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DETECTION OF NANOBACTERIA-LIKE PARTICLES IN HUMAN ATHEROSCLEROTIC PLAQUES

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Recent and historical evidence is consistent with the view that atherosclerosis is an infectious disease or microbial toxicosis impacted by genetics and behavior. Because small bacterial-like particles, also known as nanobacteria have been detected in kidney stones, kidney and liver cyst fluids, and can form a calcium apatite coat we posited that this agent is present in calcified human atherosclerotic plaques. Carotid and aortic atherosclerotic plaques and blood samples collected at autopsy were examined for nanobacteria-like structures by light microscopy (hematoxylin-eosin and a calcium-specific von Kossa staining), immuno-gold labeling for transmission electron microscopy (TEM) for specific nanobacterial antigens, and propagation from homogenized, filtered specimens in culture medium. Nanobacterial antigens were identified *in situ* by immuno-TEM in 9 of 14 plaque specimens, but none of the normal carotid or aortic tissue (5 specimens). Nanobacteria-like particles were propagated from 26 of 42 sclerotic aorta and carotid samples and were confirmed by dot immunoblot, light microscopy and TEM. [^{3}H]L-aspartic acid was incorporated into high molecular weight compounds of demineralized particles. PCR amplification of 16S rDNA sequences from the particles was unsuccessful by traditional protocols. Identification of nanobacteria-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.

Keywords: Nanobacteria-like particles – atherosclerosis – carotid artery – aorta – vascular plaque

INTRODUCTION

The "infection hypothesis" is emerging as a leading explanation for atherogenesis, the main cause of mortality of adults in the industrialized countries and the most frequent complication in patients with renal failure and patients undergoing chronic hemodialysis treatment [1–6]. While additional risk factors, such as advanced age, diabetes, hypertension, disordered lipid metabolism, and smoking are known, many of these factors and chronic diseases are intertwined with infection or exposure to

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Abbreviations: DMEM, Dulbecco's Eagle's medium; TEM, transmission electron microscopy; NB, nanobacteria; EM, electronmicroscopy, FBS, fetal bovine serum; LDL, low density lipoprotein, EDTA, ethylene-diamino-tetraacetate; BSA, bovine serum albumin; PBS, phosphate-buffered saline; FITC, fluorescein-isothiocyanate

microbial toxins [7, 10–12]. Inflammation and induction of autoimmunity (i.e., molecular mimicry) are classical components of microbe-induced pathogenesis and are present in atherosclerosis [8, 9]. Of the candidate pathogens, *Chlamydia pneumoniae* has recently emerged as an agent associated with atherosclerosis [13]. Chronic infection with *C. pneumoniae* was speculated to contribute to atherosclerosis in patients with chronic renal failure [14]. However, in spite of significant efforts, no conclusive evidence is yet available to decide whether *C. pneumoniae* is an etiological agent or only a bystander in atherosclerotic plaques [15]. Other studies suggested that *Helicobacter pylori* [16], human cytomegalovirus (CMV) [17–19] and herpesvirus infections [19–20] could also be involved in plaque formation by promoting inflammatory immune injury and elevating the low-density lipoprotein level [19]. Additional infectious agents cannot be excluded as new methodologies for microbial detection coupled with respect for historical and emerging findings of ultra-small atypical microbes [21] and infectious agents continue to reveal new pathogens, even infectious proteins (e.g. prions).

Calcification is associated with atherosclerotic plaque and positively correlated with worse cardiovascular prognosis [22–23]. Several mechanisms for vascular calcification have been suggested, including an active, regulated process similar to osteogenesis [24–25], mast cell and macrophage induced calcification [26] and stimulation of vesicle-calcification by lectins [27].

Protein-calcium phosphate complexes have recently been discovered in serum and it was hypothesized that these high-molecular weight calcified particles are the origins of soft tissue calcifications, such as atherosclerosis [43, 44].

In the context of emerging pathogens or agents in chronic diseases, nanobacteria have been suggested as candidate infectious agents capable of forming an apatite coat at physiological levels of calcium and phosphate [28]. They have been isolated from human and animal serum [28–29, 37], human kidney stones [30, 38, 39, 45], dental pulp stones [46], bile [40] and liver and kidney cyst fluids [31] and in psammoma bodies in ovarian carcinomas [47]. Hypotheses concerning a role for nanobacteria in tissue calcification and cystogenesis have also appeared [32]. However, in other studies the microbiological origin of these particles was queried [34] and the importance of biomineralization in pathological calcifications was emphasized.

Here we report the first observation of nanobacteria-like particles in human atherosclerotic plaques, thereby opening the possibility that initialization of inorganic crystallization by these particles may contribute to vascular calcifications.

MATERIALS AND METHODS

Light microscopy

During autopsy examinations, 24 carotid and 27 aortic specimens were taken under aseptic conditions from 28 subjects 4–10 hours *post mortem*. For hematoxylin-eosin and von Kossa silver staining, the samples were fixed in 4% formalin phosphate

buffer (50 mM, pH 7.0), then embedded in paraffin, and 4 μm sections were made. In order to remove paraffin, hexane and ethanol washing was applied. All other details of these staining techniques were the same as described previously [33].

Immunoelectron microscopy

Eight carotid and six aortic specimens containing highly calcified plaques were examined by immunoelectron microscopy. The specimens were fixed in 50 mM phosphate buffer (pH 7.0) containing 1% formalin and 0.25% glutaraldehyde and then embedded in paraffin. 70–80 nm-thick sections were cut and placed on Nickel grids. Sections were deparaffinated with ethanol, then washed with phosphate-buffered saline (PBS). Grids were incubated with EDTA (50 mM) containing PBS for 15 minutes, then washed three times with PBS, and incubated for 16 hours at room temperature with blocking solution [PBS and 2% bovine serum albumin (BSA) (ICN, Aurora, OH)] containing 8D10 anti-nanobacterial monoclonal primary antibody (nanobacterial porin protein epitope specific, NanoBac Ltd., Finland) in 1 : 50 dilution. The grids were washed with PBS 5 times, and the secondary antibody [5 nm gold-conjugated anti-mouse antibody (Sigma)] was applied for 3 hours, after which the grids were washed 5 times with PBS. They were then dried and analyzed under a transmission electron microscope (Philips CM10, 70KV).

Cultures

For culture of nanobacteria-like particles, carotid and aortic atherosclerotic plaques and blood samples were taken under aseptic conditions from 22 postmortem subjects. Each of the plaque samples (2–5 g) was homogenized in 5 ml sterile Dulbecco Modified Eagle's Medium and centrifuged at 1000 g for 3 min. Two ml supernate was next filtered through a 0.2 μm sterile filter, mixed with 18 ml culture medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (GIBCO) and with ampicillin and kanamycin at final concentartion of 0.1 mg/ml, and nystatin (0.04 mg/ml), irradiated with a 0.8 megarad γ-ray dose in T-25 tissue culture flasks and incubated at 37 °C (or at 4 °C, 16 °C, 22 °C for determination of the effect of incubation temperature) in a humidified 5% CO_2/95% air environment for 6–10 weeks [32, 35]. The FBS was irradiated before use with a 2×3 megarad γ-ray dose to eliminate all of the possible bacterial and biofilm forming nanoparticle contamination [28]. Blood samples were prepared for culturing by using the same process except for the homogenization. After incubation for 2 months (up to 3 months in the case of determination of the effect of the temperature) nanobacteria-like particles in the cultures were identified by means of 8D10 mAb, light microscopy and TEM.

Dot-immunoblot analysis

Dot immunoblot analysis was performed with 10-fold concentrate of the culture material (20 µl) with CDP-Star chemilumunescent detection method (Boehringer Mannheim). Nanobacteria concentrate was obtained by centrifugation of the specimen cultures at 13,000 g for 10 min. Pellets were resuspended in 2 µl PBS and dot blotted onto nitrocellulose paper (Amersham Pharmacia), blocked with 2% BSA (NB-Mab negative) and exposed to 8D10 primary antibody, washed and probed with an alkaline phosphatase-linked antimouse IgG secondary antibody. Membranes were washed with PBS, CDP-Star detection buffer (0.1 M Tris-HCl, 0.1 M NaCl, pH 9.5) and reacted with CDP-Star working solution (CDP-Star 1 : 100 in detection buffer) and incubated for 5 min. Chemiluminescent light emission was recorded on X-ray films. Cultures from assay *Escherichia coli* and *Corynebacterium glutamicum* were also tested as negative controls in dot immunoblots using the same parameters as in the case of NB cultures.

Isotope-labeled amino acid incorporation analysis

Incorporation of [^{3}H]L-aspartic acid was tested using 50 µCi (17 Ci/mmol specific activity, ICN Biomedicals Inc.) in four separate T-25 tissue culture flasks, containing 10 ml of culture. Incubation was carried out at 37 °C for two or four weeks. Nanobacteria were collected by centrifugation at 13,000 g for 15 minutes, washed three times with PBS. One half of the samples were dialysed against 50 mM EDTA for two days in total volume of 50 µl. Radioactivity was measured in 5 ml scintillation fluid with the Cerenkov method on Delta 300 Instrument (Searle Analytic Inc.).

Nucleic acids preparation and rDNA amplification

From cultures, nanobacteria-like particles were collected by centrifugation, and DNA was purified with traditional EDTA/alkali lysis or SDS method, or using FastDNA™ kit specifically designed for bone preparations (BIO 101, Vista, CA) with FastPrep™ instrument. PCRs were carried out with ultra pure water and reagents using the same amplification protocols as described before [34]. Where PCR was positive the product was isolated and cloned into pGEM-T (Promega) vectors and sequenced by dye-terminator method with an ABI Automated DNA sequencer.

RESULTS

In all 61 specimens of atherosclerotic plaques examined, light microscopy revealed extracellular crystalline calcospherule deposits 0.2–10 µm in size (Fig. 1A) that were stainable by the von Kossa method [33] (Fig. 1B). Selected specimens were examined for nanobacterial antigens.

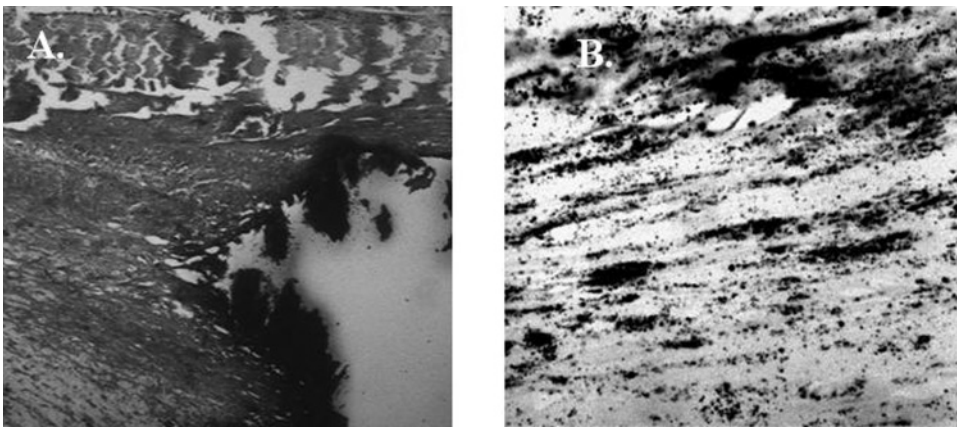

Fig. 1. Light micrographs of an atherosclerotic sample. *A)* Atherosclerotic lesion stained with hematoxylin-eosin (×400), and *B)* by the calcium-specific, von Kossa method (×800). The small black dots correspond to calcium spherules, which are almost invariable found in atherosclerotic plaques

Immunoelectron microscopy revealed specific gold-labeled areas in 5 of 8 carotid and 4 of 6 aortic plaques examined (Fig. 2A, B), indicating that nanobacterial antigens were present in the plaques. Application of a short EDTA treatment for decalcification of the native preparations led to an improved accessibility of the monoclonal antibodies to the surface of structures with thick mineral shells. Small, electron-dense dots were gathered in specific areas with an approximate size of 0.2–0.6

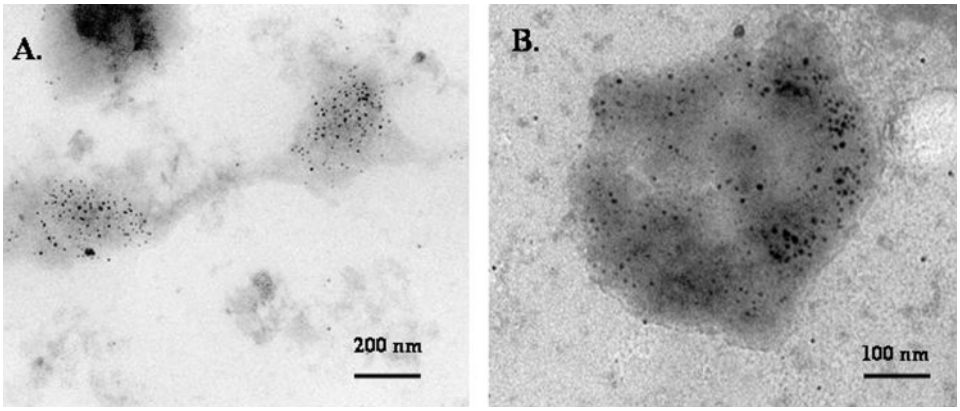

Fig. 2. Immunoelectron micrograph of a human sclerotic carotid artery and aorta. *A)* Transmission electron micrographs of a gold-immunostained carotid artery using 8D10 antibody against nanobacterial porin protein and a 5 nm gold labeled anti-mouse secondary antibody. The small, electron-dense dots are the gold particles linked to antibody. No dots can be found at the surrounding cell debris. *B)* TEM of an aorta, treated with the same procedure like in the case of the carotis *(A)*. In both cases the calcium-coat of the bacteria-like particles were removed by EDTA treatment before applying the primary antibody

Table 1
Presence of nanobacteria-like particles in cultures of atherosclerotic specimens from carotid arteries and aorta, and blood collected post mortem*

Identification number of subject	Plaque from		
	carotid artery	aorta	blood
91/00	+	+	+
671/99	+	+	+
672/99	+	+	+
675/99	+	+	+
688/99	+	+	+
109/00	+	+	+
110/00	+	+	+
111/00	+	+	–
112/00	+	+	–
122/00	+	+	–
123/00	+	+	–
95/00	–	+	–
101/00	–	–	+
97/00	–	–	+
649/99	+	–	+
135/00	–	–	–
674/99	–	–	–
102/00	–	–	–
127/00	–	–	–
130/00	–	–	–
124/00	0	+	+
92/00	0	+	+
Summary of results, (%)	60	63.63	54.54

*(+: positive, –: negative culturing results confirmed by dot immunoblot and light microscopies. 0: this kind of specimen was not taken)

mm. Dots correspond to gold particles linked to secondary antibody. No or very few dots could be found at the surrounding cell debris, which represents the specificity of the reaction. Three aortic and three carotid specimens without sclerotic plaques were analysed as controls by the same method and they did not exhibit Mab-specific gold-labeled dots. The sizes of the electron-dense spotted structures in the positive specimens corresponded to the size of the cultured agents.

Altogether 64 samples were cultivated under nanobacteria culture conditions with sublethal dose of different antibiotics (kanamycin, amicillin) as well.

At the end of the 2-month incubation period biofilm formation was observed in 38 of 64 specimens cultured (Table 1). All of these positive cultures reacted with 8D10 and G1-B8 anti-nanobacterial antibodies in dot immunoblot assays. TEM images of the positive vasculare cultures contained structures similar to nanobacteria published

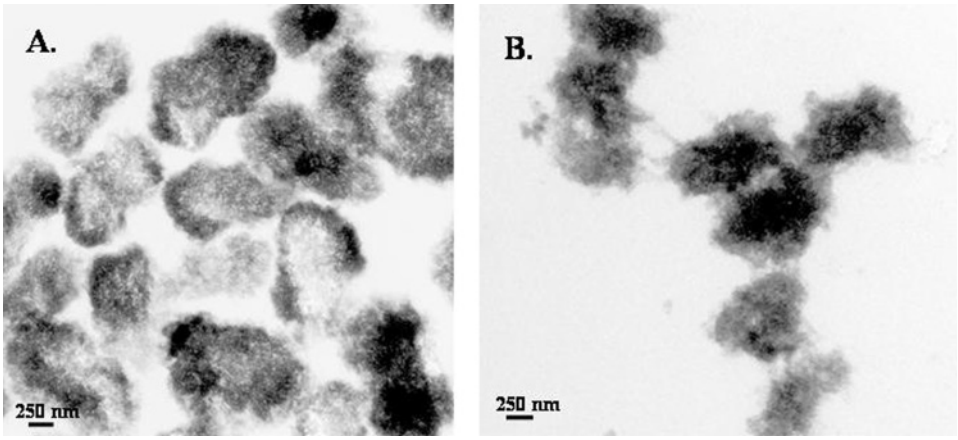

Fig. 3. Transmission electron micrographs (TEM) of biofilm containing nanobacteria-like particles. *A)* TEM of pelleted particles propagated *in vitro* (gift from N. Ciftcioglu and O.E. Kajander, University of Kuopio). (Horizontal lines (e, f) = 100 nm). *B)* TEM of nanobacteria-like particles pelleted from a culture of sclerotic carotid artery. The electron-dense characteristics of the agent is due to the thick mineral coat

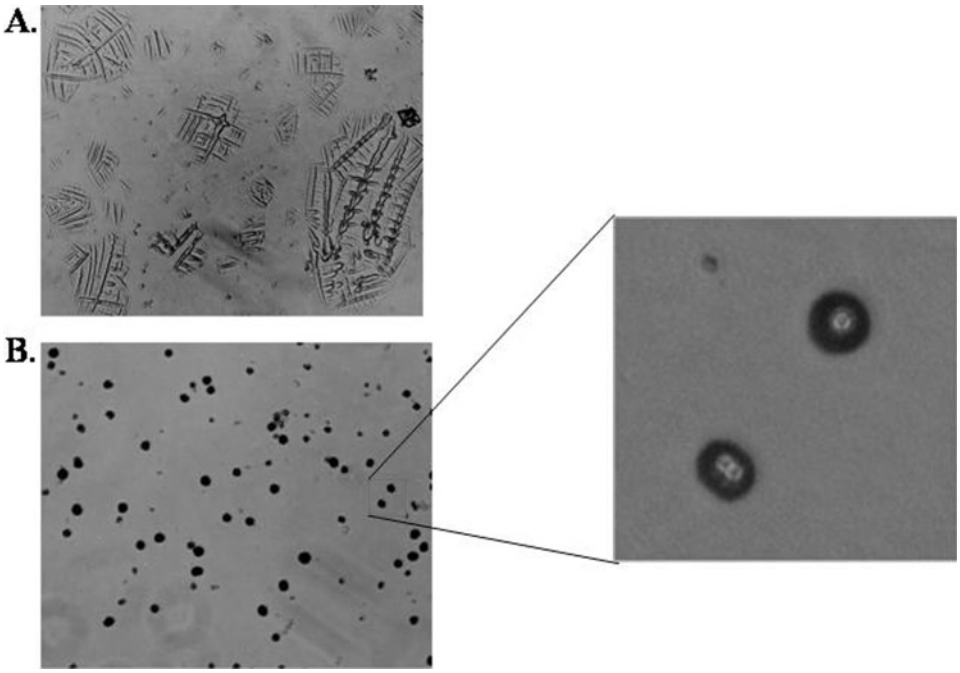

Fig. 4. Light micrographs of nanobacterial cultures. *A)* Inorganic crystallization in the bottom of the vial after 4 months incubation of the control vial. *B)* Biofilm containing nanobacteria-like particles after propagation *in vitro* for 2 months inoculated from a sclerotic carotid artery sample. Central halo and a peripheral dark rim, which is due to the thick mineral coat can be recognized in each particle (400×)

by others [28–32] (Fig. 3A–B). The culture results (Table 1) demonstrate that 12 of 20 (60%) carotid plaques, and 14 of 22 (~63%) aortic plaques, and 12 of 22 (~54%) blood samples contained nanobacteria-like particles. Taken together, 23 of the 31 (~74%) autopsy subjects examined in this study yielded immunopositivity and/or culture evidence of nanobacteria-like particles. The anti-nanobacterial monoclonal antibody used did not recognize in the dot immunoblot assay the Gram-negative *E. coli* and the Gram-positive *C. glutamicum*.

Light microscopy analysis of the biofilm revealed similar calcified particles (Fig. 4B) to those that can be found in atherosclerotic lesions stained by the von Kossa method (Fig. 1B). NB-like particles are characterized by central halo and a peripheral dark rim which correspond to calcium apatite shell. After 2 months cultivation these particles can be visible by light microscopy and could be easily distinguished from inorganic crystallization (Fig. 4A). With higher magnification in some particles double mineralized vesicles can be recognized (Fig. 4B).

The effect of temperature during incubation on biofilm formation was examined. Incubations were carried out at 4 °C, 16 °C, 22 °C, and 37 °C in three separate experiments. Temperatures lower than 37 °C inhibited the multiplication of nanobacteria-like particles. Common bacteria were not detected in these cultures at any time point or temperature; biofilm formation was not evident at the lower temperatures, even when cultures were incubated over a three months period.

Table 2
Incorporation of [^{3}H]L-aspartic acid into nanobacteria-like particles

	Without dialysis count (SD)	Dialysis against EDTA count (SD)
Two weeks incubation	1654 (389)	2978 (318)
Four weeks incubation	4543 (435)	7897 (358)
Background*	543 (298)	543 (298)

Data were obtained from 3 separate experiments. *Background was determined using PBS alone.

In order to confirm the possible metabolic activity of nanobacteria-like particles, the incorporation of [^{3}H]L-aspartic acid into the agents was examined. Cultures were examined under light microscopy in order to confirm the absence of classical microbial contamination prior to the addition of radiolabel. A time dependent increase in particle-associated radioactivity was measured (Table 2). Dialysis of the radiolabeled agents against 50 mM EDTA for two days revealed almost twofold more radiolabel incorporation.

PCR amplifications of all analyzed samples prepared from cultures with nanobacteria-like particles were carried out to determine the nucleotide sequence of the 16S

rDNA segment of the possible bacteria. In 60% of the cases amplification of contaminating template occurred while the rest of the cases no amplification product could be detected. None of the cloned and sequenced DNA corresponded to the published *Nanobacterium sanguineum* sequence [28] (data not shown).

DISCUSSION

This preliminary study of nanobacteria-like particles in vascular lesions utilized available criteria for their identification: small size, apatite coat, reactivity with anti-nanobacterial Mab, and incorporation of radiolabeled substrate [28–32]. Just as other atypical infectious agents were linked with disease before they were completely characterized, more tools and protocols for detection of nanobacterial particles will likely become available as aspects of pathological biomineralization and structure are more widely confirmed and extended. The apatite coat of nanobacterial agents has confounded structural analysis, extraction and amplification of DNA, and led some to confuse any calcified structure with nanobacteria [36]. However, in this study we failed to extract and amplify nanobacteria-specific rDNA sequences, like Cisar et al. [34], therefore we propose nanobacteria-like "agent" and "particle" terminology instead of nanobacteria until their bacterial origin will not be proved.

In the present work, small spherules visualized by light microscopy and von Kossa staining in all 61 specimens of atherosclerotic plaques examined were in the size range of nanobacteria-like particles and colonies published before [28–32]. Their size and shape corresponded to particles obtained by cultivation of human isolates as well (Fig. 3A). We found that EDTA treatment revealed both greater immuno-staining in vascular specimens and amounts of incorporated radiolabeled aspartate in cultured nanobacteria-like biofilm (Table 2). This finding is consistent with the enhanced reactivity in the Limulus Amebocyte lysate assay for endotoxin (aka lipopolysaccharide, LPS) for nanobacteria from human cyst fluids [31] and the incorporation of radiolabeled methionine into nanobacteria-like particles in vitro following EDTA treatment [36]. Mab 8D10 provisionally specific to nanobacteria porin protein [28] used in this study did not cross-react with *E. coli*, *C. glutamicum* and *C. pneumoniae*.

Our findings indicated the presence of these agents in approximately 64% of the human atherosclerotic plaques investigated by immunoelectron microscopy. Only putative nanobacteria-like structures exhibited gold-labeling while the background remained unlabeled. Control samples from non-sclerotic aorta or carotid samples were not significantly labeled by 8D10, which suggests that nanobacteria particles are found only in calcified regions. From this observation it cannot be decided whether antibody-positive regions are corresponding to intra- or extra-cellular nanobacteria-like particles. Culturing of nanobacteria-like particles (biofilm formation) was successful in about 60% of the samples, where strict aseptic conditions were used throughout. To avoid classical bacteria contamination, specimens were sterile filtered two times through a 0.2 μm filter and irradiated with medium-dose

γ-rays before any incubation; ampicillin, kanamycin and nystatin were added to the culture media to inhibit the growth of classical microbes. Biofilms were analyzed with light microscopy and double-staining technique [28] (data not shown) and tested with dot immunoblot. Classical bacteria were not observed in vessel-derived cultures under our conditions. The putative nanobacteria propagated from these specimens reacted with the anti-nanobacterial Mab and displayed nanobacterial-like shape and size in TEM imaging [28, 29, 35]. Because low temperatures inhibited plaque-derived putative nanobacteria formation and the biofilm formation needs long time (1–3 months), we think the nanobacteria-like particles visualized in atherosclerotic plaque cannot be explained by post mortem translocation to and propagation within vessels.

Lower incubation temperatures inhibited biofilm formation *in vitro*, which argues against the inorganic crystallization theory for the appearance of nanobacterial structures in the culture medium [34]. Optimal growth was observed at 37 °C, which is consistent with optimal growth temperatures of many other human pathogens, however, the bacterial origin of the nanobacterial agent is controversial.

We also verified incorporation of [^{3}H]L-aspartic acid into nanobacteria-like particles which was significantly increased by longer incubation time (Table 2). After dialysis against 50 mM EDTA for two days, most of the particles were demineralized. These demineralized samples revealed greater amounts of incorporated radioactivity (Table 2), which suggests that incorporation occurred inside the particles into high molecular weight (i.e. non-dialyzable) components and that they are not the result of adsorption to, or co-crystallization with the calcium apatite coat of the agent. The decreased radioactivity of the same sample before demineralization might be explained by the absorption of the weak beta irradiation on the thick inorganic shell of the particles or inadequate penetration of scintillation cocktail.

It was shown that the pathogenesis of calcinosis cutis is not related to nanobacteria infection [41]. In a recent study Drancourt et al. [42] failed to isolate and culture bacteria from upper urinary tract stones and could only detect nanobacteria-like structures with SEM and TEM. In our study we could detect nanobacteria antigens, isolate and culture them from calcified specimens and show their temperature dependent growth and amino acid incorporation to high molecular weight compounds. Additional studies are needed to characterize these agents in general, verify the presence of nanobacteria-like structures in pathological samples where soft tissue calcifications are found, and propose a causal or promotional role for crystallization initiation in disease. Nanobacteria-like particles have already been shown to be cytotoxic to fibroblasts *in vitro* [31, 35]. Antibiotic susceptibility studies suggest that tetracycline, a drug often used in the treatment of diseases where pathologic calcifications are present, is inhibitory on particle formation in cultures [36]. Future studies will need to consider nanobacteria-like particles as a possible contributor to initialization of calcification in atherosclerotic lesions and target of pharmacologic interventions.

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