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CONFERENCE PROCEEDINGS

Philadelphia Convention Center, Philadelphia, PA
October 26-28, 1999

A NEW PROBLEM FOR STERILE FILTRATION; NANOBACTERIA

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ABSTRACT

Nanobacteria are the smallest cell-walled bacteria, discovered only recently in human and bovine blood and commercial blood products. Nanobacterial cells are 100-500 nm in diameter and appear to produce elementary particles which, at 100 nm, are smaller than many viruses. These bacteria belong to Proteobacteria but are unique in that they produce carbonate apatite on their cell envelope. Nanobacteria are difficult or impossible to culture in microbial media and have low metabolic rates, which makes their detection difficult. They grow well under mammalian cell culture conditions and can be present in cell cultures. Mycoplasmas are the smallest classical bacteria that can grow. under cell culture conditions. These conditions also support the growth of cell wall-less bacteria (L-forms).

The sterility of cell culture supplements and related products such as vaccine materials, hormones and growth factors, depends largely on the filtration techniques that are used. All of the above-mentioned bacteria can pass through sterile filters. Filtration through 0.2 pm pore-size filters has resulted in 10^2-fold and 10^2-fold reductions in the number of mycoplasma and L-forms, respectively. In the case of nanobacteria, the reduction was less than 10-fold and they passed even through 0.1 µm pore-size filters. Novel approaches to filtration technology are therefore needed to eliminate nanobacteria, which are potentially harmful agents present in blood and biotechnology products.

NANOBACTERIA

The sterility of cell culture supplements and products such as vaccine materials, hormones or growth factors, depends largely on the efficacy of various filtration techniques. This technology has acquired new challenges as a result of recent discoveries of small bacterial life-forms. Nanobacteria, discovered ten years ago in human and bovine blood and in commercial blood products, are the smallest cell-walled bacteria described to date. The key to their discovery was to start testing the sterility of cell culture media and their supplements by using mammalian cell culture conditions and not microbiological media, as required by the official guidelines. This novel organism was named Nanobacterium sanquineum, referring to its surprisingly small size and its presence in blood, and it was reposited in the German Collection of Microorganisms (DSM No: 5819-5821). This organism was isolated from 'sterile' human and bovine sera obtained from commercial sources. Fetal bovine serum was found to be contaminated to the extent that about 80% of serum batches contained culturable nanobacteria (see Table). About 5% of human serum samples were found to contain nanobacteria [1]. As these organisms can pass even through 0.1 u.m pore-size filters, they present a new challenge for sterile filtration.

DISCOVERY OF NANOBACTERIA IN FETAL BOVINE SERUM

Bacteria-like particles that formed carbonate apatite were initially observed in long-term mammalian cell cultures and they were associated with only certain batches of serum. In these cultures the mammalian cells

became vacuolized (Figure 1A) and died within four weeks. The harmful agent could be passaged even in the absence of mammalian cells and used to infect other cells with the same outcome. Intracellular bacteria-like particles (Figure IB), similar to those cultured without mammalian cells (Figure 1C), were observed. The cultures had a white macroscopic layer of bacteria on the bottom but the pH of the medium did not change greatly.

Microbiological tests, including mycoplasma assays, were negative. Bacteriological staining methods initially failed because of difficulties in the fixation of nanobacteria with classical flame and alcohol techniques. Nanobacteria could not be cultured in standard microbiological media but grew in all commercial mammalian cell culture media at 37-45°C under 5-10% C02 - 90-95% air. There was no growth under anaerobic conditions. Serum concentrations of 10-50% supported growth optimally and very poor growth was observed on solid media. The growth rate was logarithmic after a lag period, with a doubling time between one and five days, depending on the conditions. The bacteria produced slime as shown in-Figure ID and this slime probably causes their adherence to glass, plastic and other materials. Thus, nanobacteria can form a biofilm which is associated with mineral formation.

A typical time-course of a nanobacterial culture is as follows. A small number of very tiny particles, either alone or in small groups, are seen near the bottom of the culture vessel when examined by light microscopy after about one week of culture. After two weeks they become larger and more numerous and form groups visible even to the untrained microscopist. After one month, many particles are in clumps and start to attach to the bottom and by two months most of them exist in a white-colored biofilm visible to the naked eye. The biofilm becomes bone-like after three months and sometimes detaches from the bottom after about six months. Pellicles can be seen in old cultures.

Specific monoclonal antibodies were produced against a bovine isolate of nanobacteria. These antibodies were shown to recognize all cultured human and bovine nanobacteria, which indicates the presence of common surface .antigens. Before this, nanobacteria could be distinguished readily only by electron microscopy (Figure IE), but the antibodies led to the development of a convenient detection method [2]. Cells contaminated with nanobacteria display star-like particles in immunofluorescence staining whereas healthy cells do not (Figure 1G and F).

The culture of nanobacteria under reduced serum concentration results in social growth as communities. Total removal of serum revealed a novel growth phase as seen in Figure 1H. Nanobacteria produced thick apatite walls common to a number of cells in a small community. Such 'castles' were large, sometimes even bigger than mammalian cells. Nanobacteria can be cultured in this new growth phase; they have been passaged monthly for over five years. These special forms of nanobacteria could be released from their apatite 'castles' by adding serum to the culture medium. These forms had very bizarre morphology (Figure 11), were found to react with the monoclonal antibodies and to release small, nanobacteria into the culture [3].

Table I. List of sera tested for contamination.

LIST OF SERA TESTED FOR CONTAMINATI0N

Serum	Manufacturer	lot n:o	Culture result detection'	multiplication[2]
FBS	Imperial	861163	+++	+
FBS	Imperial	460865	+++	+
HS	Imperial	260652	+++	+
NBS	Imperial	360373	+++	+
FBS	Gibco	20Q4380X	++	+
FBS	Gibco	40F0982F	+++	+
FBS	Gibco	30F0484F	+	
FBS	Gibco	10G8289Y	+++	+
FBS	Gibco	10G7572F	-	-
FBS	Gibco	10G3673Y	-	-
FBS	Gibco	10F7080F	-	-
FBS	Gibco	50Q1676X	+	
FBS	Gibco	10F0484Y	+++	+
FBS	Gibco	40F5389F	++	+
FBS	Gibco	40F8585F	+++	+
FBS	Gibco	10G7894Y	+	
FBS	Gibco	10Q3380	+	
NBS	Gibco	30A1078	+	
FBS	BM	613594	+	
FBS.	BM	210463	+	
FBS	BM	870910	+	
FBS.	Nord Vacc	Moo 196-7	+	
FBS	Sera-Lab	701112	+	
FBS	Sera -Lab	701113	+	
FBS	Sera-Lab	601129A	+++	+
FBS	Sera-Lab	801114	+++	+
Hum S	FCR	2059-7001	++	+
HS	Flow Lab	026017	+	+
FBS	Flow Lab	028011	+	
FBS	BI	593811	++	+
FBS	BI	593410	++	+
NBS	BI	311237	+	
NBS	BI	411126	+	
HS	BI	VE3585	+	
HS	BI	407669	+	

Detection
+++: coccoid particles after cultivation of one day
++: coccoid particles after cultivation of five days
+: coccoid particles after cultivation of ten days

Multiplication +: means an increase in number over 10-times the initial observed during 1 month test

Abbreviations:

NBS	Newborn bovine sera
FBS	Fetal bovine sera
HS	Horse sera
Hum S	Human sera
Imperial	Imperial Laboratories, U.K.
Gibco	Gibco Ltd. Paisley, Scotland
BM	Boehninger Mannheim, F.R.C.
Nord Vacc	Nord Vacc, Skärholmen, Sweden
Sera-Lab	Sera-Lab Ltd, Crawley Down, Sussex. England
FCR	Finnish Red Cross, Helsinki. Finland
FlowLab	Flow Laboratories Ltd. Ayshire. Scotland
BI	Biological Industries. Kibbutz Belh Haemek, Israel

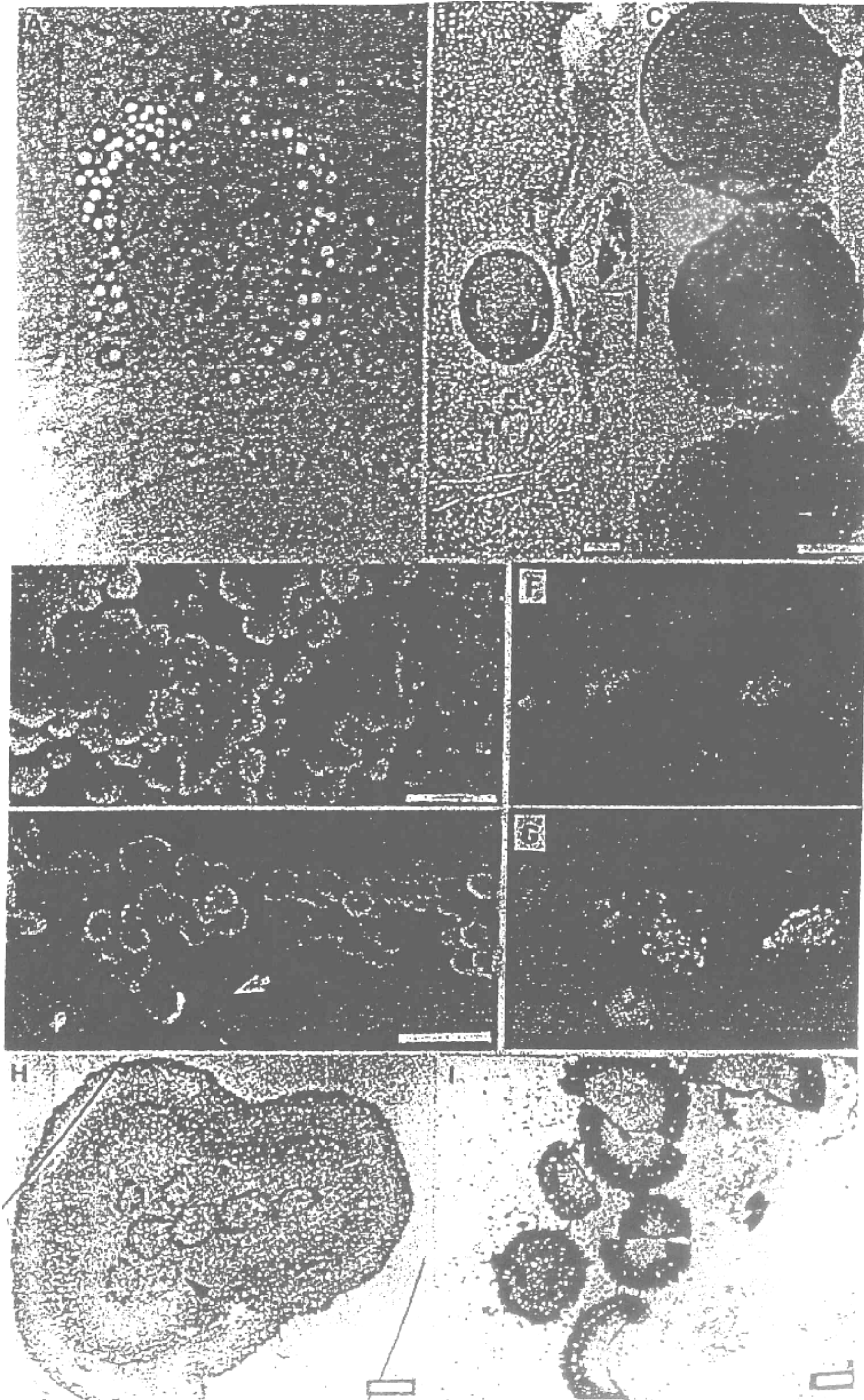

Figure 1. Ultrastructure of nanobaclcria and their interaction with fibroblasts. (A) Perinuclear vacuolization of an infected 3T6 cell under phase contrast microscope (B) TEM image of' a nanobacterium in a BHK cell, (C) cultured nanobacteria (bars 200 nm). (D) SEM image of nanobacteria attached to culture vessel: (E) nanobacteria on a fibroblast surface (arrow: the fibroblast: bars 1 µm). (F) Indirect immunofluorescence staining of healthy 3T6 cells with a monoclonal antibody against nanobacteria; (G) 3T6 cells inoculated with nanohacteria: (H) TEM of a nanobacterial population in a serum-free culture (arrow shows a D-shaped nanobacterium): (I) D-shaped nanobacteria after culture in serum-containing medium (bars 1 µm). Reproduced from Vaccines 97, Cold Spring Harbor Laboratory Press.

EVIDENCE SUPPORTING THE EXISTENCE OF NANOBACTERIA

1. Nanobacteria can be cultured with a doubling time of about three days, and they can be passaged apparently indefinitely. At present, they have been passaged monthly for over six years.
2. They produce biomass at a rate of about 0.0001 times that of E. coli.
3. Their biomass contains novel proteins and 'tough' polysaccharides.
4. SDS-PAGE of nanobacterial samples shows over 30 prominent protein bands. Amino terminal sequences are available from six different proteins and one of them has been shown to be a functional porin (unpublished work in collaboration with Dr. James Coulton, McGill University). Porins, a hallmark of gram-negative bacteria, are located in the outer membrane where they function in the transport of relatively small molecules. In nanobacteria, the porins seem to be located in the mineral layers. Muramic acid, a major component of peptidoglycan, has also been detected. Thus, nanobacterial cell walls have typical gram-negative components, although the ultrastructure of these bacteria is unique and varies as a function of the growth phase.
5. Nanobacteria contain material tentatively identified as nucleic acid and Us components have been analyzed by mass-spectroscopy [4]. A tentative 16 S rRNA gene has been identified by PCR and its sequence (EMBL Entries X98418 and -X9841?) suggests an affiliation of nanobacteria with the alpha-2 subgroup of Proteobacteria.
6. Nanobacterial growth can be inhibited with small concentrations of tetracycline antibiotics or with high concentrations of aminoglycoside antibiotics. Both antibiotics stop bacterial protein synthesis at the ribosomal level.
7. Nanobacterial growth can be inhibited with small concentrations of cytosine arabinoside or fluoro-uracil, both of which are antimetabolites that prevent nucleic acid synthesis in all types of cells.
8. Nanobacteria can be detected with metabolic labeling using methionine or uridine.
9. Nanobacteria have unique strategies for social behaviour and multiplication, including communities, budding and fragmentation.

THE PHYLOGENETIC POSITION OF NANOBACTERIA

According to a phylogenetic analysis based on a tentative 16 S rRNA sequence from nanobacterium, its nearest relatives are in the genera Phyllobacterium, Thiobacillus, Brucella, Bartonella, Rhizobium and Agrobacterium [4]. This phylogenetic placement is further supported by other findings. Nanobacteria are highly resistant to gamma irradiation and heat, which suggest the possibility of a recent habitat in hot-springs. One of its closest relatives, Thiobacillus sp., is known to form iron and sulphur mineral deposits in hot springs [5]. Furthermore, antibodies against nanobacteria cross-reacted with Bartonella henselae and B. quintana and antibodies against B. quintana cross-reacted with nanobacteria in immunoassay. Thus, known human pathogens Bartonella and Brucella seem to be relatives of nanobacteria. All these bacteria share certain common properties; they are very fastidious, impermeable to stains and invade mammalian tissues, and they even have a preference for fetal tissues. Brucella is known to pass into urine and the Kuopio research group has recently shown that this also happens with nanobacteria [6]. Brucella and Bartonella invade animal and human cells and tissues and other members of the group exist in soil and invade plant cells, except for some Thiobacilli which inhabit hot-springs. Interestingly, it has been speculated that -mitochondria originated from an ancestor of this group.

UNIQUE PROPERTIES OF NANOBACTERIA

The average diameter of nanobacteria, measured with electron microscopic; • techniques, is 0.2-0.3 µm, smaller than that of any known cell-walled organism. Several viruses (e.g. Vaccinia) are larger than nanobacteria, which can be as small as 0.1 urn. Ultrafiltration methods produce even smaller estimates of size, as nanobacteria readily pass through O.I um pore size filters. Apparently, nanobacteria can pass through pores smaller than their own size and must therefore have flexible cell walls. We have also shown that during filtration nanobacteria lose part of their apatite mineral layer. The theoretical minimum diameter of a cell, based on the size of those macromolecular components now considered to be necessary and sufficient for a living cell, is about 0.14 um [7j. The measured size of nanobacteria approaches the theoretical lower limit for the size of a living organism. The very slow growth rate and requirement of a very rich medium may be adaptations to the very small size of the cell.

Nanobacteria are highly resistant to heat and gamma-irradiation and thus provide the first example of such organisms isolated from mammalian tissues. Bacteria with these properties are found in environments where these traits are essential. Therefore it might be speculated that nanobacteria have emerged from an extreme environment, perhaps only relatively recently in evolutionary terms.

High doses ofaminoglycosides(l mg/mL) effectively block the replication of nanobacteria, which indicates the presence of bacterial type of protein synthesis. The relative resistance to these antibiotics may be due to the impermeable cell wall, which also makes their microbiological staining very difficult; In addition to the cell wall, a second permeability barrier is provided by mineral deposits composed ofbiogenic carbonate or hydroxyl apatite. These apatite shells, which can be several micrometers thick, form "castle" structures, which are made and inhabited by a small community of nanobacteria. The "cave" in the apatite indicates the biogenic origin of the "castle" structure. Under the protection of such "castles" nanobacteria are almost indestructible. It is noteworthy that nanobacteria cannot be detected by standard sterility-testing methods that employ DNA stains. We have developed improved methods to unravel the nature of the nanobacterial nucleic acids, which appear to be unusual in that they show a UV-absorbance maximum at 270 run [4, 8] instead of 260 nm, the absorbance maximum of conventional nucleic acids.

Nanobaeteria have a distinctive way of invading mammalian fibroblasts. They trigger cells that are not normally phagocytic to engulf them. After receptor-mediated adherence followed by intemalization, apoptotic cell death occurred in all tested fibroblasts in a dose-dependent way. The cytotoxic potential depended on culture passage number, primary isolates being the most cytotoxic. Adherence and cytotoxicity are rare traits among cell culture contaminants [2].

NANOBACTERIA, MYCOPLASMA AND BACTERIAL L-FORMS; PROBLEMS FOR STERILE FILTRATION

Nanobacteria seem to be a serious problem in sterile filtration as they are found in animal and human sera which are used in processes that require sterility [9]. These bacteria can find their way into cell culture materials that are used.to make therapeutic products which rely on filtration for sterility. Recent data from environmental studies suggest that very small bacteria, possibly nanobacteria, are widely distributed on our planet. Particles resembling the tiniest nanobacteria were discovered in sedimentary rocks by Dr. Folk, who named them nannobacteria [10] and suggested that they may contribute to the formation of carbonate minerals. This raises the possibility of environmental sources of nanobacterial contamination. Ultramicrobacteria, which are able to pass through sterile filters, were found in soil and natural water sources some 20 years ago. They are difficult to culture and therefore have remained largely uncharacterized [11] and their possible connection to nanobacteria is not known. Normal bacteria may

acquire a dormant state and will not grow on subsequent culture [11]. The size of such starved ells can be only a fraction of the size of actively growing cells. No dormant state has been detected in nanobacteria.

Cell wall-less bacteria, L-forms, have small and large forms. Conventional culture methods do not support the growth ofL-fonn microbes. L-forms can pass through sterile filters after which they can be easily lysed and their nucleic acids and proteins extracted [12]. Mycoplasma, Chlamydia and Rickettsia are smallest 'classically known' bacteria with sizes approaching 200 run and they can be cultured with mammalian cells under cell culture conditions, but only mycoplasma can grow autonomously. All three bacteria can be a problem in sterile filtration as they can be present in biological material and have been shown to pass through sterile filters. Filtration through 0.2 urn pore-size filters results in over 100-fold reduction in the numbers of these bacteria, bacterial L-forms are reduced by a factor of 10^6 [12], whereas with nanobacteria the reduction is typically less than 10-fold [4]. When nanobacteria were subjected to filtration through 0.2 11 m pore-size filters, the temperature and back-pressure were found to influence the number of cells that are able to pass through. Only 2% went through the filter at 4°C and at low back-pressure, whereas at 56°C the numbers reached up to 50%. Nanobacteria could pass through 0.1 but not 0.05 u.m nominal pore size filters. In filtration, cell fragmentation may result in very tiny forms that can pass through filters, as has been observed with mycoplasma. Such fragmentation may also occur when nanobacteria are subjected to filtration.

Cell size is considered to be a stable characteristic of a given bacterial species, but examples of changes in the size, shape or morphology of the organism in response to environmental and social stimuli, have been described. Myxococcus xanthus has a life cycle, carefully controlled by cell density and nutrient levels, which includes tiny forms, actively moving large forms and huge social formations that produce mushroom-like fruiting bodies. Rapidly growing mycoplasma "forget" cell division and form very long multicellular structures. Nanobacteria can have several growth forms, sizes and social formations depending on the culture conditions. The small size of an organism does not necessarily imply a small genome. The Myxococcus xanthus genome, at 9.4 Mb [13], is among the largest bacterial genomes, whereas mycoplasmas have the smallest known genomes, at 0.58-1.6 Mb [14]. Chlamydia and Rickettsia have genomes of 1 Mb. The size of the nanobacterial genome is unknown but quantitative Hoechst staining suggests it to be smaller than that of mycoplasmas.

STERILE-FILTRATION PRINCIPLES

The microporous sterilizing-grade filters are one of the most widely used filter types for the elimination of bacteria from different types of biological liquids. There are three types of mechanisms by which these membranes eliminate the microbes: size exclusion, adsorption and cake filtration. The microporous membranes can employ any of these mechanisms in different combinations and to varying degrees.

In size exclusion mechanisms the critical opening in the path of the particle is prohibitively small and therefore prevents the particle from passing through the microporous membrane. The mechanism is based on the mechanical blockage of the particle from further movement through the filter. If this critical opening is located on the surface of the filter, the filter acts as a sieve. If the opening is within the depth of the filter, the filter acts by entrapment. In practice, most sterilizing grade filters use a combination of sieving and entrapment.

The absorptive removal of bacteria is based on physicochemical, noncovalent bonding or interaction between the filter medium and the particle to be removed. The efficiency of the adsorptive removal is

dictated primarily by the surface chemistry between the membrane and the organism, the physico-chemical properties of the liquid vehicle and the processing conditions. The adsorption of the organism requires a series of events that can be divided into three phases: physical approach of the organism to the filter matrix, formation of a noncovalent bond and the maintenance of this bond throughout the filtration process.

Cake filtration refers to a situation in which a heavily loaded filter relies on the build-up of contaminant particles on the surface for the removal of subsequent particles. This filtration technique is mainly used with heavy particulate loads which is not usually the case with cell culture supplements and products.

The currently used sterile-filters reduce the numbers of mycoplasma and bacterial L-forms considerably but are inadequate for nanobacteria. As nanobacteria are common in biological materials for which such filtration is routinely used, we must ask how to improve the filtration technology. Obviously filter structures and materials should be improved so that more of the tiny nanobacteria can be retained by adsorption. Possible sample pretreatment strategics could be employed as well. These might include physical and chemical treatments for increasing nanobacterial size and adsorptivity, or trapping.

IMPROVED STERILE-FILTRATION IS NEEDED

Spreading harmful agents in 'sterile' products: can be the consequence of inadequate sterile-filtration. Mycoplasma infections can be lethal to AIDS patients. Bacterial L-forms can revert to normal bacterial forms and cause severe diseases. Nanobacteria are found in human and bovine blood and blood products. This means, that bacteremia was present in the donors, but may not have caused a manifest acute illness. Bacteremia may persist for a long time; we have followed a human case with positive nanobacterial blood tests for several years. However, bacteremia would be expected to cause disease(s) in at least some of the infected individuals, as there are no known examples of long-lasting bacteremia which does not cause harmful effects. The apatite coat of nanobacteria may protect them from the immune system as apatite is a normal bodily constituent and also binds serum proteins further hiding the bacteria. This might explain the long-lasting bacteremia. The apatite coat might also play a role in special pathogenic calcifications. In cell culture, nanobacteria attach to fibroblasts and are internalized so that intracellular calcifications are also observed. Nanobacteria can induce apoptotic cell death. Are fibroblasts also attacked by nanobacteria in vivo?

In rabbits, nanobacteria labeled with ^{99m}Tc and injected intravenously, showed a tissue-specific distribution with kidneys being the major-target. Surprisingly, no significant numbers of nanobacteria were found in muscle, skin, bone or-other tissues with fibroblastoid cells. Nanobacteria were found to pass through the glomerular and tubular cells and nanobacteria could be isolated from the urine [6]. This implies that nanobacteria may cause diseases in the urinary tract.

Nanobacteria produce apatite which is a mineral commonly found in kidney stones. In fact, apatite may play a key role in the formation of all kidney stones. The crystalline components of urinary tract stones arc calcium oxalate, calcium phosphate, bacterial related, purines or cystine. The majority of urinary stones arc admixtures of two or more components, with the primary admixture being calcium oxalate with apatite [15]. Furthermore, fermentor model studies have shown that calcium phosphate nidi are always initially formed and may subsequently be coated with calcium oxalate or other components. There is remarkable similar]!." in the size and morphology of nanobacterial apatite and the apatite found in human kidney stones. Nanobacteria were found in 70 out of 72 human kidney stones that were screened [16].

Immunologically similar bacteria could also be found in samples of dental pulp stone [17]. If a bacterial cause for stone formation could be proven, these diseases may lend themselves to treatment with antibiotics.

Tissue calcifications are found in several diseases such as ovarian serous tumor, papillary adenocarcinoma of the endometrium, breast carcinoma, papillary carcinoma of the thyroid, duodenal carcinoid tumor and craniopharyngioma [18]. Many malignant cells have receptors for nanobacterial adherence [2] and therefore could introduce nanobacteria into the tumor with subsequent calcification. Furthermore, some dividing cells under inflammatory stimuli may also have receptors for adherence. This may be the case in atherosclerotic plaques, which are known to accumulate calcium phosphate.

Slow bacterial infection has been suggested to play a role in autoimmune diseases [19]. Nanobacteria are a new example of slowly growing organisms that infect humans for extended periods of time. The apatite structure and anomalous nucleic acids may contribute to abnormalities in the immune response to this infection.

CONCLUSIONS

Sterile-filtration of serum and other biological materials is an enormously demanding task and further improvements are needed, especially for the removal of nanobacteria. Nanobacteria are novel, emerging pathogens widely present in blood and other biological materials. Nanobacteria injected into the circulation were shown to penetrate through kidney cells and pass into urine. They were found to cause apoptotic cell death in the kidney and they showed a strong association with kidney stones. They have been found to infect many types of cultured cells under in vitro conditions. Thus, nanobacteria may present a wide risk in sterile-filtered products.

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