Extraordinary growth phases of Nanobacteria isolated from mammalian blood

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ABSTRACT

Nanobacteria, novel sterile-filterable coccoid bacteria inhabiting mammalian blood and blood products, have different growth phases depending on the culture conditions. These minute organisms produce biogenic apatite as a part of their envelope. This becomes thicker as the cultures age, rendering them visible in microscopy and resisitant to harsh conditions. Mineral deposits were not formed without live nanobacteria. Apatite formation was faster and more voluminous in serum-free (SF) medium, and within a week, several micrometer thick 'castles' formed around each nanobacteria. These formations were firmly attached to the culture plates. Nanobacteria multiplied inside these thick layers by turning into D-shaped forms 2-3 μm in size. After a longer culture period, tens of them could be observed inside a common stony shelter. The apatite shelters had a hollow interior compartment occupied by the organisms as evidenced by SEM and TEM. Supplementing the culture medium with a milk growth-factor product, caused the castles to grow bigger by budding. These formations finally lost their minerallayer, and released typical small coccoid nanobacteria. When SF cultures were supplemented with sterile serum, mobile D-shaped nanobacteria together with small 'elementary particles' 50-100 nm in size were found. Negative results in standard sterility testing, positivity in immunofluorescence staining and ELISA tests with nanobacteria-specific monoclonal antibodies, and 98% identity of l6S rRNA gene sequences proved that all of these unique creatures are nanobacterial growth phases.

Keywords: nanobacteria, nannobacteria, sterile-filterable, apatite formation, biogenic apatite, calcinosis

1. NANOBACTERIA AND STERlLE CELL CULTURE SERA

During the last 70 years mammalian blood has been suspected at various times to contain bacteria-like but uncharacterized organisms.[1-3] Our group has isolated and characterized a new bacterium initially from 'sterile' cell culture sera, subsequently from horse and human blood[4,5], and used SEM and TEM methods[6,7] to observe their morphology. These sterile filterable, minute, immunogenic, multiplying organisms were generally coccoid with a size of 80-300 nm (Fig. lA). Unknown according to classical microbiological criteria they were tentativeley named as Nanobacteria.[4] When we modified and adapted the classical methods in microbiology and molecular biology, we proved that nanobacteria were culturable, have nucleic acids and are very different than all other known bacteria. More important, they are evidence that many of the methods used to detect the presence of bacteria, cells etc. may be overlooking other forms of living organisms.
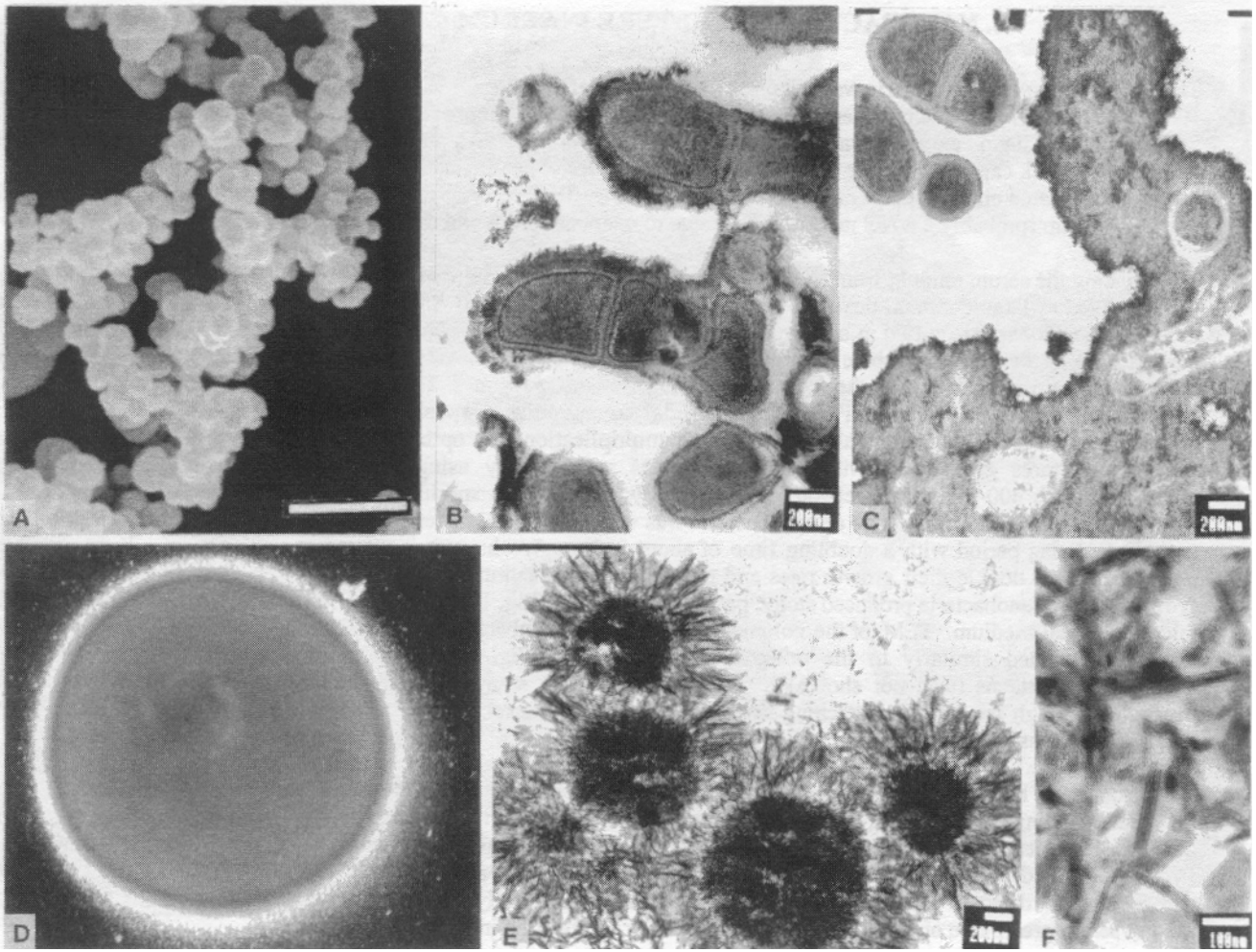
Figure 1. Scanning electron micrograph of nanobacteria (A) showing size and shape variation. Their diameter is between 80-300 nm, bar is 1μm. Transmission electron micrograph of nanobacteria (B and C). In (B), bizare septal divisions between the cells in different morphology, and partia1 coverage of the cells by hydroxyl apatite (hariy structure) can be seen. (C) Depicts nanobacteria free and inside the thick apatite layer. The macroscopic (D), magnification 25X, and electron microscopic (E) structure of nanobacteria1 colony on modified Loeffler medium after culture for 6 weeks. TEM image of reference apatite crystals (F). Similar staining results were obtained both in the TEM of the reference apatite crystals (F) and apatite structure on the surface of the nanobacterial particles (E).

SEM work was performed as described before[6]. For TEM, nanobacteria were harvested by centrifugation, fixed with 3% glutaraldehyde overnightly, prestained with 1% OsO_4 for 1 h, dehydrated with ethanol, embedded in epon, cut and stained with uranyl acetate and lead citrate, and examined with Joel 1200 EX electron microscope at 80 kV. For the reference apatite crysta1s (Hydroxyapatite type 1, Sigma), the same method was applied.

2. NANOBACTERIAL CULTURE IN SERUM CONTAINING MEDIA

The first isolation of nanobacteria was obtained by using RPMI 1640 or DMEM medium with sterile cell culture serum at 10-50% concentration as a supplement and as the suspected source of nanobacteria. They were proven to be derived from the serum by a γ-irradiation experimentation[5]. Cultured nanobacteria revealed a pleomorphic shape, with a structure resembling a cell wall, a thick capsule partly or totally covered with hydroxyl apatite, and with division septa (Fig. 1B). The division septum was located either in the central part, indicating binary fission, or at the end of the cell suggesting budding with a terminal septum (probably a novel mechanism unique to nanobacteria) as mechanisms for multiplication.

Reducing the serum amount from the culture medium still supported growth although at a slower rate and often growth was observed as large communities in a slime-like material attaching to the culture vessel. TEM experiments revealed multiplying nanobacteria buried in a hydroxyl apatite layer (Fig. 1C). It was very difficult to obtain good morphology of nanobacteria inside the apatite since fixatives, stains and epon do not penetrate into mineral. The most common indicator of the existence of nanobacteria was the appearance of holes in the preparates. This may be a major reason why nanobacteria have not been previously seen in TEM. Nanobacterial communities grew slowly and were easily visible even in light microscopy because of their mineral deposits. Their multiplication was optimal at 37°C under 5-10% CO_2 -90-95% air. Anaerobic cultures gave no growth. The growth was measured by using several methods including phase contrast microscopy with 400-fold magnification, optical density, nanobacteria specific ELISA, and metabolic labelling with ^{35}S methionine. The multiplication rate of nanobacteria was very similar in all tested serum samples and followed logarithmic growth after a lag period with a doubling time of about 3 days. We have found two different kinds of culture supplement preparations that double their growth rates and make them more culturable in a classical sense. In the presence of these growth factors, nanobacteria produced stony, passagable colonies (Fig. 1D) on solid Loeffler's medium which is a standard microbiological medium. TEM of the colonies revealed large nanobacteria-like bodies covered with apatite crystals (Fig. 1E) which stained similarly to the reference apatite crystals (Fig. 1F). Immunostaining indicated the presence of nanobacterial antigens (data not shown) in these colonies. The stony colonies were about 1-2 mm in diameter, and penetrated through the medium attaching firmly to the culture vessel. Thus, nanobacteria could produce large biogenic apatite formations in vitro.

Nanobacteria have such bizarre morphology, culturing and nucleic acid properties (see E. 0. Kajander et al., in this issue) that findings on this area, unfortunately, proved to be difficult to publish in international journals. It was hard to convince the scientific community that such an 'odd contaminant' might be present in sterile cell culture sera that are slrictly controlled with the best known sterility control methods. Sceptics tended to try to explain our results by stating that though the detected particles did look like bacteria, were multiplying and immunogenic, they might represent some kind of serum precipitate or some other debris formed during the long incubation period.

3. NANOBACTERIAL CULTURE IN SERUM-FREE MEDIA

When we added cultured and washed viable nanobacteria to SF cell culture medium (DMEM or RPMI-1640), the initial observation was the appearance of yeast-sized budding organisms, firmly attached to the bottom (Fig. 2A). These differed extensively in morphology from the coccoid nanobacteria. They grew about half of the rate as assessed by the increase in their numbers and metabolic incorporation of ^{35}S methionine and (5-^{3}) uridine. Phase contrast (Fig. 2B) and TEM experiments (Fig. 2C) revealed that within a week, there was apatite formation in several micrometer thickness around each live nanobacteria. We could observe an increase in the amount of nanobacteria attached to the bottom and, additionally, multiplication inside these attached formations where nanobacteria changed to having D-shaped forms, 2-3 μm in size (Fig. 2D-F). These apatite shelters were also shown in SEM to have a hollow interior compartment, apparently the dwelling of the organisms (Fig. 3A). The size of the cave-like structure seemed to depend on the number of nanobacteria inside (Fig. 3B). It is clear that the opening of the caves faces the bottom of the culture vessel. Thus, the apatite shelter provides total protection to the organisms. This formation may be a general stress reaction against unfavourable culture conditions, since we have observed it to be initiated after application of heat, or γ-irradiation, or addition of antibiotics, e. g., gentamycin. The cultures could be passaged 1: 11 each month for over 5 years and they always followed the same kind of growth pattern.

Nanobacteria are very difficult to fix on glass slides for microbiological staining, obviously because of the apatite coat. Surprisingly, they can adhere to glass themselves during the culture period. The apatite formation and adherence of nanobacteria to glass surfaces was comparable with that on plastic cell culture vessels. Sterile, 500 μm sized glass beads were incubated with SF-nanobacteria for two weeks and observed with SEM (Fig. 3C, D). With the greater magnifications, tiny tubular structures, about 50 nm sized (Fig. 3E), were seen adjacent to the typical hydroxyl apatite coated globular
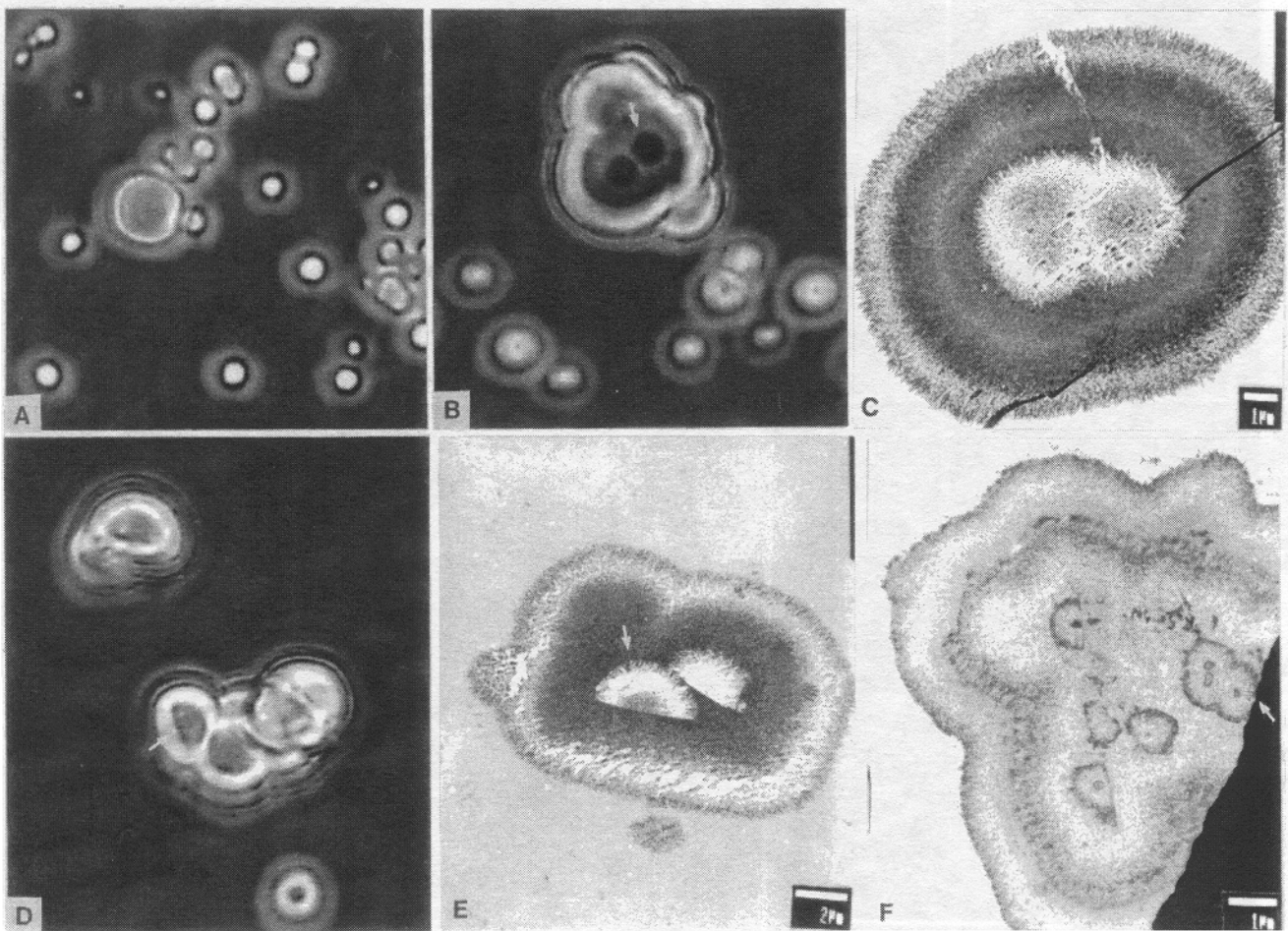
Figure 2. Light microscopic (A, B, and D) and TEM (C, E and F) micrographs of nanobacteria cultured in cell culture medium without serum. In (A), the size of the smallest coccoid particle is comparable with yeast size. The bigger particles, as seen in the central area, showed budding. With phase contrast microscopy, it was possible to see the nanobacterial location and change in their shape during the culture period in the large stony shelters (B and D) almost as well as TEM (C, E, and F). Nanobacteria are coccoid or D-shapped organisms shown by white arrows.

structures (Fig. 3D). The same experiment was controlled by culturing with the γ-irradiated nanobacteria, and this resulted in very poor attachment and no growth (Fig. 3F and G).

Nanobacteria grew in SF culture conditions. Thus, they cannot be serum protein or lipid precipitates. In the presence of milk growth-factor preparation (Valio Bio-Products, Finland), nanobacteria grew better than in SF conditions but revealed all the same formations and forms. Under these conditions, they produced large numbers of extracellular particles by budding. These 20-100 nm sized particles produced hydroxyl apatite on their surfaces although they seemed to lack the full cell structure. In this way, relatively few nanobacteria could produce large amounts of hydroxyl apatite. This finding may be relevant to both enviromental and bodily conditions, e.g., in the sedimentary mineral formations described by Dr. Folk[8,9] or mineral deposits such as urinary stones. Our preliminary work indicates that human urinary stones often contain bacteria resembling nanobacteria (data not shown).
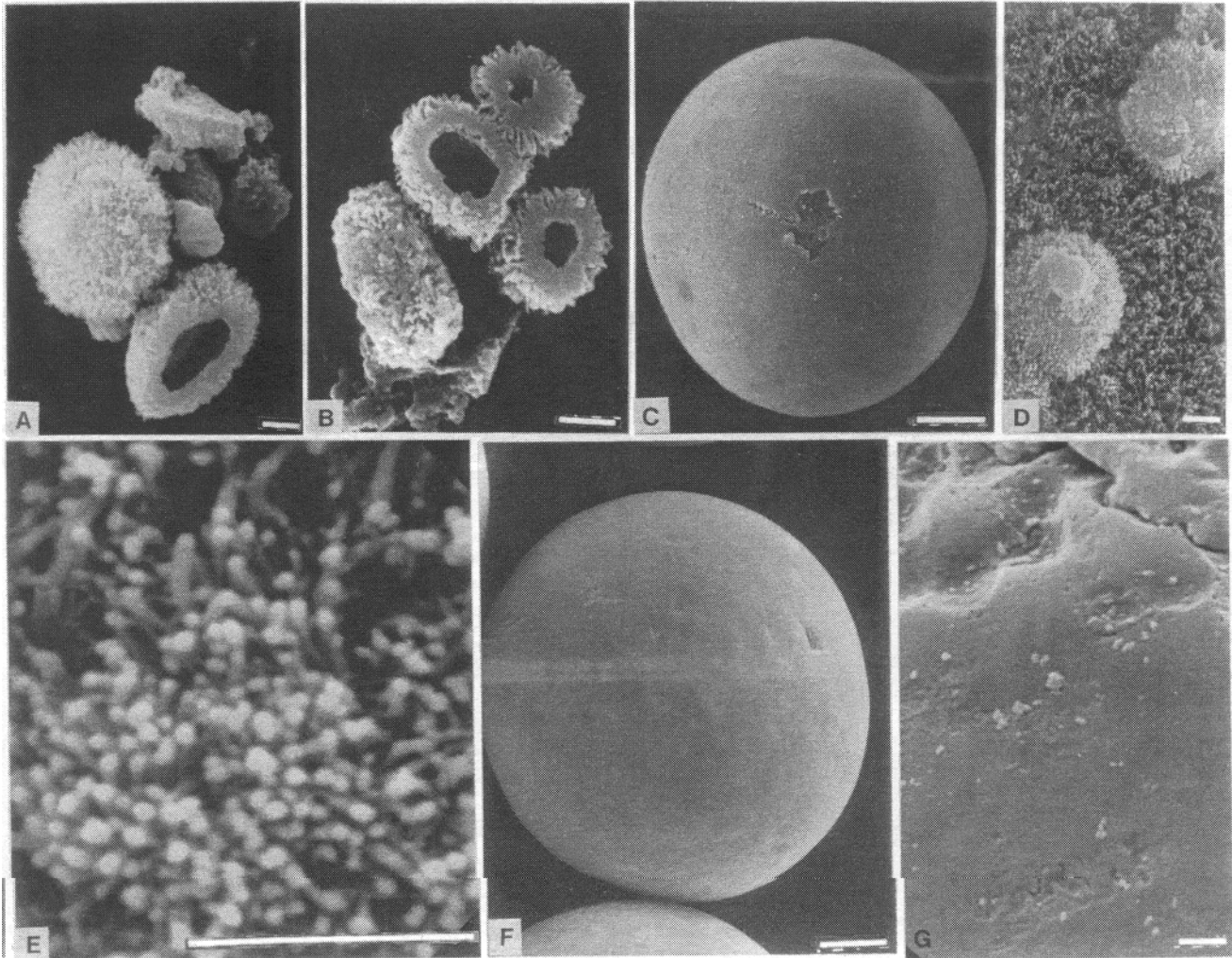
Figure 3. Scanning electron micrographs of SF-nanobacterial dwellings after scraping from the bottom of the culture vessel (A and B), and SF-nanobacterial attachment to a glass surface (C-E), and negative controls (F and G). Bars: A, B, D, E, and G 1 μm, C and F 100 μm. (A) and (B) shows the interior compartments and the surfaces of the stony shelters of SF- nanobacteria. (C) is a SEM image of a glass bead which was incubated with SF-nanobacteria for two weeks. In the center of the bead, a small area of the SF-nanobacterial coat has been removed. (D) Shows the surface attachment of the SF- nanobacteria, and (E) shows the detail of an area having tiny tubular structures. These structures were not present in the control incubation that was carried out using γ-irradiated nanobacteria (F and G).

Twenty-four hours after addition of sterile serum to the SF-nanobacterial cu1tures their 'hydroxyl apatite castles' lost the mineral layer, and characteristic small coccoid nanobacteria later appeared in the same cultures with the mobile, D-shaped nanobacteria (Fig. 4A). Additionally, there were fruiting bodies (Fig. 4B), containing approximately 50 nm sized coccoid, 'elementary particles' surrounded by a membrane (Fig. 4C).
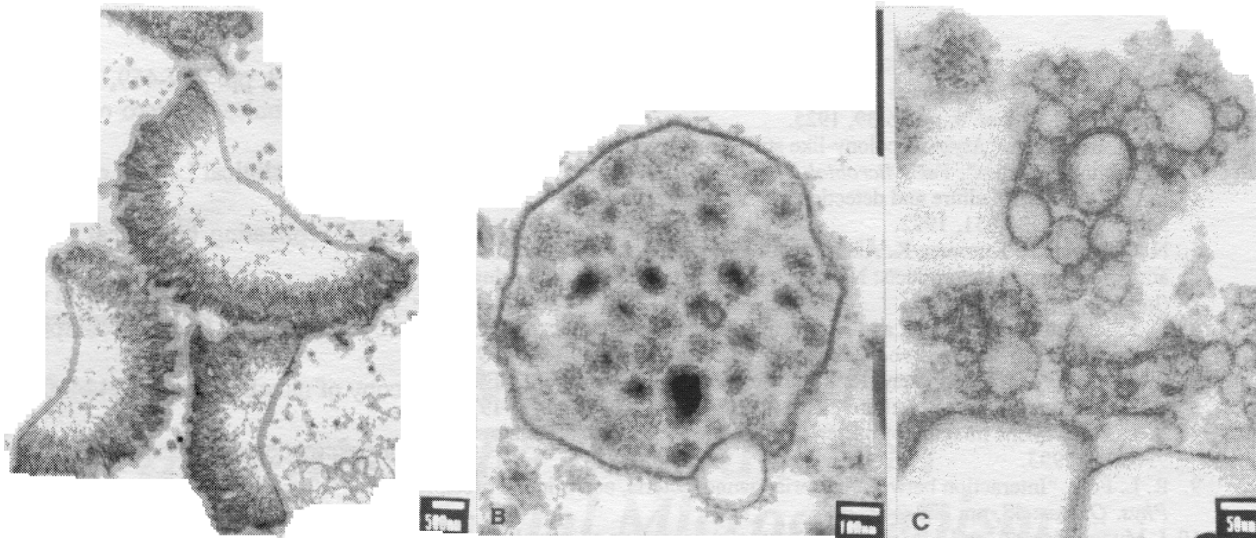
Figure 4. TEM of D shaped nanobacteria (A), and a fruiting body (B), and 'elementary particles' (C) 24 h after addition of sterile serum to the SF -nanobacteria culture.

   SF-nanobacterial culture was scraped with a cell-culture scraper, and 10% γ-irradiated FBS was added. 24 h later, D- shaped and mobile nanobacteria were seen with phase contrast microscopy as also depicted with TEM (A). In the same culture there were other growth phases of nanobacteria such as a fruiting body and approximately 50 nm sized elementary particles. After continuation of the culture for one week, only typical small coccoid nanobacteria were seen (not shown).

4. IDENTIFICATION OF NANOBACTERIA

Negative results with the standard steri1ity detection methods were obtained from all kind of nanobacteria1 cu1tures. We have several po1y and monoclona1 antibodies against nanobacteria. Using these specific antibodies in ELISA and immunostaining, positive results were obtained with a1l forms of these unique organisms. Adherence and internalization properties of nanobacteria to the cu1tured fibrob1asts were simi1ar a1so in the different growth phases. Fina1ly, 98% identity in 16S rRNA sequences (1406 bases sequenced) proved that a1l these unique apatite forming organisms represent different nanobacteria1 growth phases.

5. CONCLUSION

In the natural environment, there are social micro-organisms such as Myxobacteria that undergo a spectacular cell cycle that allows them to adapt to various enviromental conditions.[10] Additionally, under starvation conditions, they form aggregates which resemble macroscopic fruiting bodies, inside which the cells differentiate into myxospores. Apparently, nanobacteria also possess comparable reactions to stress conditions. Their hydroxyl apatite surface makes them resistant to heat, freezing. antibiotics, radiation, drying, and to the immune response of the mammalian body. It also provides shelter against UV irradiation and since nanobacteria are also resistant to γ-irradiation, in theory , they should endure, cosmic conditions even better than bacterial spores.[11]

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