Kajander, E.G., Bjorklund, M. and (^ift^ioglu, N.: Nanobacteria and man. In: Enigmatic Microorganisms and Life in Extreme Environments. Ed. J. Seckbach, Kluwer (the Netherlands), in press 1997 (An invited chapter), 7 pp.

NANOBACTERIA AND MAN

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1. Introduction

Nanobacteria are very small mineral forming bacteria recently discovered in mammalian blood and tissues. They are the first mineral forming bacteria found in blood, and the first heat and gamma irradiation resistant bacteria detected in man. They seem to have a genetic organization which permits them to compensate for their small size and slow metabolism, and endure in their environmental niche. These properties together with their very slow growth rate meant that they were not detected until about 10 years ago. Their extraordinary nature ako hindered attempts to publish this data. These autonomously replicating microorganisms were approaching the theoretical limit of self-replicating life with a size of only one hundredth of that of usual bacteria (Kajander, 1992; Akermanet al, 1993, Kajander et al, 1994). Morphologically similar organisms had been discovered by Dr. Folk (1993) in sedimentary rocks and in hot-springs in travertine, and these he had given the name nannobacteria. Such bacteria seem to contribute to the formation of many kinds of minerals. These bacteria may secrete molecules acting as nucleation centers for depositing biogenic minerals, e.g., apatite, around them in aquatic environments (Mojzsis et al, 1996). In a similar way, nanobacteria from blood can surround themselves with mineral deposits.

2. Nanobacteria

Nanobacteria were named as *Nanobacterium sanquineum* referring to their surprisingly small size and apparently vivid movements, and their existence in blood. The organism was reposited in the German Collection of Microorganisms (DSM No: 5819-5821). These organisms were isolated from 'sterile' commercial human and bovine sera. Fetal bovine serum was found to be ubiquitously contaminated with nanobacteria so that about 80% of serum batches contained culturable organisms. Nanobacteria were found in about *5%* of human serum samples (Ciftyioglu et al, 1997a). Thus nanobacteria are the most common cell culture contaminant and a major cause for bacteremia. Yet they have remained undiscovered, most likely because of their unique properties.

2.1. DISCOVERY

E. Olavi Kajander, helped by several enthusiastic students, discovered these carbonate/hydroxyapatite mineral-forming organisms in blood and sera for cell culture. Bacteria-like particles were initially observed in long-term mammalian cell cultures. It

was only with some batches of serum that the panicles could be seen. The cultured mammalian cells became vacuolized (Figure 1A) and died within four weeks. The harmful agent could be passaged and infected other cells with the same outcome. Intracellular bacteria-like particles were observed (Figure 1B) that were similar to those cultured without mammalian cells (Figure 1C). These cultures had a white macroscopic layer of bacteria at the bottom but the medium pH was not greatly changed. The novel bacteria replicated whether mammalian cells were present or not.

Microbiological tests including mycoplasma assays were negative. Bacteriological staining methods initially failed because of difficulties in their fixation with classical flame and alcohol techniques. Nanobacteria could not be cultured in standard microbiological media, but grew in all commercial mammalian cell culture media at 37-45°C under 5-10% COi - 90-95% air. Anaerobic cultures produced no growth. Serum concentrations of 10-50% supported growth optimally. Very poor growth was obtained on solid media. Their multiplication rate followed logarithmic growth, after a lag period, with a doubling time between 1 to 5 days. They produced a slime as shown in Figure ID. This slime explains their observed adherence to glass, plastic and other materials. Thus, nanobacteria can form a biofilm, which is associated with mineral formation.

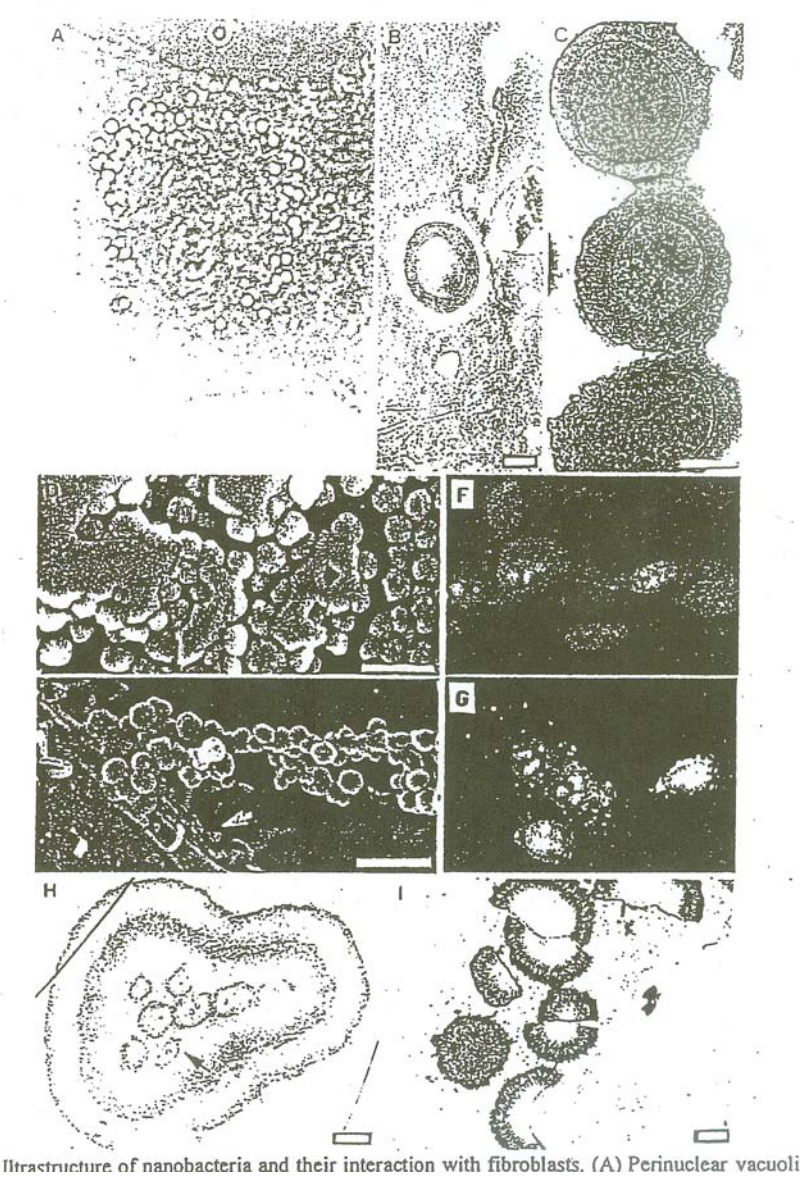
A typical time-course of a. nanobacterial culture is: Near me bottom of the culture' vessel, a few very tiny particles, either alone or in small groups are seen with light microscopy after about a one week culture period. After two weeks, they become more numerous, bigger and form groups visible even to the untrained microscopist. After one month, many are in clumps and start to attach to the bottom, and by two months most exist in a white-colored biofilm visible to the naked eye. The biofilm becomes bone-like after 3 months but sometimes it becomes detached after half a year. Pellicles can be seen in old cultures.

Specific monoclonal antibodies were produced against a bovine nanobacterial isolate. These were shown to recognize all cultured human and bovine nanobacteria thus indicating the presence of common surface antigens. Before then, nanobacteria could be well distinguished with electron microscopy (Figure IE). However, antibodies let to the development of a convenient detection method (Ciftcioglu and Kajander, 1997). Contaminated cells show the presence of star-like particles in immunofluorescence staining whereas healthy cells do not (Figure 1G and F).

Decreased serum concentration in nanobacterial culture resulted in their social growth as communities. The total removal of serum revealed a novel growth phase as seen in Figure 1H and 2A. Nanobacteria produced thick common apatite walls around small communities. These 'castles' were large, sometimes even bigger than mammalian cells. Nanobacteria were truly culturable in the new growth phase: they have been passaged monthly for over five years. These special. forms of nanobacteria could be released from their apatite 'castles' by adding serum to the culture medium. These forms had very bizarre morphology (Figure II), were found to react with the monoclonal antibodies and to release small nanobacteria later into the culture (Ciftcioglu et al, 1997b).

2.2. PHYLOGENETIC POSITION

Based upon 1406 base long 16S rRNA gene sequences, deposited now in a gene bank (EMBL Entry X98418 and X98419), nanobacteria belong to the alpha-2 subgroup of



Ultrastructure of nanobacteria and their interaction with fibroblasts. (A) Perinuclear vacuoli

Figure I. Ultrastructure of nanobacteria and their interaction with fibroblasts. (A) Perinuclear vacuolization of an infected 3T6 cell under phase-contrast microscope; (B) TEM image of a nanobacterium in a BHK cell; (C) cultured nanobacteria (bars 200 run). (D) SEM image of nanobacteria attached to culture vessel; (E) nanobacteria on a fibroblast surface (arrow: the fibroblast; bars 1 *[im).* (F) Indirect immunofluorescence staining of healthy 3T6 cells with a monoclonal antibody against nanobacteria; (G) 3T6 cells inoculated with nanobacteria; (H) TEM of a nanobacterial population in a serum-free culture (arrov? shows a D-shapcd nanobacterium); (I) D-shaped nanobacteria after culture in serum-containing medium (bars 1 urn). Reproduced from Vaccines 97, Cold Spring Harbor Laboratory Press.

Proteobacteria distinct from their nearest relatives which are Phyilobacterium, Thiobacillus, Brucella, Bartonella, Rhizobium and Agrobacterium genera (Kajander et al, 1997). The phylogenetic location is further supported by other findings: nanobacteria

are highly resistant to gamma irradiation and to heat, suggesting a recent habitat in hot-springs. *Thiobacillus* sp., one of their nearest relatives, is known to form iron and sulphur mineral deposits in hot springs (Wood and Kelly, 1985). Furthermore, antibodies against nanobacteria cross-reacted with *Bartonella henselae* and *quintana* and antibodies against *B. quintana* cross-reacted with nanobacteria in immunoassay. Thus, known human pathogens Bartonella and Brucella are relatives of nanobacteria. All share certain common properties: they are very fastidious, impermeable to stains and invade mammalian tissues, with even a preference for fetal tissues. Brucella is known to pass into urine and the Kuopio research group has recently proven that this also happens with nanobacteria (Akerman et al, 1997). Brucella and Bartonella invade animals and human cells and tissues, other members of the group exist in soil and invade plant cells, except for some Thiobacilli which inhabit hot-springs. Interestingly, mitochondria have been speculated to be derived from an ancestor of this group.

3. Unique properties

Nanobacteria are one, of the most distinctive organisms (Kajander et al, 1997), as summarized in Table 1. Their poor culturability and extremely long doubling time can be compared only to some Mycobacteria, such as *M. leprae.* The average diameter of nanobacteria measured with electron microscopic techniques, 0.2-0.3 (im, is smaller than that of any known cell-walled organism. Several viruses, e.g. Vaccinia, are larger than nanobacteria that can show sizes as small as 0.1 μm. Ultrafiltration methods produce even smaller estimates of size, since nanobacteria pass readily through 0.1 μm pore size. Apparently, nanobacteria can pass through pores smaller than their own size and they must therefore have flexible cell walls. We have also shown that during filtration they lose part of their cell wall (apatite mineral layer). The theoretical minimum diameter ofa cell, based on size of those macromolecular components now considered to be necessary and sufficient-for a living cell, is about 0.14 μm (Mushegian and Koonin, 1996). Nanobacteria have a size approaching the lower limit of the theoretical size for a living organism. The very slow growth rate and requirement of a very rich medium may be their way of adapting to make their small size compatible with life.

Nanobacteria are highly heat and γ-irradiation resistant and thus the first example of such organisms isolated from mammalian tissues. Bacteria with these properties are found in environments where these traits are essential. Nanobacteria must have emerged from a special environmental source, perhaps only recently in evolutionary terms.

High doses of aminoglycosides, 1 mg/ml, effectively blocked their replication indicating the presence of bacterial type of protein synthesis. Their resistance to these antibiotics may be due to the impermeable cell wall, which also makes their microbiological staining very difficult. The impermeability of the cell wall is further increased by mineral deposits on the cell wall. These deposits are composed of biogenic carbonate or hydroxyl apatite. Figure 2A shows the several micrometer thick apatite formation made and inhabited by a small community of nanobacteria. The cave in the apatite formed indicates its biogenic origin. Under the protection of such 'castles' nanobacteria are almost indestructible. It is noteworthy that nanobacteria cannot be detected by standard sterility testing methods using DNA-stains. We have developed improved methods and started to unravel the nature of nanobacterial nucleic acids, e.g., they show a UV-absorbance shift from 260 to 270 run (Kajander, 1992; Kajander et al, 1997).

Nanobacteria have a distinctive way of invading mammalian fibroblasts. They trigger cells that are not normally phagocytic to engulf them. After receptor-mediated adherence, followed by intemalization, apoptotic cell death occurred in all tested fibroblasts in a dose-dependent way. The cytotoxic potential depended on culture passage number, primary isolates being the most cytotoxic. Adherence and cytotoxicity are rare among cell culture contaminants (Cifti;ioglu and Kajander, 1997),

TABLE 1. Extraordinary characteristics of nanobacteria

Doubling time	° *3 days*
Culturability	mammalian cell culture medium
Size and shape	0.08-0.50 u.m, coccoid
Cell wall	very thick, flexible
Filtcrability	0.1 p.m in high yield
Heat resistance	90°C 1 h
•y-iiradiation resistance	1.5 Megarads
Antibiotic sensitivity	resistant to aminoglycosides at 100 ^g/ml
Mineral formation	biogcnic apatite formations
Resistance to lysis	most lysis methods fail
Nucleic acids	need special methods
Interaction with mammalian cells	adherence, intemalization, apoptosis

4. Do nanobacteria have a role in human diseases?

Nanobacteria are found in human and cow blood and blood products. This means that bacteremiawas present but seems to have caused no manifest acute illness. Bacteremia may last for a long time, since we have followed a human case for several years with positive nanobacterial tests from blood. However, bacteremia would be expected to cause disease(s) at least in some of the infected individuals, since there are no known examples of long-lasting bacteremia which do not cause harmful effects. The apatite coat of nanobacteria may protect them from the immune system since apatite is a normal bodily constituent and also binds serum proteins further hiding the bacteria. This might explain the long-lasting bacteremia. On the other hand, it might indicate special pathogenic role in calcifications. In cell culture, nanobacteria attach onto fibroblasts and are internalized so that intracellular calcifications are also observed. Nanobacteria can induce apoptotic cell death. Are fibroblasts attacked by nanobacteria also *in* vrvo?

4.1. TISSUE DISTRIBUTION IN VIVO

In rabbits, nanobacteria labeled with """Tc and injected intravenously showed a tissue-specific distribution with kidneys being the major target. Surprisingly, there was no significant amounts of nanobacteria in muscle, skin, bone and other tissues with fibroblastoid cells. Nanobacteria were found to pass through glomerular and tubular cells, so that nanobacteria could be isolated from urine (Akerman et al, 1997). This implies that nanobacteria may cause diseases in the urinary tract. Is there any evidence for this proposal?

4.2. NANOBACTERIA IN HUMAN STONES

Nanobacteria produce apatite minerals which are very common in kidney stones. In fact, apatite may play a key role in the formation of all kidney stones. The crystalline components of urinary tract stones are: calcium oxalate, calcium phosphate, bacterial

related, purines, or cystine. The majority of urinary stones are admixtures of two or more components, with the primary admixture being calcium oxalate with apatite (Mandel, 1996). Furthermore, fermentor model studies have shown that calcium phosphate nidi are always initially formed, and may subsequently be coated by calcium oxalate (Leusmann and Sabinski, 1996) or other components. There is a remarkable similarity in the size and morphology of nanobacterial apatite and apatite found in human kidney stones (Figure 2A and B, respectively). Nanobacteria were found commonly in the 30 human kidney stones that we have now screened. Immunologically similar bacteria could be found also in samples of dental pulp stone (our unpublished data). If a bacterial cause for stone formation could be proven, the disease may lend itself to treatment with antibiotics.

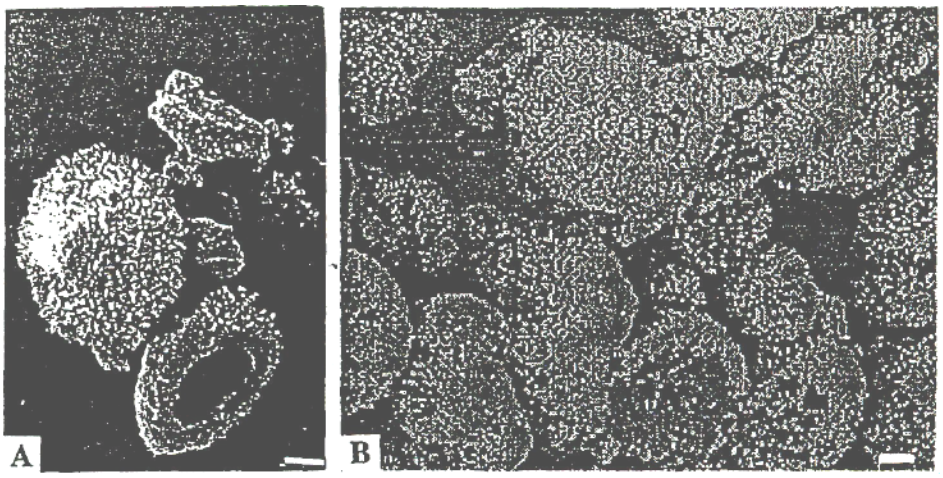


Figure 2. Scanning electron micrographs of nanobacterial dwellings (serum free culture) after scraping from the bottom of the culture vessel (A) and calcium phosphate human kidney stone (B). Both pictures arc presented in the same scale, bar 1 μm. (B) is reproduced from http://www.herringlab.cotn/scms/sems2.html. Energy dispersive X-ray microanalysis (Kajander ct al, 1997) revealed that material in (A) was also composed of calcium phosphate.

4.3. TISSUE CALCIFICATIONS AND AUTOIMMUNE DISEASES

Malacoplakia is a rare chronic inflammatory disease of unknown cause, but a bacterial factor has been strongly implicated. The disease typically occurs as tumoral growth in urogenital system characterized by an intensive infiltrate of histiocytes containing intra and extracellular calcospherules, so called Michaelis-Gutmann bodies which are composed of apatite (Ho, 1989). The structure of these spherules closely resembles our calcified nanobacteria. We propose that malacoplakia may be caused by nanobacteria.

Tissue calcifications are found in several diseases such as ovarian serous tumor, papillary adenocarcinoma of the endometrium, breast carcinoma, papillary carcinoma of the thyroid, duodenal carcinoid tumor, and craniopharyngioma (see Ho, 1989). Many malignant cells have receptors for nanobacterial adherence (Ciftcioglu and Kajander, 1997). They could introduce nanobacteria into the tumor with subsequent calcification. Furthermore, some dividing cells under inflammatory stimuli may have receptors for adherence, e.g., in atherosclerotic plaques known to have calcium phosphate accumulation.

Alzheimer plaques have been found to be labeled with anti-nanobacterial polyclonal antibodies (Kajander et al, 1993). These polyclonal antibodies contain some autoantibodies and we have also obtained some monoclonal autoantibodies in nanobacterial immunizations. Slow bacterial infection has been suggested to play a role in autoimmune diseases (Rook and Stanford, 1992). Nanobacteria are a new example of slowly growing organisms infecting man for long periods of time. The apatite structure and anomalous nucleic acids may contribute to abnormalities in immune response to this infection.

5. Conclusions

Nanobacteria are novel emerging pathogens and may be related to small mineral forming bacteria found in sedimentary rocks, linking medicine to geology. They produce biogenic apatite *in vitro and* also seem to do so *in vivo.* Since apatite is considered to be the main nidus initiating the formation of most kidney stones, nanobacteria seem to be excellent candidates for triggering this process. Nanobacteria injected to blood circulation were shown to penetrate through kidney cells and pass into urine. In urine, apatite formation by nanobacteria is further increased. Other minerals may thereafter bind onto this nidus. The focus of future work should be to clarify their role also in intracellular calcifications and tissue calcinosis, processes involved in many major diseases. Since nanobacteria are cytotoxic, their possible role in kidney damage also warrants further work. Our data indicate that the pathophysiology of stone formation in human diseases has now to be re-evaluated to include nanobacteria in the etiology and this may lead to new treatment strategies and therapies for stones and calcifications.

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