Zbl. Bakt. Suppl. 26, 1994.

Comparison of Staphylococci and Novel Bacteria-Like Particles from Blood

E.O. Kajander, E. Tahvanainen, I. Kuronen and N. Ciftcioglu
Department of Biochemistry and Biotechnology, University of Kuopio, Kuopio, Finland.

Summary

Our group has discovered autonomously replicating bacteria-like particles in sera sold for cell culture and in human blood samples. The novel agent was typically coccoid or coccobacillar, variable in size (0.2 to 0.6 um) and passed through 0.2 |im filters. The agent was difficult to culture but had division septa resembling that of Staphylococci. Long-term serial cultures were established to see if this organism would be a special state reverting back to a normal bacteria. During 1 year culture,2 cultures out of 20 gave Staphylococcus epidermidis. Therefore, we used several bacteriological staining methods, EM techniques, chemical and nucleic acid analysis and immunomethods to compare the novel agent with S. epidermidis. Results indicatethat the novel agent is not related to Staphylococci and has quite exceptional, unique properties, e.g. filterability, growth requirements and resistance to gamma irradiation and antibiotics. Its bacterial nature is favoured by its structure and because high doses of gamma irradiation and aminoglycoside antibiotics prevented its multiplication. The novel agent has been named tentatively as *Nanobacterhim sanquineum.*

Key words: Cell culture, strility tests, unculturable microorganisms, serum, sterile-filtration.

Introduction

Our group has studied the sterility of mammalian cell cultures for several years. In the early phase of these studies a surprising discovery was made: autonomously replicating biological particles were detected in long term cell cultures, even in the absence of mammalian cells. The particles multiplied with a doubling time of 1 to 5 days. They did not form colonies on any commonly used microbiological media. The particles could be passaged in serial cultures in liquid cell culture media. They were coccoid or coccobacillar and appeared as free particles or were in small groups or in big clumps. A special characteristic was ability to pass through sterile filters of pore-size of 0.2 um. Their origin was shown to be the commercial cell culture serum. They could also be detected from some human serum samples.

Microscopic data including EM showed that the novel particle may resemble a staphylococci, that are common on human and animal skin and may contaminate blood samples and processed blood. Furthermore, cell cultures are often contaminated with Staphylococci which grow aerobically and are relatively resistant to the antibiotics used in cell culture. Also L-forms of Staphylococci are common. L-forms can pass through sterile filters and may lack typical bacterial components and behave quite anomalously (4). For these reasons, we compared the novel autonomously replicating particle with Staphylococci.

Materials and Methods

Autonomously replicating particles were isolated from one or two month old cultures established by culturing 10% FBS (for the bovine isolates) or 10% human serum (for the human isolates) in RPMI-1640 (Gibco, Paisley, Scotland) under an athmosphere of 5% C02-95% air (90% humidity) at 37°C. Culture and subsequent preparation conditions used refer to US patent No:
5,135,851 and European patent application No: 91107318.7. After harvesting by centrifugation, particles were washed three times with PBS. For microscopy, the pellets were suspended in PBS, air dried, fixed and stained. S. epidermidis (cat. No: 16) was from Scott Lab., Carson, CA and was cultivated in RPMI-1640.

Transmission EM was carried out as described before (6). DNA was quantified with the Burton method (2). RNA was assayed using D-ribose as a standard with the orsinol reaction. Protein was assayed with Coomassie brilliant blue (5) from acid hydro-lyzates. Bacteriological stainings included the gram stain for pale staining bacteria, simple staining methods with safranin 0, methylene blue and crystal violet, Giemsa stain, spore stain (Wirtz-Conklin), Ziehl-Neelsen acid-fast stain, Gimenez stain for Chlamydiae and Rickettsiae, and capsule stains including Anthony method, India ink method and negative stain.

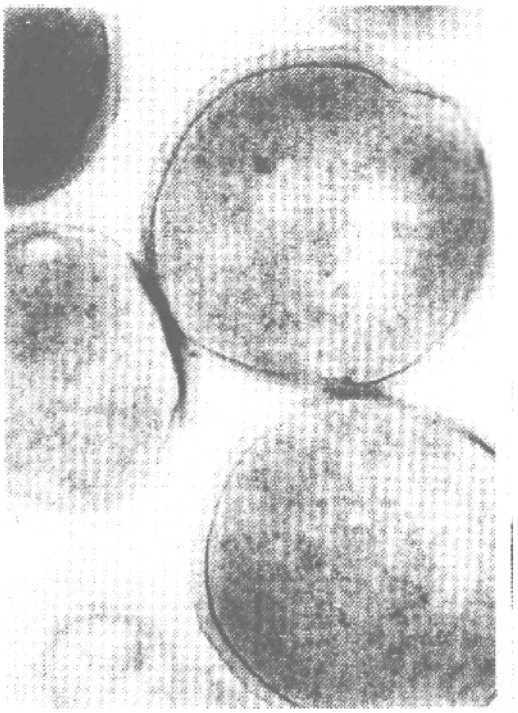
Results

Autonomously replicating particles could be cultured from tens of sterile cell culture sera. They appeared best under phase-contrast microscopy and thus were optically dense particles, differing from mycoplasmas and L-forms. Their numbers multiplied typically at about 3 d, but their maximal densities were low. The particles became larger during the first two weeks of culture remaining then the same corresponding with EM results of a diameter of 0.2 to 0.6um, see Fig. 1. The novel particles possessed a bacteria-like structure and had thicker cell-wall than that ofS. epidermidis, often surrounded by a capsule and slime-like material, see the dividing particle in Fig. 1. Even the cultured particles depicted could not grow on blood agar. Could they ever gain ability to grow like normal bacteria? Long-term cultures using IX (isotonic) and 5X (hyperosmolar) media were established for detection of "revertants". The cultures were monthly serially passaged for one year. During this time, two cultures started to grow rapidly and gave S. epidermidis on culture. The possible revertants showed very different protein patterns in SDS-PAGE and immunoblotting with particle-specific antibodies was negative. The particles, when compared per dry weight with S. epidermidis, contained about a twentith part of DNA, tenth of RNA and equal amount of protein. No DNA could be isolated from the particles with the standard DNA isolation methods. This may relate to the fact, that the particles could not be broken. They were resistant to the action of lysosome and otherproteolytic enzymes, and SDS. Their acid hydrolyzates, however, contained a small amount of UV-absorbing material similar to that ofS. epidermidis (Fig. 2). The UV-absorbing material was released with 1 N HCL, which hydrolyzed the particles rapidly. S. epidermidis was more resistant and was broken only after boiling, releasing the UV-absorbing material (nucleosides).

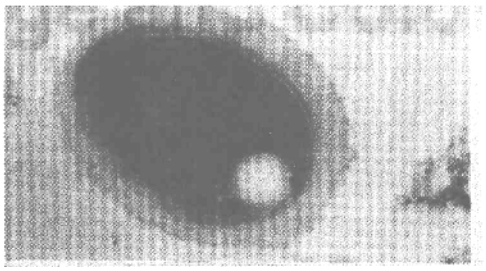
EM revealed that the novel agent had a thick cell-wall and civision septa (Fig. 1). So, the novel agent was not an L-form of S. epidermidis. This was supported by bacteriological stainings. Stainability was generally poor. The presence of slime and capsule could be verified. The human isolate had the biggest size and was visualized best, especially after enzymic splitting of surface carbohydrates. This and the capsule stainings indicated thick capsule. Furthermore, the novel agent differed from staphylococci in being resistant to 0.5 megarad dose of gamma irradiation and to bacteriocidal antibiotic levels. Normal sterilization dose (2.5 megarad) and high aminoglycoside antibiotic levels prevented the growth of the novel agent.

Discussion

The results proof that the novel agent is not a staphylococci or its specific state. Thus the two "revertants" obtained were contaminants. Are the novel particles bacteria or not? Clear evidence is difficult to get since yhey did not form typical colonies and the harvested material is scarce and contains serum-derived precipitates. "Pseudo-organisms" have been previously described in long-term cultures with serum (1). Pseudo-organisms have been regarded as non-living soap-like aggregates and no cell structures were found (3). The novel organism has cell-wall and division septa that clearly indicate it as a living organism. Its properties differ from known eubacteria and archaebacteria. We have very tentatively named it as Nanobacteria, species Nanobacte-rium sanquineum. Clearly, this intriguing finding deserves much more research.

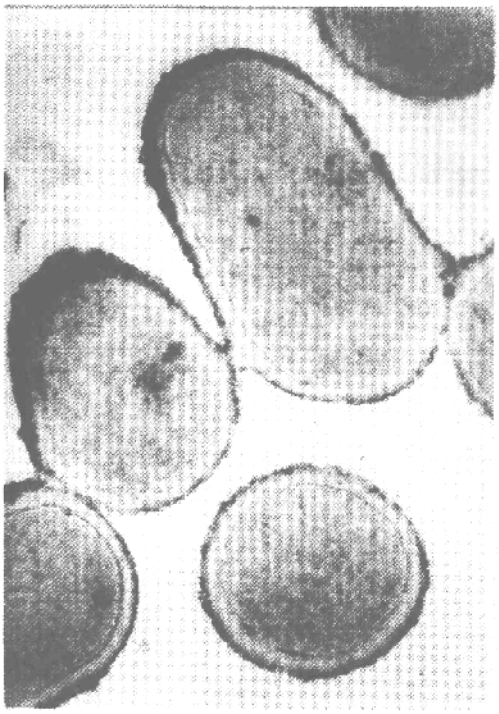


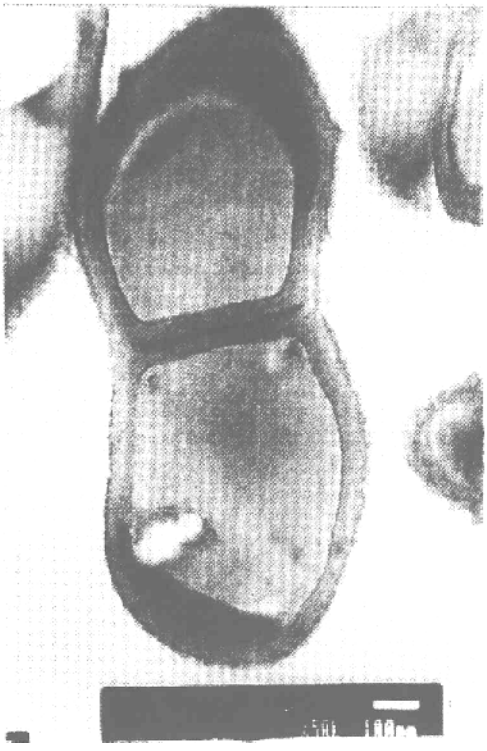
A) Transmission EM of S. epidermidis



B) Transmission EM of the novel particles

C) Transmission EM of the novel particles

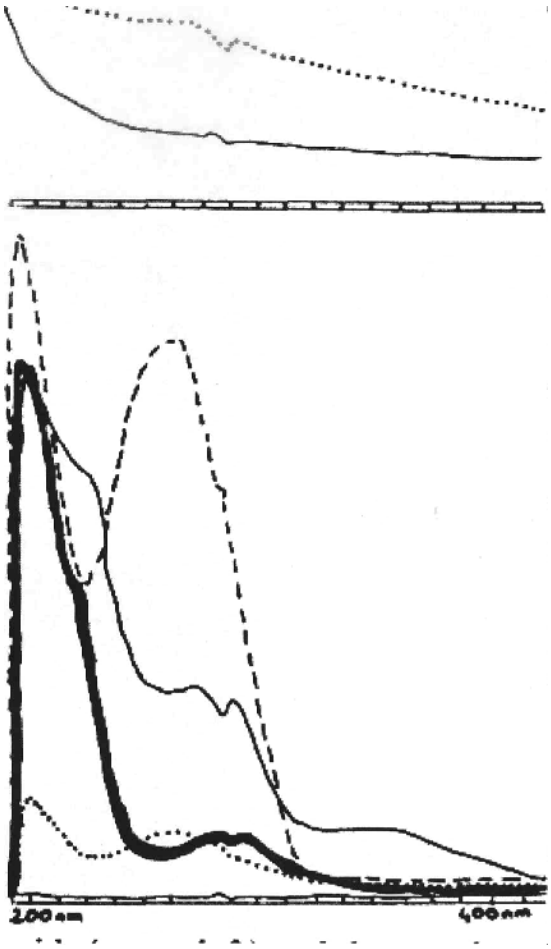




D) Transmission EM of the novel particles

Fig 2.

Spectral analysis (200-400 run) of acid hydrolyzates ofS. epidermidis and the novel particles. Lyophilized S. epidermidis (0.5 ug/ml) or particles (0.5 mg/ml) were suspended in water (upper picture, ...= S.epid., ___=particles) or in 1 N HC1 (lower picture) before (symbols as above) and after boiling (——— = S.epid., ___=particles)



Acknowledgements:This work was supported by the University of Kuopio, The High Technology Foundation ofSavo, Juho Vainio Foundation and Abcell Oy.

References

1. Buchanan, A. M. Atypical colony-like structures developing in control media and in clinical L-form cultures containing serum. Vet. Microbiol. 7: 1-18,1982
2. Giles, K. W. and A. Myers. An improved diphenylamine method for the estimation of deoxyribonucleic acid. Nature 206: 93,1965.
3. Hijmans, W., C. P. A. van Boven and H. A. L. Clasener. Fundamental biology of the L-phase of bacteria. In The Mycoplasmatales and L-phase of Bacteria (ed. Hayflick L.). Appleton-Century-Crofts, New York, pp. 118-121, 1969.
4. Kenny, J. F. Role of cell-wall defective microbial variants in human infections. South. Med. J. 2: 180-190, 1978.
5. Spector, T. Refinement of the Coomassie blue method of protein quantitation. Anal. Biochem. 86: 142-146, 1978.
6. Tedeschi, G. G. and I. Santarelli. Electron microscopical evidence of the presence ofunstbile L-forms ofS. Epidermidid in human platelets. In Spheroplasts, Protoplasts and L-forms of Bacteria (ed. Roux, J.). Inserm, Paris, pp. 341-344, 1976.