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A New Potential Threat in Antigen and Antibody Products: Nanobacteria

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Several vaccines are currently being produced by using cultured mammalian cells. Microbiological sterility of such vaccines is of great importance since several examples indicate potential safety hazards in vaccines contaminated with unknown organisms. Fetal bovine serum (FBS) used as a supplement in cell culture is a known safety risk (Hodgson, 1995). Obviously, not all of the risk factors of FBS are yet known and thus cannot be controlled. It is commonly known that only about 10% of FBS batches support cell cloning well (Liddel and Cryer, 1991) but the reasons for this have remained unclear. As with many other cell culturers, we faced a problem about 10 years ago of poorly thrivingo cells not attributable to any known contaminant. In this report, we describe the discovery of a new bacterium from mammalian blood and blood products, tentatively named as *Nano-bacterium sanguineum* gen. et sp. nov., and show that this agent is common and harmful.

DISCUSSION

Culture and Diagnosis of Nanobacteria

The discovery of Nanobacteria came about because we had a problem with cell cultures namely vacuolized cells (Fig. 1A) and poorly thriving cultures without any contaminant detectable by standard methods. Transmission electron microscopy (TEM) made from these poorly thriving cell cultures indicated the presence of internalized procaryotic organisms (Fig. 1B). That their source was the commercial "sterile" FBS was proven by gamma-irradiating all the culture components (Table 1). This experiment also indicated that sterile culture media for detection of new organisms can be made by using gamma-irradiated serum as a supplement. The new organisms passed through 100 nm (but not 50 nm) filters and were called nanobacteria, since no other bacteria are known that can pass through filters with such small pores. This ability to pass through such small-pore filters was most remarkable since they were shown to have a cell wall and yet were able to surpass the filterablity of cell-wall-less bacteria. They were unculturable in microbiological media but could be cultured under cell culture conditions (with or without mammalian cells, C02 5-10%). These minute generally coccoid organisms had a diameter of 200 to 300 nm in serum, and their size increased during the culture due to the production of a very thick cell envelope (Fig. 1C, D). The thick and calcified envelope made them visible even by light microscopy. The doubling time of nanobacteria was 1-5 days (Fig. 2). Their multiplication could be detected by specific ELISA, optical density, microscopic counting, SDS-PAGE or methionine and uridine incorporation, and the multiplication could be prevented with high doses of aminoglycoside antibiotics, EDTA, cytosine arabinoside and gamma-irradiation. Considerable evidence suggested the presence of nontraditional DNA. 16S rRNA gene sequence results (data will be published elsewhere) placed them into the alpha-2 subgroup of Proteobacteria which includes *Brucella* (which are also pathogens of mammalians with preference to the fetus) and *Bartonella.* Nanobacteria were isolated from more than 80% of commercial FBS and newborn bovine sera and are the most common contaminant present in cell cultures. In addition, we isolated nanobacteria from the blood of 4% of medical students at our university. Positive identification of nanobacteria involved growth in cell culture medium with typical growth rate and optical properties, specific stainability with Hoechst 33258 using the high dye concentration and positive immunoassay results with immunofluorescence and/or ELISA using monoclonal anti-nanobacteria antibodies.

Cytotoxicity of Nanobacteria

Nanobacteria are cytopathic in cell cultures and invade mammalian cells in a distinctive manner: They trigger cells that are not normally phagocytic to engulf them. These novel organisms are one of the causes for cell vacuoliza-tion, poor thriving and unexpected cell lysis, problems often encountered in mammalian cell culture. Several mammalian fibroblast lines were cultured in MEM medium as described previously (Kajander et al., 1990), and were infected with nanobacteria. Electron microscopy and FITC staining with specific monoclonal antibodies indicated that nanobacteria were bound on the surface of the fibroblasts (Fig. 1E-G). We concluded that they were internalized either by receptor-mediated endocytosis or by a closely related pathway. After the internalization, fibroblasts showed apoptotic abnormalities and died if subjected to a high dose (>100 nanobacteria/cell).

Different Growth Phases of Nanobacteria

Washed nanobacteria added to serum-free medium grew very slowly as evidenced by increase in their numbers and protein level and were firmly attached to the culture plates. These cultures progressed to large multicellular formations covered by layers of a firm protective material several micrometers thick (Fig. 1H). After addition of sterile serum, the layer disappeared, with typical small coccoid nanobacteria later appearing in the same cultures with the mobile, larger D-shaped ones (Fig. II). Specific monoclonal antibodies indicated the presence of the same anti-genic sites in both D-shaped and coccoid nanobacteria, and their 16S rRNA gene sequences were 98% identical.

How can Cell Culture be Possible with Nanobacteria-contaminated Fetal Bovine Serum?

Although more than 80% of cell culture serum batches are contaminated with nanobacteria, many cell culturers have not faced this problem with their cell cultures. We have experienced a major problem with nanobacteria in cell culture only when they are present at high concentrations relative to cells. This can occure typically in cell cloning and in long-term experiments where mammalian cells do not multiply. Internalization of numerous nanobacteria by a cell results in cytotoxicity. Importantly, most cell lines multiply faster than nanobacteria. Thus, cyto-toxic concentrations may be avoided.

Why is Nanobacteria a Potential Threat?

Nanobacteria can cause a chronic infection in laboratory animals and in humans. The agent could be isolated from blood of one peron for 5 years despite the presence of antibody. When nanobacteria were injected into rabbits, the agent was initially isolated from urine and then from cerebrospinal fluid after one year. Nanobacteria multiply very slowly and, if pathogenic in humans, might cause slow chronic autoimmune-like disorders (compare with leprosy or brucellosis). Sofar, there are no chronic bacteraemia known that would not be harmful. Thus, the posibility that nanobacteria may be present in vaccines made with cell culture, or in gammaglobulin or other antibody preparations, must be controlled.

SUMMARY AND CONCLUSIONS

Nanobacteria are novel microorganisms that are not detectable with present sterility testing methods, but they are detectable with new culture and immunomethods. They are commonly present in bovine and blood products and thus in cell cultures and antigens, including vaccines derived therefrom, and may be present in antibody and gammaglobulin products. Nanobacteria are a potential risk because of their cytotoxic properties and ability to infect fetuses, and thus their pathogenicity should be scrutinized.

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REFERENCES

- Hodgson, J. 1995. To treat or not to treat: That is the question for serum. *BioTechnology* **13:** 333.
- Kajander, E. 0., R. J. Harvima, L. Kauppinen, K.K. Akerman, H. Martikainen, R. L. Pajula, and S. 0. Karenlampi. 1990. Effects ofselenomethionine on cell growth and on S-adenosylmethionine metabolism in cultured malignant cells. *Biochem. J.* 267: 767.
- Liddel, J. E., and A. Cryer. 1991. *in A practical guide to monoclonal antibodies,* p. 25. Wiley, New York.

Table 1. The Effect of 60Co Gamma-Irradiation of Culture Components on Multiplication of Nanobacteria

Culture	Multiplication
FBS RPMI	+
FBS *RPMI	+
*FBS RPMI	-
*FBS RPMI	+
*FBS RPMI	-

The material marked with asterisk (*) received a sterilization dose of 3 megarads during 16 h at room temperature. Cultures were established using 10 % serum and nanobacterial counts were followed for 4 weeks.

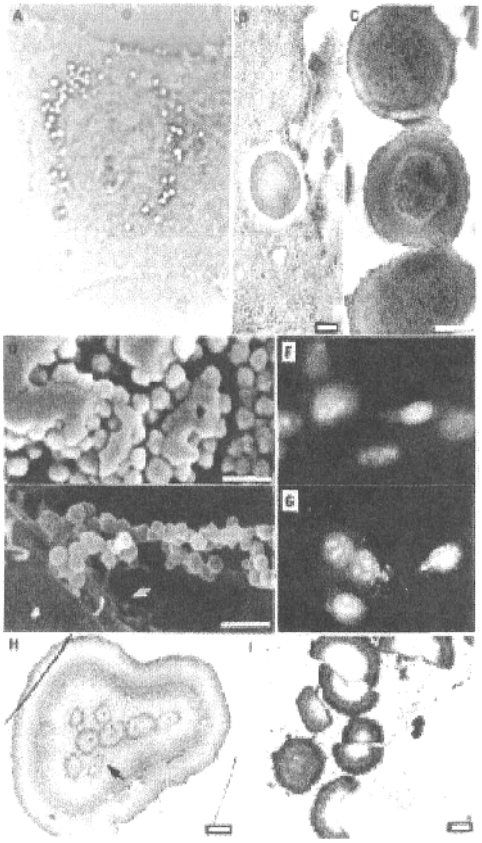

Figure 1. Ultrastructure ofnanobacteria and their interaction with fibroblasts.

(A) Perinuclear vacuolization of an infected 3T6 cell under phase-contrast microscope;

(B) TEM image of a nanobacterium engulfed by a BHK cell;

(C) cultured coccoid nanobacteria (bars 200 nm).

(D) SEM image ofnanobacteria attached to culture vessel;

(E) nanobacteria attached to a fibroblast surface (arrow shows the surface of the fibroblast; bars 1 um).

(F) Indirect immunofluorescence staining of cultured healthy 3T6 cells with a monoclonal antibody (8/0) against nanobacteria;

(G) 3T6 cells inoculated with nanobacteria;

(H) TEM of a nanobacterial population in a serum-free culture (arrow shows a D-shaped nanobacterium in this population);

(I) D-shaped nanobacteria after culture in serum-containing medium (bars 1 μm).

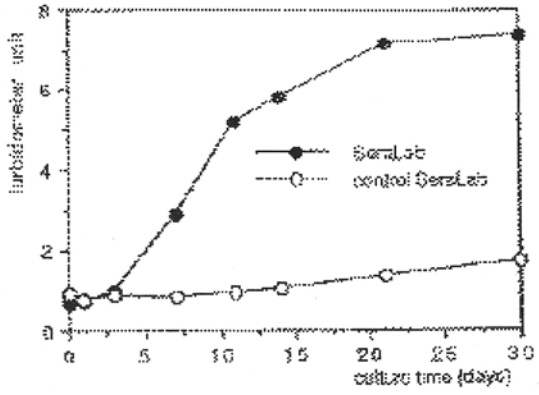

Figure 2. Growth-curve of nanobacteria. As a control, gamma-irradiated FBS was used. At each time point, samples from triplicate incubations were taken, frozen and analyzed by turbidometer at the end of the experiment. Turbidometer units are means of three measurements from 1/6 dilutions of cultures.