Stone formation and calcification by Nanobacteria in human body

Neva Ciftcioglu, Mikael Björklund and E. Olavi Kajander

Department of Biochemistry and Biotechnology , University of Kuopio, P.O.Box 1627, FIN- 70211, Kuopio, Finland

ABSTRACT

The formation of discrete and organized inorganic crystalline structures within macromolecular extracellular matrices is a widespread biological phenomenon generally referred to as biomineralization. Recently, bacteria have been implicated as factors in biogeochemical cycles for formation of many minerals in aqueous sediments. We have found nanobacterial culture systems that allow for reproducible production of apatite calcifications *in vitro.* Depending on the culture conditions, tiny nanocolloid-sized particles covered with apatite, forming various size of aggregates and stones were observed.

In this study, we detected the presence of nanobacteria in demineralized trilobit fossil, geode, apatite, and calcite stones by immunofluorescence staining. Amethyst and other quartz stones, and chalk gave negative results. Micro-organisms are capable of depositing apatite outside the thermodynamic equilibrium in sea water. We bring now evidence that this occurs in the human body as well. Previously, only struvite kidney stones (25% of all kidney stones) composed of magnesium ammonium phosphate and small amounts of apatite have been regarded as bacteria related. 90% of demineralized human kidney stones now screened. contained nanobacteria. At least three different distribution patterns of nanobacteria were observed in the stones. SEM revealed great similarity in the size and morphology of nanobacteria cultured in serum fire conditions, and human kidney stones that are formed from small apatite units. Prerequisites for the formation of kidney stones are the supersaturation of urine and presence of nidi for crystallization. Nanobacteria are important nidi and their presence might be of especial interest in space flights where supersaturation of urine is present due to the loss of bone. Furthermore, we bring evidence that nanobacteria may act as crystallization nidi for the formation of biogenic apatite structures in tissue calcifications found in, e.g., atherosclerotic plaques, extensive metastatic and tumoral calcifications, acute periarthritis, malacoplakia, and malignant diseases. In nanobacteria-infected fibroblasts, electron microscopy revealed intra- and extracellular needle-like crystal deposits, which were stainable with von Kossa stain and resemble calcospherules found in pathological calcifications. Thus bacteria-mediated apatite formation takes place in aqueous environments, in humans and in geological sediments.

Keywords: Nanobacteria, nannobacteria, calcification, stone formation, apatite, biomineralization

1. INTRODUCTION

Nanobacteria, approaching the theoretical limit of the self-replicating life with a size of only one hundredth of that of usual bacteria, were isolated from mammalian blood and blood products.[1] We have identified with energy-dispersive X-ray microanalysis and chemical analysis that nanobacteria produce biogenic apatite on their cell envelope. The thickness of the apatite depends mostly on the culture conditions of the nanobacteria (Kajander *et al.,* in this issue).

Several aspects of biogenic apatite nucleation, crystal growth and morphology have been determined both *in vivo* and *in vitro* studies.[2] However, many details remain unresolved, including the specific nature of the initial precipitating phases, the mechanism and factors which control the incorporation of ionic impurities into the crystal lattice, details of the crystallographic ultrastructure and morphology in mineralized tissues (bone, dentine), and the relationship of the inorganic components with the complex collagen based matrix. The reason behind the calcium phosphate deposition in many diseases remain speculative. It has been shown that an accumulation of calcium in mitochondria, which is presumably dependent upon residual substrate for energy production, appeared to cause calcification:[3,4] Amorphous calcium phosphate in the form of spheroids, and possibly fine fibrils and granules, also appeared to play a role in calcification by their transformation into apatite. The seemingly simple phenomenon of tissue calcification is complex.[3]

In the biosphere, bacteria can function as geochemical agents, promoting the dispersion, fractionation and/or concentration of matter processes only recently started to be understood. These have given rise to the field of geomicrobiology .It is known that dead bacteria, and bacterial remains, act as heterogenous nuclei of crystallization (biomineralization).[5] Micro-organisms that are capable of depositing apatite outside thermodynamic equilibrium in sea water can segregate Ca from Mg under conditions of pH<8.5 and [Mg]:[Ca]>0.1.[6] Such conditions are also present in human body. In this paper, we provide evidence that nanobacteria can act as crystallization centers (nidi) for the formation of biogenic apatite structures in the mammalian body, and in environmental sources.

2. CALCIFICATION CAUSED BY NANOBACTERIA IN A CELL CULTURE MODEL

We have proven that nanobacteria are cytotoxic *in vitro*[7] and *in vivo*[8]. 3T6 fibroblastoid cells infected for 48 hours with nanobacteria (cultured in serum free condition, SF-nanobacteria), showed altered cell morphology due to internalized SF- nanobacteria (Fig. IA and B). von Kossa staining revealed intra- and extracellular calcification in the infected cells (Fig. IC). Heavily infected cells showed nuclear abnormalities, e.g., macronucleus.[9] There was no calcification and nuclear abnormalities in the control cells stained with the von Kossa method (Fig. ID).

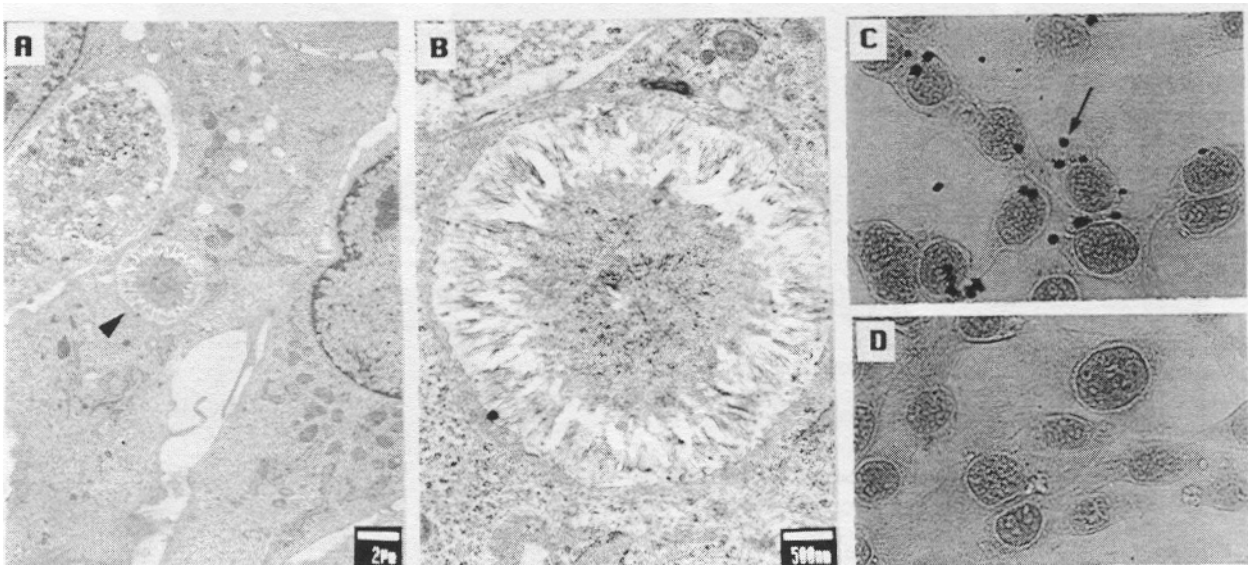

Figure 1. Interaction of SF-nanobacteria with fibroblasts (3T6 cells). (A) SF-nanobacteria internalized by a fibroblast (arrow head shows the nanobacteria inside a vacuole), (B) higher magnification showing the needle-Iike apatite structure of the internalized SF-nanobacteria, (C) von Kossa staining result of the nanobacteria infected fibroblasts, and (D) negative control. Magnification in (C and D) is 270X. Arrow in (C) shows stained nanobacteria after staining with the von Kossa method which is a standard calcification detection method used in pathology.

3. NANOBACTERIA AND ENVIRONMENTAL STONES

The evidence obtained by McKay *et al.*[10] of possible nanofossils in the ancient Martian meteorite ALH84001 brought wide interest to the subject of role of bacteria in biogeochemical cycles. SEM observations made from different sorts of minerals and rocks have shown minute, spherical to worm-shaped cells named as Nannobacteria.[11] These tiny micro-organisms were observed in different mineral sources by the other geologists, too.[12] There is no evidence for apatite formation by these SEM observed organisms so far .Recently, we have proven that nanobacteria isolated from mammalian blood and blood products by us, produce an apatite layer on their cell envelope during the culture period. [1,9,13]

In this study, we screened six different kind of environmental stones (trilobit, geode, apatite, amethyst, chalk and calcite) for the existence of nanobacteria. The stones were demineralized by using 1 N HCl, and immunostained by using anti-nanobacteria monoclona1 antibodies. Except in amethyst and chalk, we detected positive stained, nanobacteria-sized cocci at various concentrations in the other stones. Although this study needs further investigation, e.g., nanobacterial culture, we are confident, based on our results from kidney stones, that nanobacteria exist in these environmental stones and participate in mineral formation in nature.

4. NANOBACTERIA AND KIDNEY STONES

Urinary tract stone formation is a multifaceted process. Urinary tract stone crystalline components are of five types: 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. The viability and location of bacteria within infection stones (struvite [$MgNH_4PO.6H_2O$] and/or carbonate apatite [$Ca_{10}(PO_4)_6CO_3$] stones) have been investigated. It was found that large numbers of bacterial impressions and bodies were existing in the interstices surrounded by crystals of apatite and struvite from the nuclei to the peripherallayers.[14] The presence of bacterial colonies even in the nuclear portion of the stones suggests that bacteria participate in the initial stone formation, as well as in growth of infection stones. Though some other research groups could find no bacteria within the nuclear parts of the kidney stones, they detected calcium phosphate at the central core which obviously had been acting as a nidus for stone formation.[15] In our work, bacteria of similar size and morphology (Fig. 1 A and C) as nanobacteria (Fig. 1 B and D) were found with TEM in human kidney stones.

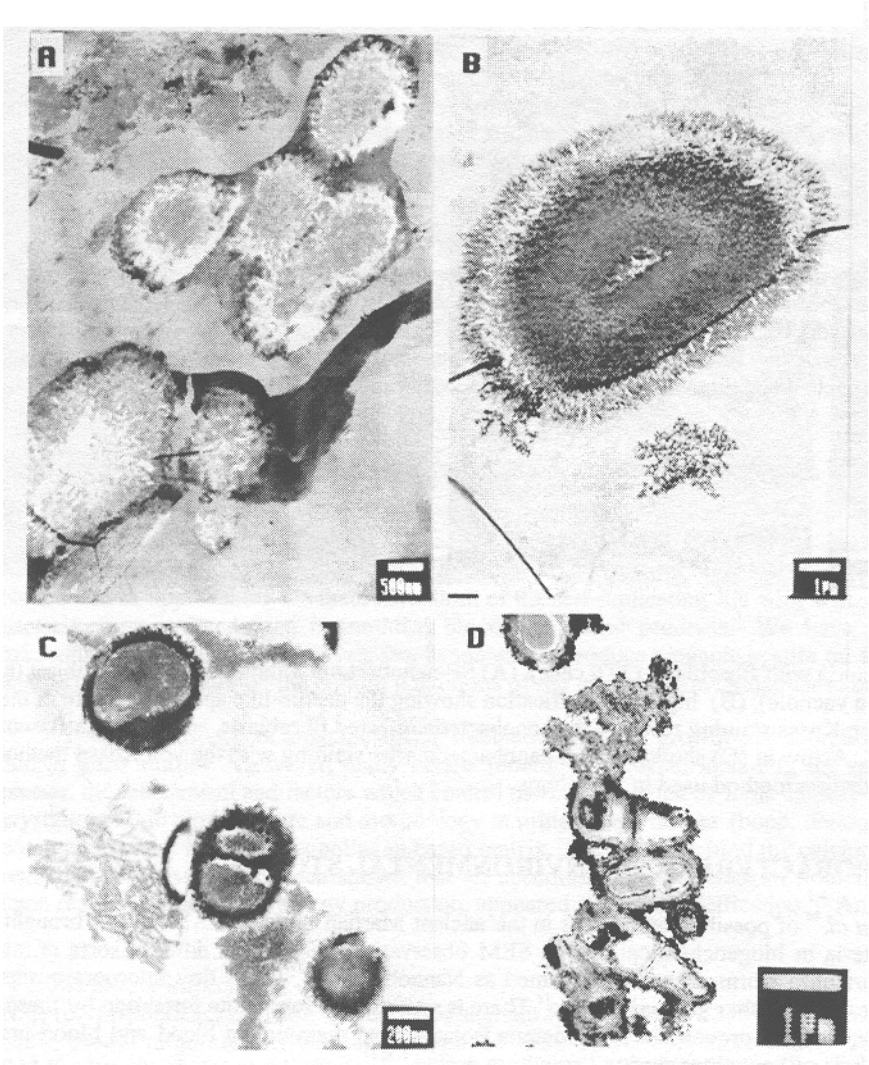

Figure 2. TEM micrographs of a carbonate apatite human kidney stone and nanobacteria. (A) A kidney stone before demineralization, (B) SF- nanobacteria cultured for one month, (C) the same kidney stone after demineralization, (D) nanobacteria cultured in serum containing medium for 2 months. The kidney stone (C) was demineralized by incubating the smashed stone in lN HCl for 10 min at room temperature, neutralized with NaOH and potassium phosphate buffer, and epon embedded. Both cultures of nanobacteria (B and D) adhered to the bottom of their culture vessels.

We screened 60 human kidney stones for nanobacteria positivity by using immunofluorescence staining and culture methods. Nanobacteria show a thick apatite envelope layer on their surface in TEM (Fig. 3A). Demineralization by using a very harsh condition such as incubation with 1 N HCI did not affect their epitopes recognized by our monoclonal antibodies. Nanobacteria-specific monoclonal antibodies revealed positive, small cocci at various concentrations in all demineralized stone samples (Fig. 3 C-E) and nanobacteria (Fig. 3B). Different distribution patterns of nanobacteria were observed in the stones, e.g., central and/or peripheral location (Fig. 3E) in the small stone units, or random distribution (Fig. 3 D). Specificity of the staining was further proven with negative staining results with four different monoclonal antibodies detecting nonrelevant antigens.

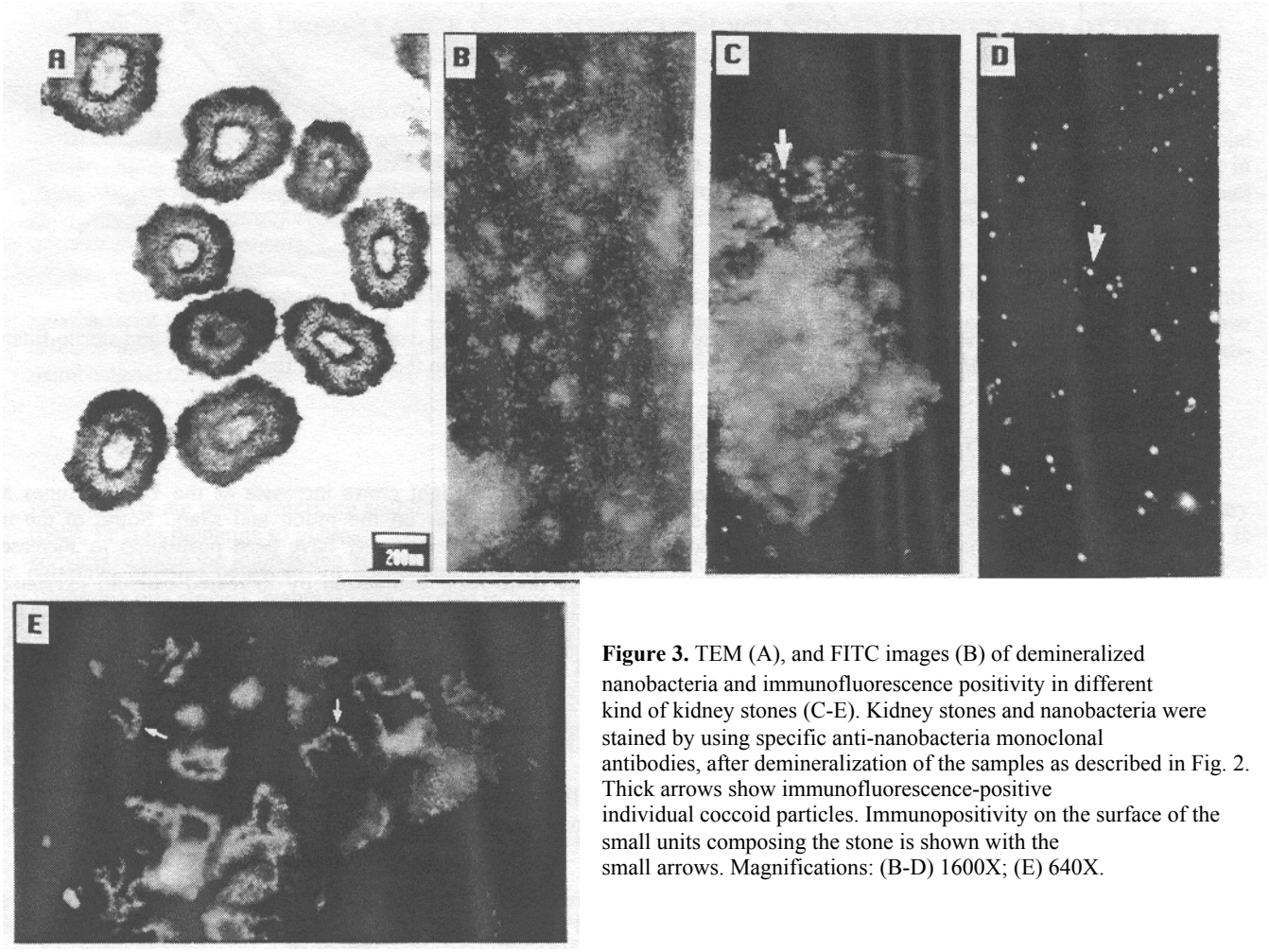

Figure 3. TEM (A), and FITC images (B) of demineralized nanobacteria and immunofluorescence positivity in different kind of kidney stones (C-E). Kidney stones and nanobacteria were stained by using specific anti-nanobacteria monoclonal antibodies, after demineralization of the samples as described in Fig. 2. Thick arrows show immunofluorescence-positive individual coccoid particles. Immunopositivity on the surface of the small units composing the stone is shown with the small arrows. Magnifications: (B-D) 1600X; (E) 640X.

The demineralized, screened kidney stone samples were sterile-filtered through a 0.2 μm filter, and cultured in the nanobacterial culture conditions for three weeks as described.[7, 16] Gamma irradiated serum at 10% concentration was used as a culture supplement. In each experiment, only gamma irradiated serum culture was used as a negative control, and no growth was observed.

Interestingly, we observed nanobacterial growth in 90% of the stone samples despite of the very harsh demineralization step. In addition, the stones had been stored at room temperature for more than one month before screening. Demineralized control nanobacteria, the positive controls, multiplied well (Fig. 4 A and B). Nucleic acid staining by using Hoechst (#33258) stain proved no other kind of bacterial growth was present in the cultures. For further proof, 3T6 cells were infected with the nanobacteria cultured from stone samples, and stained with anti-nanobacteria monoclonal antibodies. Five different kind of nanobacteria-cell interaction was observed (data not shown).

In recent years, microbiologists and geologists have started to search the extreme limits of life on earth. A group d scientists have found organisms trapped almost 3 kilometers beneath Virginia for millions of years, microbes living off bare rock and water a kilometer down in the Columbia Plateau. Obviously, these extraordinary microbes have adapted with exotic metabolisms and very slow rates of reproduction.[17] It seems that wherever a source of energy exists, life is present. Survival of nanobacteria in dried, and HC1-treated kidney stones may be an example to that.

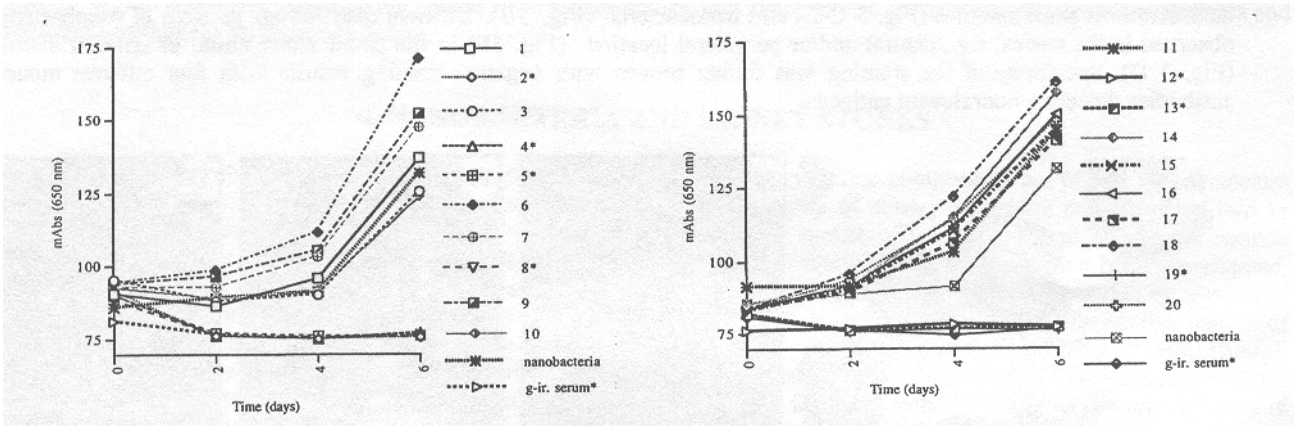

Figure 4. Graphics showing the nanobacterial growth in the subcu1ture of the demineralized, neutralized and sterile-filtered 20 different human kidney stones, and nanobacteria. * Indicates no growth in the followed first 6 days.

4.1. KIDNEY STONE RISK FACTORS DURING SPACE FLIGHT

The importance of maintaining the long-term health and well-being of flight crews increases as the United States space program develops a pennanently manned space station and potential bases on the moon and Mars. Some of the many metabolic changes that occur in the human body upon exposure to microgravity have been postulated to increase the potential for renal stone formation.[18] One such reaction is bone loss, manifested by increased calcium excretion and a negative calcium balance.[19] Stone formation typically is the result of hypercalciuria, hyperphosphaturia, hyperoxalauria or hypocitraturia.[20] The crystal formation easily occurs in this kind of supersaturated urine if there is crystallization nidi. In the light of our results, showing the nanobacterial positivity of the human kidney stones, we here propose that blood samples of astronauts should be screened for possible nanobacteria infection before space flights.

5. OTHER PATHOLOGIC CALCIFICATIONS IN RUMAN BODY

We found nanobacteria positivity in dental stones in a pilot survey. Interestingly, a very high percentage of the patients and/or the parents of the patients, were having kidney stone, gallstone and/or tissue calcification (Ciftcioglu *et al.,* in this issue).

Malacoplakia is a rare chronic inflammatory disease of unknown cause, but a bacterial factor has been strongly implicated.[21] It may be fatal. The disease is characterized by von Kossa staining positive, calcified laminated or target- shaped bodies termed Michaelis-Gutmann bodies which are composed of apatite.[22] The structure of these calcospherules closely resembles our calcified nanobacteria.[1]

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 Ref. 23). In many malignant tumors, needle-shaped crystals are found in epithelial cells.[24] To detect this kind of calcification it is necessary to use electron microscopy, since the crystals are too small to be seen with the light microscope, and their origin is unknown. Many malignant cells have receptors for nanobacterial adherence.[7] 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. In this disease, although electron probe analysis showed that the surface and interior of the mineral deposit had the same chemical composition, SEM revealed different kinds of structures such as spherical particles and fibres25 which are resembling nanobacteria. Similarly, in acute periarthritis, in the joints, hydroxyapatite crystals have been shown.[26,27]

Alzheimer plaques have been found to be labeled with anti-nanobacterial polyclonal antibodies.[28] 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.[29] Tissue calcification is often present in these diseases. 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.

6. MEDICAL IMPORTANCE OF NANOBACTERIA IN KIDNEY STONES AND OTHER PATHOLOGICAL CALCIFICATIONS

Urinary tract infection causing struvite and carbonate apatite formation is the commonest cause of stones in Europe.[30] Conventional therapy has usually consisted of surgical removaI of the stone, combined with a short course of antimicrobial therapy. Such treatment is curative in about 50% of cases. Recurrent stone formation and progressive pyelonephritis occur in those who are not cured.[31] The morbidity and expense that result from this disease are great. Long-term (perhaps life-time) chemotherapy with antimicrobial agents and expert surgical intervention can be expected to significantly improve the plight of these unfortunate patients.[31]

Since our findings show the high association of nanobacteria with kidney stones, further research for the anti-nanobacterial therapeutics is needed. If successful, most kidney stone disease could be eradicated. Some 20% of human diseases have potential nanobacterial features in their pathogenesis, such as stone formation and calcification. The anti- nanobacterial therapy might thus prove to be highly important.

7. 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 of laboratory animals, were shown to penetrate through kidney cells and pass into urine.[8] 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 the 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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