing prevalence.

Microbiological characteristics

Drancourt el al.[26] have reported on their attempts to isolate nanobacteria from upper urinary tract stones. Their findings and opinions are valuable for the nanobacteria research. However, we want to point out difficulties that any researcher will face when working with nanobacteria: lack of published data and working instructions, lack of tested commercial culture media and identification tools, and lack of readily available positive and negative controls. Novel paradigms are difficult to publish. Manuscripts on nanobacteria have so far been returned from Nature, Science, etc. Lack of publications on basic findings and methods used leads to two important consequences. (i) Scientists will waste their time trying to work with well-established routine methods, which unfortunately need modifications or must be replaced by new technologies. (ii) Negative results are obtained and accepted as such. Although the results were not properly controlled (culture media were neither pretested for growth promotion of nanobacteria nor controlled by positive test cultures), people and journals may choose the easiest way.

Any microbiological classification of tentative nanoorganisms, such as nanobacteria proposed by Kajander and Ciftcioglu[7,14,17,18,36,39] and nanobes proposed by Uwins et al.[69], is difficult because they are not typical bacteria. They have also virus-, fungus-, and prion-like characteristics and thus cannot fit into any existing class of microorganisms. They should be considered as their own entity. Research tools and techniques for nanoorganisms require new attitudes and ideas. Recent findings have indicated that there are many surprises to come[31,35].

Isolation of Nanoarchaeum equitans, a symbiont of hyperthermophilic bacteria, required extra efforts in characterization because standard PCR techniques failed in detecting the organism's genetic material, the presence of which was revealed with DNA stains[31,35]. Interestingly, both nanobacteria and nanobes contain nucleic acid material detectable with DNA and RNA stains[7,14,17,18,36,39,69].

It is of utmost importance to realize the limits of our current methodologies with respect to detection and culture of novel nanoorganisms, as exemplified by nanobacteria. Many so-called negative reports have been able to repeat the morphological finding of calcium phos-

phate self-propagating units[6,21,26].

Nanobacteria-like organisms have been found in human atherosclerotic plaques[63]. Atherosclerosis is a burden to billions of people. Clinical and microbiological laboratories should not take the easiest way and judge the calcium phosphate particles as artifacts. Who would like to carry self-propagating nanocrystalline apatite in their blood, blood vessels, stones, and tissues? Evaluation of nanobacteria phenomena should not be based just on routine bacteriological criteria but rather on muisciplinary efforts by innovative and open-minded scientists.

Aho and Kajander[3,4] are commenting on the negative attempts in the previous study by my laboratory to grow nanobacteria. My coworkers and I tried to reproduce their technique without success. Since 1998, we have tried to obtain the strain from Kajander. Here we failed to confirm their work. The putative "Nanobacterium" strain is protected and not available, but they sell products to detect nanobacteria.

To the best of my knowledge, nobody has reproduced this work. I would be happy to test their strain and change my mind if the data are convincing. The authors cite references on Nanoarchaea[31,35], which have no correlation with this topic but the name.

The main problem is that in science, the exact method and the obtained strains should be exchanged to allow other investigators to reproduce and confirm the work. Regarding my alleged reluctance to find new microorganisms, I suggest that the authors consider previous studies from my laboratory, including reports of the culture of the biggest virus, that of Tropheryma whipplei[46,61] and that of other microorganisms, including Rickettsia species, which are small bacteria[51,62].

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CONCLUSIONS

NB remain controversial agents that mediate apatite nucleation and crystal growth. They are renotropic, cause apoptotic cell death, are present in human kidney stones and occasionally in urine. They may trigger renal pathology involving damage to tubular epithelium, biomineralization, and perhaps tubule obstruction and chronic infection resulting in defective tissue repair and stone formation.

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