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Scanning Electron Microscopy of Nanobacteria - Novel Biofilm Producing Organisms in Blood

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Our group has scrutinized cell culture contaminats for several years and discovered a most unusual and apparently novel type of contaminant from several commercial sterile cell culture sera. These autonomously replicating particles are tentatively named nanobacteria. The particles divided under cell culture conditions, even without mammalian cells, with a doubling time of about 3 days. During culture their size and coccobacillar shape resembled the smallest bacteria under a light microscope. Yet they could not be easily stained with bacteriological stains, and all common bacteriological media tested failed to support their growth. The novel agent was cultuvable in all common cell culture media for mammalian cells, caused a specicif immune response, and could be immunostained with specific antibodies.(1) In long-term culture, the novel agent formed biofilm and attached on surfaces. Formation of thick white biofilm clearly distingguished nanobacteria from e.q. mycoplasma and other known contaminants present in animal sera after sterile filtration. We have now visualized the novel agent and its biofilm.

Nanobacteria cultures were established by mixing fetal bovine serum at 20% final volume in DMEM medium without antibiotics, followed by incubation under standard cell culture conditions (5% C02 - 95% air, 37;C) up to 4 months. Nanobacteria were centrifuged at 20,000 g for 30 min and subcultured in serum-free DMEM on cover glasses for biofilm detection. Additionally, 3T6 fibroplasts (ATCC CCL 96) in logarithmic growth phase were let to attach on cover glasses in 4-well plates followed by inoculation with nanobacteria, 1-3 ul wet pellets per ml 3T6 culture medium (suspended in 50 ul medium). For EM, the cover glasses were washed and fixed with 2% glutaraldehyde in PBS (phosphate buffered saline) for 16 h. For SEM the glasses were dehydrated with ethanol and dried in a critical point dryer and layered with gold before being examined with JEOL JSM-35. TEM samples of centrifuged nanobacteria were processed as described for other bacteria.(2,3) Ultrathin sections were cut, placed on formwar covered 200 mesh copper grids and stained with uranyl acetate and lead citrate before microscopy with JEOL 1200EX.

Uder a light microscope we could see tiny coccoid particles, usually within a week from the inoculation. During culture, the particles became more visible, optically opaque, and bigger. They multiplied in numbers. The shape and size of nanobacteria did not change much in prolonged culture but they formed a biofilm and attached to the bottom of their culture vessel within 3-4 months. The nonadherent nanobacteria were slightly irregular shaped, rough coccoid particles (Fig. 1A) with a diameter of 200-500 nm. The size varied largely and the smallest particles had a diameter of 50 nm. Their ultrastructure revealed a thick cell env-velope surrounded by slime-like material that was washed away by sample preparation procedure (Fig. 1B) but could be seen **if** the sample was only air-dried. The surface of the envelope was rough. In old cultures this structure became extremely thick and showed fibrils and crystals resembling hydroxyapatite. These nanobacteria became immobilised in the biofilm thet slowly became bone-like (Fig. 2A). The biofilm contained particles with comparable size to those in the nonattached culture (Fig. 1A).

The novel agent was discovered as a cell culture contaminant. However, it originates from blood and we have detected nanobacteria in humans and cows by culture and immunoassay.(4) They share many features with pathogenic bacteria, e.g. they form a biofilm and have thick protective capsule structure. In culture, nanobacteria were rapidly bound and attached as clusters an patchy areas of the cell membrane of several mammalian cells (Fig. 2B). Infected cells internalized the bound particles into ly-sosomal vesicles and concomitantly cytotoxicity appeared. Unculturable organisms resembling nanobacteria have been previously detected dy EM in association with human and plant diseases.(5,6) We have started screening for possible association of these most unusual particles with human diseases.

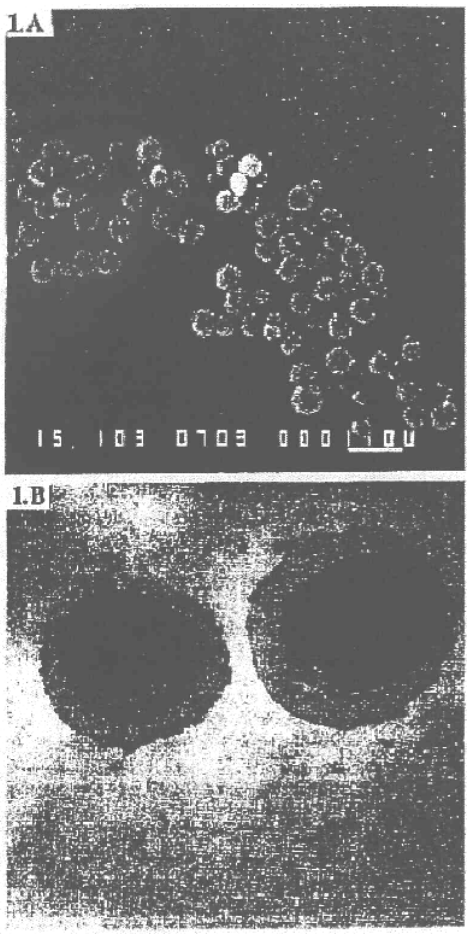

FIG. 1 (A) SEM of cultured nanobacteria. Nanobacteria were obtained from 2-month old Gibco fetal bovine serum (lot N:010G8289Y)culture by centrifugation. The washed pellet was resuspended in serum free medium and fixed on a cover glass. Bar: 1.0 µm (B) TEM of cultured nanobacteria. Nonadherent washed nanobacteria were fixed and stained as described in the text. Bar: 0.2 µm

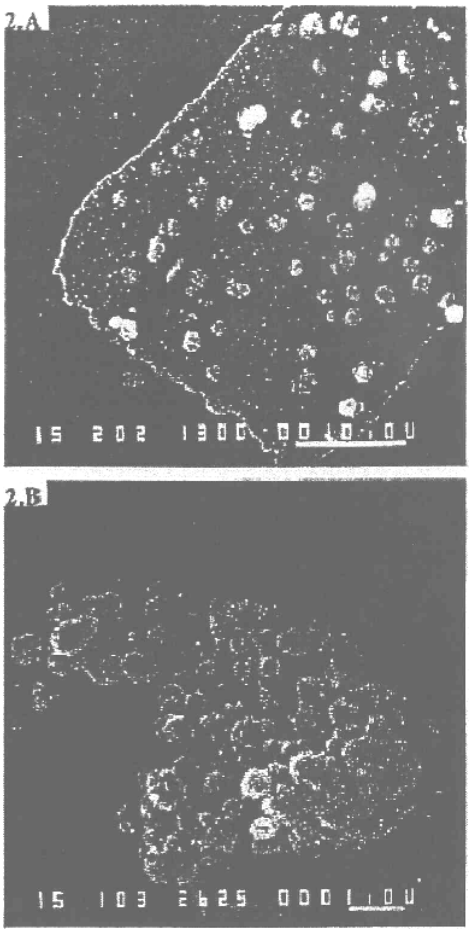

FIG. 2 (A) SEM of the biofilm formed by nanobacteria. Nanobacteria were cultured for 4-months without serum and then the formed biofilm was detached with a spatula and fixed on a cover glass. Bar: 10.0µm (B) Nanobacteria on the cell membrane of mouse 3T6 fibroblast. Cultured nanobacteria were added to the 3T6 cells 2.5 h earlier. Nanobacteria could be seen attached on patchy areas on the cell surface as seen in this detail. Bar: 1.0 µm

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