Invited Paper

Nanobacteria from blood, the smallest culturable autonomously replicating agent on Earth

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ABSTRACT

Nanobacteria are the first mineral forming bacteria isolated from blood and blood products. They are coccoid cell-wa11ed organisms with a size of 0.08-0.5 μm in EM, occure in clusters, produce a biofilm containing carbonate or hydroxyl apatite, and are highly resistant to heat, gamma-irradiation and antibiotics. Their growth rate is about one hunredth that of ordinary bacteria and they divide via several mechanisms. Taq polymerase was able to use their nontraditional nucleic acid as a template. 16S rRNA gene sequence results positioned them into the alpha-2 subgroup of Proteobacteria.

Nanobacteria are smallest cell-walled bacteria since they can pass through 0.07 μm pores. In low-serum cultures, they form even smaller elementary particles or tubular units. How can blood be infected with such slow growing, heat and radio- resistant bacteria? The answer may lie in their phylogeny: alpha-2 subgroup has organisms from soil exposed to radiation and heat, that can penetrate into eukaryotic cells. Nanobacteria grow so slowly that they require a niche 'cleaned' with heat, radiation or immunodefence. For surviva1 they cloak themselves in apatite, a norma1 constituent of mamma1ian body. This may link nanobacteria to nannobacteria discovered from sedimentary rocks by Dr. Folk. Both have similar size, size variation, clustering and mineral deposits. They may resemble the probable ancient bacterial fossils in the Martian meteorite ALH84001.

Key words: nanobacteria, nannobacteria, carbonate apatite, hydroxyl apatite, hydroxyapatite, life-forms

1. INTRODUCTION

Recently, polymerase chain reaction (PCR) results with 16S rRNA gene have lead to the remarkable discovery that the bulk of microbes in the environment are refractory to in vitro cultivation by current techniques. There is little reason to doubt that also mammals play host to yet undiscovered and uncharacterized microbes. We have tried to report for five years, our identification of such a microbe in blood and blood products, undetectable with present microbiologica1 methods. The organisms were tentatively named as Nanobacterium sanguineum, referring to their small size and their blood habitat [1,2] and were reposited in the German Collection of Micro-organisms (DSM No: 5819-5821). Phylogenetic analysis based on comparisons of 16S rRNA sequences is now routinely used in the determination of taxonomic relationships. This analysis placed Nanobacteria isolated from fetal calf serum (FCS) into the a1pha-2 subgroup of Proteobacteria.

During the 10 years this work has been in progress, we were able to develop culture, immunoassay, identify protein sequences and devise several new staining methods for the organisms in addition to the nucleic acid work. All findings emphasized their distinctive nature. To us their special properties and unique qualities importantly differentiated nanobacteria from known bacteria. Regretably, this was not the case for the (micro)biological journals: all our eight manuscripts were rejected because at least one of editors or reviewers offered one of two major criticisms: I do not believe that such organisms can exist, or, the findings should be published first somewhere else. Such a negative attitude preventing the spread of information is difficult to understand since the new organism, nanobacteria, may be of major importance for our understanding about life in general, for medicine (causative agent in several diseases, risks in blood products and transfusion, tissue transplantation, vaccines), for basic research (contaminants in cell repositories and cell cultures all over the world) and for the quality of biotechnology products.

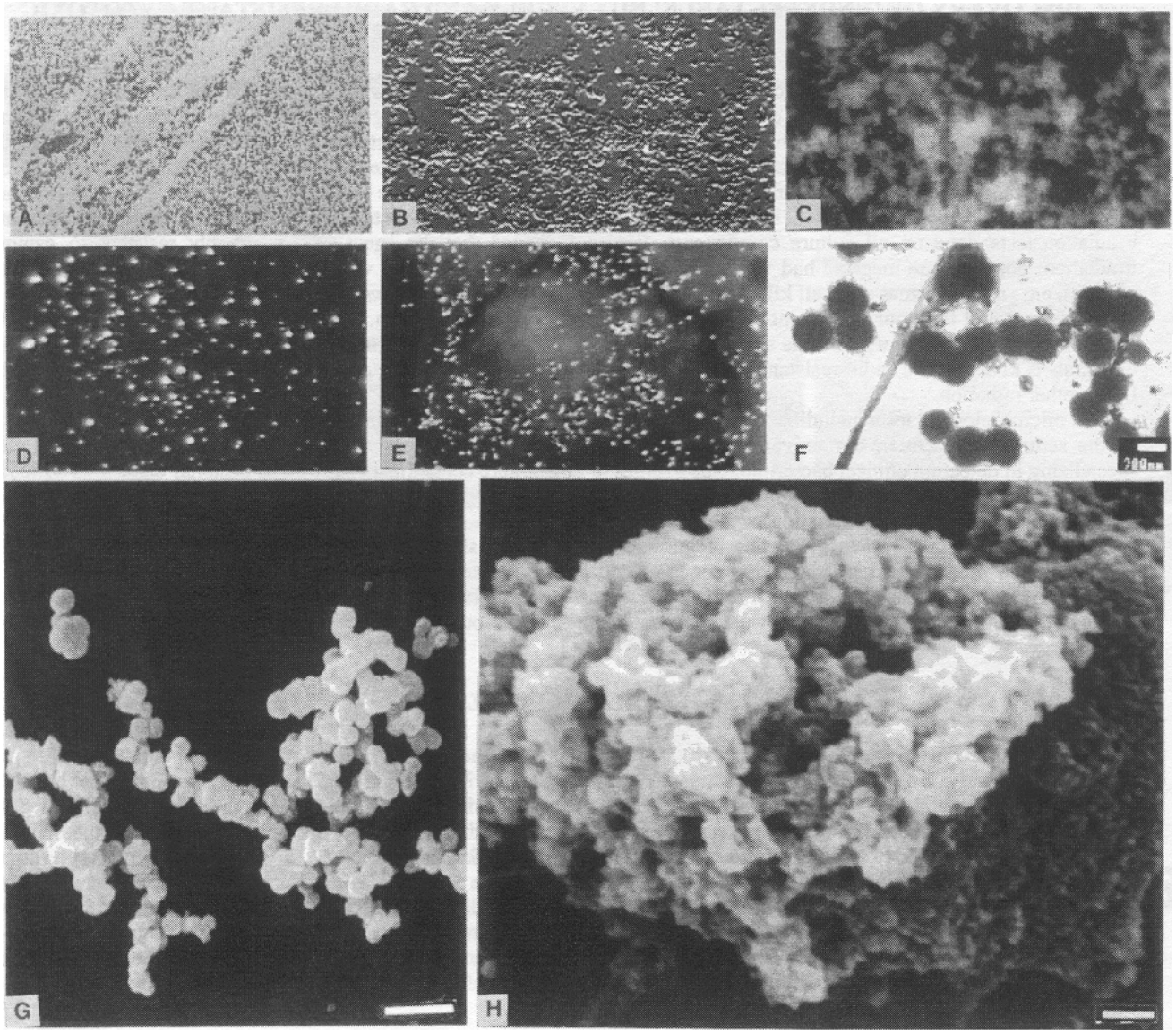

Figure 1. Visualization of nanobacteria. (A): Nanobacteria1 biofilm attached to the culture vessel, magnification 800x. (B): One month cultured nanobacteria starting to attach to the culture vessel, magnification 1600x. (C): Hoechst 33258 staining of the same area as in (B). (D): Immunostaining of nanobacteria with a specific anti-nanobacteria monoclonal antibody. (E): Immunostaining of the nanobacteria attached on 3T6 cells utilizing the same antibody, magnifications in (D) and (E) 1600x. (F): negative staining of nanobacteria isolated directly from serum, bar 200 nm. (G and H): SEM of nanobacteria, bars 1 μm.

2. DISCOVERY OF 'UNDETECTABLE' BUT VISIBLE AND RADIO-RESISTANT CYTOTOXIC NANOBACTERIA

Bacteria-like particles were initially observed 10 years ago in long-term mamma1ian cell cultures (a nongrowing cell mutant). The initial clue to the nature of the problem was the fortuitous cu1turing of the same ce1ls in two media differing only in their serum supplement. In the presence of one serum batch, which contained the particles, the mamma1ian cells died within four weeks. These wells had a white macroscopic layer of bacteria-1ike particles at the bottom (Fig. 1A), but the medium remained otherwise clear and the pH was not greatly a1tered. The particles multiplied similarly whether mamma1ian cells were present or not. They were passageable. Serum was proven to be their only source of origin by gamma irradiation tests done on all culture components. It a1so indicated that the agent was remarkably resistant to gamma irradiation: doses of one megarad had very little effect on them, three megarads were needed to ensure their destruction, whereas, e.g., mycoplasmas were all killed with a 0.05 megarad dose. *Deinococcus radiodurans* is known to survive even 6 megarad dose[3] and thus our new agent is not the most radio-resistant bacterium. The observed radio-resistance, however, eliminated most of the known bacteria as potential candidates for our agent, most importantly, none of the bacteria infecting mammals is known to be radio-resistant. The known radio-resistant bacteria come from environmental sources exposed to UV or other radiation.

All microbiological tests including mycoplasma assays were negative as performed in our own laboratory and repeated by the serum manufacturer, our university microbiology laboratory, a pharmaceutica1 company and several laboratories specializing in detecting clinical bacteriological samples or cell culture contaminants. Standard bacterial cultures were made on sheep blood agar and mycoplasma media. The traditiona1 culture and staining tests for sterility gave negative results in all experiments. Positive identification of the new agent involved its characteristic growth in cell culture medium with typical growth rate and optical properties including generally poor stainability , specific stainability with a DNA stain Hoechst 33258 modified using a high dye concentration and with immunofluorescence staining using monoclonal anti- nanobacteria antibody developed by us. From the very onset, these observations pointed to the distinct and extraordinary nature of the new agent. These criteria were sound and robust to us but not to the microbiological experts reviewing our work.

3. SMALLEST BACTERIUM

Serum is 'steri1ized' using steri1e filtration. Can the nove1 agent pass these filters? Fi1ters with nomina1 pore-size of 0.1 μm cou1d not effective1y remove them. No growth was observed after filtering through 0.05 μm or sma11er pore-size, but 0.07 μm pores cou1d not e1iminate the organism. We did further studies using 0.2 μm filters since these are genera1ly used for steri1ization.lnitia11y, about 3% of nanobacteria in the solution passed through. A high back-pressure increased the numbers passing through up to 50%, whi1e fi1tration temperature had 1itt1e effect on filtrability. In fact, at a high back pressure, nanobacterial counts coming out of the fi1ter surpassed those in the feeding 1iquid. Nanobacteria were 1itera1ly being squeezed through. After the fi1tration, no particles cou1d be seen by direct microscopica1 survey as was the situation a1so initially with sera. They became visible within 24h of culture regaining their origina1 size. Serum samples needed 1-2 weeks cu1ture to reach a simi1ar size. Thus, they lost their optica11y dense (apatite) coat as they were squeezed through the pores. As shown in this issue by Ciftciog1u et a1., the sma11est unit of nanobacteria retaining membrane has a diameter of on1y 50 nm.

4. SLOW GROWING AND RESISTANT TO HEAT AND ANTIBIOTICS

Their growth was readily measured by optical density , particle counting, microscopy, by an increase in their proteins and antigens and by metabolic labelling with radiolabelled methionine and uridine. Their doubling time was between 1 to 5 days, generally about 3 days. This is remarkable, one of the slowest recorded for bacteria (some Mycobacteria seems to grow more slowly). Thus obviously microbiological methods aimed at detecting bacteria by metabolic tests cannot detect them since their metabolic rate is about a hundredth that of common organisms. They were observed to utilize amino acids but not glucose to any significant extent. In particular, they used glutamine, asparagine and arginine from their culture medium. Antibiotics routinely used in cell culture could not prevent their multiplication. Aminoglycosides (streptomycin, kanamycin, gentamycin) at high concentrations prevented multiplication as assessed microscopically or with specific ELISA, but only in a bacteriostatic way. Growth inhibition was seen with cytosine arabinoside, an inhibitor of DNA synthesis, and with a calcium chelator, EDTA. Heating at 100°C for 30 min prevented their growth in subculture but lower temperatures did not. Those forms that had the thickest apatite coat, serum-free cultured forms, were even more resistant to heat, and could not be killed by one hour exposure at 100°C .

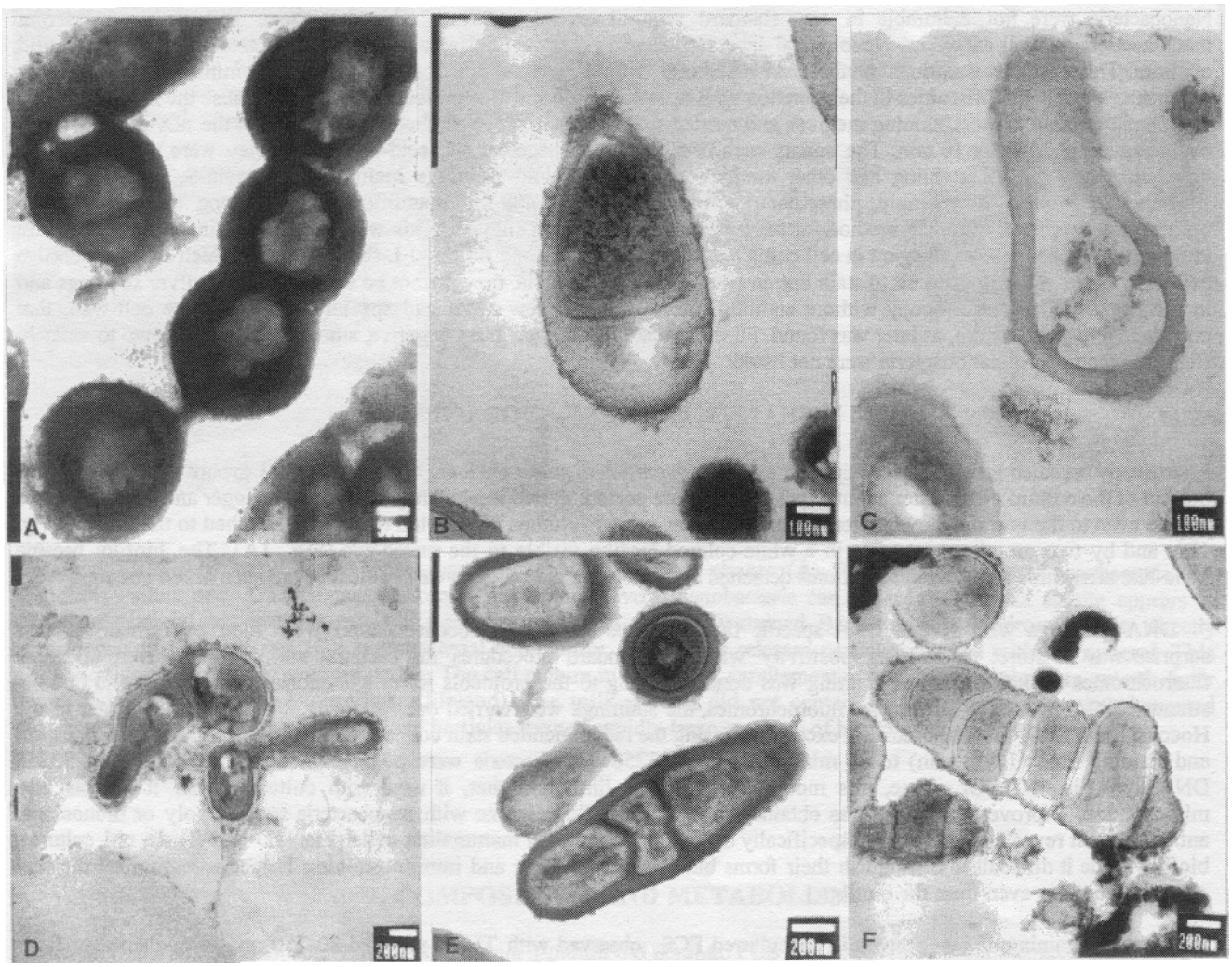

Figure 2. TEM images of nanobacteria. Coccoid morphology (A), bar 50 nm; special form showing budding of the capsular structure (B), bar 100 nm; multiplication with an unevenly located septum (C); budding forms (D); distinct forms showing multicellular appearance (E and F) of nanobacteria. (F) shows nanobacteria-specific structure, budding with a terminal septum (see upper comer of the multicellular form).

5. TRADITIONAL MICROBIOLOGICAL METHODS FAIL

Nanobacteria were not culturable in any standard microbiological medium. This has been proven by the serum manufacturers as well as by microbiological laboratories using commercial sera. They do not grow well on any solid medium. This prevents traditional analysis for metabolic typing. Bacteriological staining methods initially failed to show organisms because of difficulties in their fixation with classical flame and alcohol techniques, and because the new organisms were impermeable to most staining methods and needed special visualization methods. We could fix the novel agent using oven fixation at 70°C for 10 min. The agents were very tiny gram negative and acid-fast cocci. They were we1l visualized only with Jones' silver staining and other methods used for amyloid stainings including thioflavin-S, Congo red and Bielschowsky silver. Surprisingly, phase contrast microscopy under 400-fold magnification using long working distance optics proved to be an exce1lent method for fo1lowing the new agent in cultures. This was remarkable since the most likely known candidates for such an agent in cell cultures were mycoplasmas and bacterial L-forms both of which have a defective cell wall. These are also difficult to stain but, in contrast to nanobacteria, they cannot be seen at all with silver stainings and in the phase contrast microscopy without staining. Obviously, the new agent had special optica1ly dense ce1l-wall, that contained hydroxyl apatite, as later was found. Furthermore, no acid-fast, gram negative, small cocci were known to exist in Bergey’s Manual. So nanobacteria were not listed!

6. VISUALIZATION OF NANOBACTERIA

Microscopy revealed growth of very tiny optically dense particles, either on their own or in small groups moving near the bottom of the culture vessel after about a one week culture period. In two weeks, they had become bigger and formed groups visible even to the untrained eye. After one month, many were in clumps and started to become attached to the bottom (Fig. 1B), and by two months most were in a white-colored biofilm visible to the naked eye (Fig. IA). The biofilm became bone-like after three months but sometimes detached and autolyzed after half a year. Pellicles were seen in old cultures.

DNA stainings with several DNA specific fluorochromes (Dapi and Hoechst stains) were then performed Another surprise was in store: no staining positivity with the standard procedures for bacterial staining. However, all these fluorochromes worked wel1 when staining was done according to the protocols for mitochondria. chloroplasts and double- stranded DNA viruses. With Hoechst fluorochromes, the stainings were carried out fol1owing the instructions given in the Hoechst Stain Kit, Flow Laboratories, except increasing the recommended stain concentration (0.05-0.5 μg/ml) to 5 μg/ml and staining time (10-30 min) to 45 min for Hoechst 33258. Nanobacteria were positively stained with Hoechst 33258 DNA dye (Fig. 1C). Of course, this modification has the limitation that, if used with cultured cells, it detects also mitochondria. Improved specificity was obtained using immunofluorescence with nanobacteria-specific poly or monoclona1 antibodies that revealed nanobacteria specifical1y on and in the cultured mammalian cells (Fig. 1D and E). In old cultures, biofi1m made it difficult to distinguish their forms but Hoechst staining and immunostaining helped to visua1izel the tiny coccoid structures even from the biofi1m.

Negative-staining of nanobacteria in noncu1tured FCS, observed with TEM, revealed 80-250 nm cocci-shaped particles showing septa and attaching to each other with a slime-Iike material (Fig. lF). This resu1t is very important, because it shows the size of nanobacteria directly isolated by centrifugation from blood, their habitat. The negative staining is most reliable in analysis of their original size, since the sample is in a native state and is not covered with a layer of gold, as in SEM. SEM of the in vitro-cultured organisms revealed a pleomorphic procaryotic shape, variable size similar to the negative-staining result, and rough surfaces (Fig. 1 G). The apatite mineral formations are clearly seen with SEM in older cultures (Fig. lH).

TEM revealed surprisingly thick cell wall and a capsule of variable thickness, and divisions with a septum (Fig. 2A-D). The sizes and shapes were pleomorphic and even elongated chains of tightly attached organisms were seen. These have apparently special ce1ls in the group, or even appear as multice1lular (Fig. 2 E-F). The ce1l wall and capsule could be clearly seen as separate structures only in the division septum. Their total thickness varied from 20 nm to over 200 nm. The division septum was located either in the central part indicating binary fission, or at the end of the ce1l (see upper part of 2F) suggesting budding with a terminal septum as mechanism for multiplication. Budding seems to start by formation of a capsule for a new cell fo1lowed by transfer of cytoplasmic contents into this sheltered compartment.

In old cultures, many nanobacteria were surrounded by a biofilm (Fig. 3A) which was 'bony' and contained both compact and fine crystals of hydroxyl apatite or carbonate apatite. The nanobacteria were located inside this biofilm which prevented their fixation, embedding and staining, often leaving a hole in the preparates visualized with TEM. Apatite grew directly on the surface of nanobacteria (Fig. 3B). Under serum-free culture conditions, these apatite castles were fast growing and released new apatite-forming units by budding (Fig. 3C). Elongated tubular structures, only 20-50 nm in diameter, were present in these cultures, see Ciftcioglu et al. in this issue.

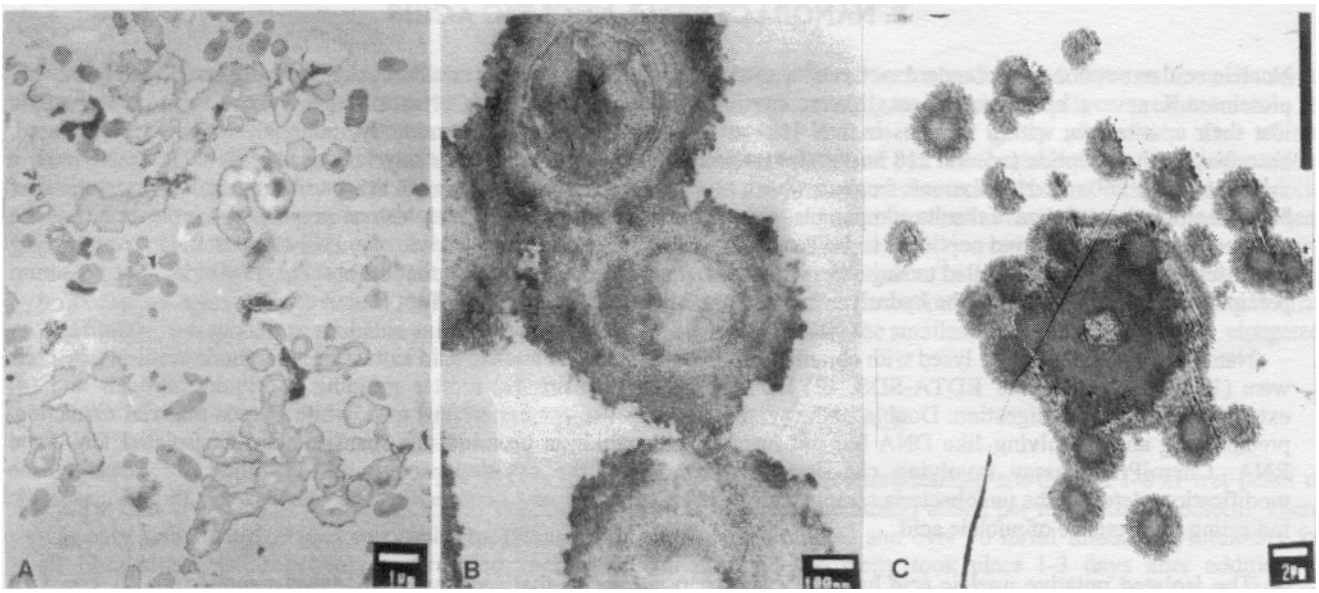

Figure 3. TEM of hydroxyl apatite formations in nanobacterial growth phases. (A): Bottom attachment of nanobacteria after 2-months culture period. Large apatite formations around severa1 nanobacteria can be seen. Compact apatite appears as unstained material, but apatite crystals, in a more porous form, are darkly stained. Bar 1 μm. (B): Hydroxyl apatite directly on nanobacteria, bar 100 nm. (C): Nanobacterial community, castle, that released particles covered with the mineral. This formation was from culture without serum. The cell culture medium was supplemented with bovine colostrum growth factor preparation made by Valio Bioproducts (Finland). The milk product did not contain any nanobacteria but supplemented their growth. in these cultures, the budded particles formed rapidly large amounts of hydroxyl apatite. Bar 2 μm.

7. COMPOSITION AND METABOLISM

Elementary analysis of nanobacteria harvested from a 3-month old SeraLab FCS culture revealed a high content of inorganic materia1. The pellet dry weight varied from 23% to 39% and consisted of: nitrogen 1-1.3%, phosphate 12.3-14.6%, calcium 23.4-23.5%, magnesium 1.4-1.9%, potassium 0.1%, and sodium 1.2-1.4%. The calcium-phosphate ratio is quite similar to that of hydroxyapatite and the overall composition resembled that of bone. The high phosphate concentration was verified with capture-ELISA where specific monoclonal antibody bound the nanobacteria, followed by a standard phosphate assay. The ca1cium and phosphate contents were verified also with energy dispersive X-ray microanalysis[4] under the scanning electron microscopy.

When compared (results expressed per dry weights) to Staphylococcus epidermidis grown in the same medium, the Burton method (deoxyguanosine as standard) revea1ed 'DNA' (0.014-0.045%, n=7; 0.3%, n=2), orcinol reaction ribose attributable to RNA, nucleotides and other sources (6.8 nmol/mg; 18 nmol/mg) and Coomassie protein (10%; 10%) from 1 N HCl treated nanobacteria and S. epidermidis, respectively. Nanobacteria1 hydrolyzates also contained glucose, ga1actose, their amino derivates, mannose and unidentified compounds by HPLC. Fatty acids after methanolysis[5] revealed no tuberculostearate typica1 of mycobacteria. The same fatty acids as found in serum were seen but in remarkably different ratios (data not shown). All common L-amino acids were present. The luciferase system revea1ed the presence of ATP . Nanobacteria did not possess catalase but milk-coagulating activity was present. The amino acids consumed mostly from the culture media were glutamine, asparagine and arginine. When three 1-ml cultures of nanobacteria were labelled for three days with 70 μCi/ml of macromolecule synthesis labels, the following results as cpm were obtained: leucine incorporation 209±65, leucine control 25±1, thymidine 160±51, thymidine control 28±2, uridine 556±112, uridine control 31±4. In longer labelling experiments, nanobacteria incorporated thousands of cpms of methionine and uridine but again minimal thymidine. This may be because thymidine was not taken up, or it could not be used in this form, or thymidine is not a constituent of the nucleic acid in nanobacteria.

8. NANOBACTERIAL NUCLEIC ACIDS

Nucleic acid extraction with standard methods[6] was unsuccessful. Nanobacteria could not be effectively lysed with lysozyme, proteinase K, several other proteinases, lipases, amylases, alkali, ultrasound, X-press, detergents or solvents. Nanobacteria lost their apatite coat within minutes in 1 N HCl at room temperature. Concomitantly, material started to be released, absorbing both at peptide (around 218 nm) and at nucleic acid wavelengths. Previously known nucleic acids absorb with a maximum near 260 nm. HC1-extracts from nanobacteria showed absorbance maximum at around 270 nm. Representatives of bacteria and eukaryotes gave results comparable to those obtained for isolated nucleohistone preparations indicating that the presence of nucleic acids and peptides can be detected in crude acid extracts. Accordingly, HC1-extracts from a nonthriving nanobacterial culture pellet failed to show significant increase at 270 nm verifying that the absorbing material is not a serum precipitate. Further analysis of the hydrolyzates were not done, because they gelled at pH above 2-3.

Nanobacteria could not be lysed with common standard methods for nucleic acid extractions. Methods resu1ting in lysis were (1) double boiling in EDTA-SDS, (2) EDTA-EGTA-subtilisin, (3) mortar grinding in liquid nitrogen and (4) extensive papain-SDS digestion. Double boiling in EDTA-SDS was the easiest. All gave white fibrous material extracting, precipitating and dissolving like DNA but not reacting with ethidium bromide, the standard stain for isolated DNA and RNA. ChemiProbe assay involving chemical derivatization of the cytosine moiety and antibody for detecting the modification, detected the nanobacteria sample weakly positively this was nonspecific. The binding was likely nonspecific indicating no presence of nucleic acid.

The isolated putative nucleic acid had a UV spectrum similar to that seen with HCl. lysed nanobacteria. It could be isolated from all tested 12 nanobacterial cultures representing various origins and cultura1 ages. Its absorbance maximum was at 269-270 nm, absorbance corresponding to thymine derivates (maximum 264-267 nm) and deoxycytidine derivates (271-275 nm) was high but that of guanine-adenine at 250-260 nm low. It was not hydrolyzed to free nucleobases in a standard formic acid treatment hydrolyzing control DNA as evidenced by HPLC. Boiling in 3 or 5 N HCl for 1 h yielded a pattern of putative nucleobases (spectral maxima between 240-280 nm). Surprisingly, HPLC peaks collected and reinjected gave peak(s) with a different retention time. Mass spectra revealed that the peaks showed, in addition to possible nucleobase fragments, also higher molecular mass fragments, especially an about 550 dalton fragment It possibly derives from a compound that might act as an ion-pair or aromatic-pair reagent changing HPLC-peak retention. After the isolated nucleic acid was subjected to Sephadex G-50 chromatography, the hyrolyzates gave relatively pure nucleobase.like peaks. Two of them gave characteristic mass fragments identical with methyldeoxyadenosine and 7-deazaadenosine.

9. PHYLOGENETIC POSITION

Nucleic acids were isolated from nanobacteria and used as a template in PCR to amplify the small subunit rRNA gene. A universal primer, 1492 RPL (GGCTCGAGCGGCCGCCCGGGTTACCTTGTTACGACTT), complementary to regions of rDNA that are conserved among all known organisms, and the bacteria-specific primer 8FPL (GCGGAT CCGCGGCCGCTGCAGAGTTTGATCCTGGCTCAG) were used in PCR.[7-8] rRNA gene sequences retrieved from serum- containing and serum-free cultures revealed two 1406 base long sequences (between the PCR primers) having 98% identity with each other. A phylogenetic tree was constructed using nucleic acid sequence maximum likelihood method and reference sequences from the Ribosomal Database Project. Phylogenetic tree positioned the nanobacterial forms as a new genus in the alpha-2 subgroup of Proteobacteria. Similar results were obtained with both isolated nucleic acid and nanobacteriallysate as samples in PCR. That the rRNA gene clones were derived from the nanobacteria is supported by their repeatability , sequence uniqueness, positive identification with monoclonal antibodies and lack of other culturable micro-organisms in the starting material, the use of UV irradiated materials and hood and negative results in PCR with all solutions used, including culture media, all PCR reagents and Taq polymerase. Although Taq polymerase can accept abnormalities possibly be present in the nanobacterial DNA, fidelity of PCR remains unknown. *Nanobacterium sanguineum* represents isolate one (EMBL Entry X98418) and isolate two is called Nanobacterium sp. (EMBL Entry X98419). The phylogenetic placement suggests a new genus in a most interesting area: bacteria closely related to nanobacteria inhabit soil, hot-springs, mammals and plants. Some of them can penetrate plant cells resulting in symbiosis (Rhizobia) or cancer and other disease states (Agrobacteria, Phyllobacteria). Culture properties of Brucella and especially Bartonella, also close relatives of nanobacteria, are similar to nanobacteria, and they are human/mammalian pathogens causing bacteraemia and several diseases. The phylogenetic location of nanobacteria is suppotted by cross reactivity of antibodies against Bartonella and nanobacteria. This cross reactivity was observed poly and monoclonal antibodies. (data not shown). Finally, the closest relative was Thiobacillus, which has been found from hot-springs where traventines, carbonate hot-spring deposits, are formed.[9,10]

Dwarf bacteria (ultramicrobacteria) passing through 0.2 μm filters have been found from soil and natural water sources. They are difficult to culture and their nature is unknown. Better culture results were obtained in highly diluted media.[11] Dr. Folk[9,10] has found using SEM small-sized bacteria in carbonate sediments and rocks, and named them as nannobacteria. Both nanobacteria and nannobacteria have similar size, size variation, clustering and probably play a prominent role in catalyzing the precipitation of carbonate materials. Nannobacteria may include several group of bacteria: One of the candidates is Thiobacillus, which we have now shown to be the closest relative of our nanobacteria. Thus, our independent findings may link geology with human diseases. The unique properties of nanobacteria: heat, radiation and antibiotic resistance and mineral formation are strong indications that nanobacteria originate from soil or rock bacteria exposed to heat and radiation in their environment and only recent1y inhabited mammals. Their small size may indicate presence of a very small genome. Interestingly, oldest bacterial fossils found on Earth are in apatite grains [12] resembling those apatite formations produced by nanobacteria in serum-free cultures. On the other hand, the formations found in the Martian meteorite ALH84001, interpreted as probable ancient bacterial fossils,[13] resemble great1y the smallest nanobacteria and their elongated tubular formations.

10. BIOLOGICAL SIGNIFICANCE

Cultured nanobacteria were cytotoxic towards six fibroblast lines in a three day test, starting at a dose of 1-8 nl wet pellet of washed nanobacteria (10 nl = one million nanobacteria with microscopic counting) added per ml culture medium. There was only a slight variation in susceptibility. Doses of 16 nl/ml caused marked and over 30 nl/ml massive cytotoxicity for all fibroblasts (human primary and murine transformed cell lines). Cytotoxicity took place 1-3 days after addition of nanobacteria, but if addition was 1000 nl/ml, all cells were lysed after two hours. Thus cytotoxicity depended on nanobacterial concentration and exposure time. Nanobacteria could be cultured from more than 80% of bovine serum lots tested (50 lots tested). Similar structures were present in some horse sera and in all three tested bottles of commercial sterile-filtered human sera pooled from Finnish blood donors by the Finnish Red Cross. In preliminary tests, we have found nanobacteria and their antigens in a number of local cows and in 4 out of 100 medical students attending Kuopio University. Nanobacteria are often present in high numbers in commercial bovine serum which is pooled from thousands of animals. Results were similar for all major manufactures obtaining crude serum from distinct geographical areas. This suggests that nanobacteria may be present in cows, may be transmitted transplacentally and may contaminate cell cultures all over the world.

Nanobacteria grow slowly. If pathogenic, one would expect them to be associated with chronic diseases, e.g., autoimmune disorders proposed to be caused by extremely slow-growing bacteria, or in mineral deposits, such as kidney stones. Our preliminary results indicate that nanobacteria are associated commonly with human urinary stones.

CONCLUSIONS

1. Nanobacteria, a new agent, is isolatable from mamma1ian blood and blood products. 2. Human blood and blood products may contain them. 3. New culture tests or detection of their antigens should be adopted. 4. Commercial cell culture sera may contain this adventitious infectious agent and should be handled as infectious material. 5. Cell cultures and cell culture products may contain this multiplying agent and it may explain serum cytotoxicity commonly observed. 6. Sterilization methods for sera should be improved. 7. Their role in adult and fetal life should be scrutinized. Their pathogenicity should be clarified. 8. Their genetic material, its code and genomic organization may revea1 new adaptations of life. Finally, we would like to suggest that a forum for presenting extraordinary discoveries should be established. Publishing tota1ly new ideas should not face the fate of Ga1ileo Galilei anymore.

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