

Radiolabeling and in vivo distribution of nanobacteria in rabbit

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ABSTRACT

Nanobacteria are minute bacteria recently isolated from mammalian blood. They encapsulate themselves with apatite mineral. Cultured nanobacteria were radiolabeled with ^{99m}Tc, using a method which has been previously used for labeling red blood cells with ^{99m}Tc, and in vivo distribution of nanobacteria was followed with Single Photon Emission Computed Tomography (SPECT) imaging. The labeling yield was over 30%. Two rabbits were studied using dynamic planar imaging performed in the AP-position immediately after injection. Serial SPECT scans were acquired up to 24h and one planar image was taken at 45h. A control study was performed administering a similar dose of [^{99m}Tc] labeled albumin nanocolloids. Regional nanobacteria-to-nanocolloid ratios were calculated along with time and tissues (45 h) were analyzed for radioactivity and for nanobacteria. The main finding was that radiolabeled nanobacteria remained intact and showed a tissue specific distribution with a high accumulation in the kidneys and also in urine. Spleen, stomach, heart and intestine also showed increased uptake. Excretion into urine started 10-15 min after injection. These were live nanobacteria in the urine, which had better capabilities to penetrate into cells in vitro. The nanobacteria accessed the urine via tubular cells since nanobacteria were found in their cytoplasm and tubular surfaces. The results suggest that nanobacteria utilize endocytic transport of tubular cells and may be involved in the pathogenesis of mineral formation in mammalian kidney stones.

Key words: nanobacteria, nannobacteria, radiolabeling, SPECT, biogenic apatite, ^{99m}Tc

1. INTRODUCTION

Nanobacteria are minute bacteria recently isolated from mammalian blood. They divide under cell culture conditions, even if there are no mammalian cells present, with a doubling time of 1-5 days. All commonly used microbial stains gave only weak staining results¹. Nanobacteria could be stained weakly gram negative. They belong to difficult to the stain group of bacteria but similarly can be stained well with Jones' methenamine silver. Nanobacteria could not be cultured with common bacteriological media. During culture in cell culture medium their shape and size resembled the smallest bacteria under the light microscope but they could be visualized much better, because of the optical density of hydroxyl apatite. Nanobacteria can provoke a normal immunoresponse², and specific antibodies obtained³ can be used for immunostaining of them. In long-term culture, a thick white biofilm containing apatite mineral was formed around nanobacteria. This distinguished nanobacteria from mycoplasma and other known bacterial forms present in animal sera after sterile filtration.

The structure of nanobacteria has been investigated with light and electron microscopes⁴. In the light microscope, they were seen as tiny coccoid particles, during culture they became more optically opaque and larger. In the electron microscope, the nonadherent nanobacteria were irregular-shaped, rough coccoid particles with a diameter of 80-500 nm. In older cultures, the nanobacteria were surrounded with a rough envelope showing fibrils and crystals of hydroxyl apatite.

Our in vitro work indicated, that nanobacteria are harmful and cytotoxic (see Kajander et al. in this issue). Thus their in vivo distribution and effects must be unraveled. Now we have investigated the in vivo tissue distribution of cultured nanobacteria in rabbits. The nanobacteria were initially cultured in vitro, radiolabeled with ^{99m}Tc and injected intravenously and their distribution was followed with Single Photon Emission Computed Tomography (SPECT) imaging. For verification of the vivo tissue distribution, rabbits were sacrificed and tissues were removed, counted and stained. These animals served as their own controls because their distribution of ^{99m}Tc-labeled nanocolloid particles was also analyzed.

2. MATERIALS AND METHODS

2.1 Animals and reagents

Two rabbits approximately 6 months old weighing 3.5 kg each were studied. Midazolam 1 mg/kg was injected intra- peritoneally and 40 µg fentanyl intramuscularly to anaesthetize them prior to SPECT imaging. Before removing the tissues, the rabbits were sacrificed under halotane anesthesia with an overdose of fentanyl. ^{99m}Tc labeled nanocolloid particles were obtained using a commercial kit (SOLCO, Birsfelden, Switzerland) and ^{99m}Tc-generator (MAP Medical Technologies Oy, Tikkakoski, Finland) according to the manufacturer's instructions. All other reagents were of analytical grade.

2.2 Culture of nanobacteria

Nanobacteria were cultured in RPMI-1640 medium, supplemented with L-glutamine and 10% gamma irradiated FBS (dose 3 megarads), at 37°C in an atmosphere of 5% CO₂-95% air for three weeks. After the incubation period, nanobacteria were washed and stored at -20°C until used for radiolabeling. Nanobacteria are not damaged by freezing.

2.3 Radiolabeling

For radiolabeling, 25 µl wet pellet of nanobacteria from bovine origin was incubated with 2 mg SnCl₂ x (2H₂O) in aqueous solution for 20 minutes. The pellet was separated, washed with 0.9% saline and incubated with 30 mCi of ^{99m}TcO₄ for 30 minutes. subsequently, the suspension was separated by centrifugation and the nanobacterial pellet was washed twice with 0.9% saline and suspended in a small volume of 0.9% saline. A dose of 4-8 mCi was injected into the rabbit ear vein. A control study in rabbit was performed administering a similar dose of ^{99m}Tc-labeled albumin nanocolloids. Two rabbits were studied with both tracers: radiolabeled nanobacteria and radiolabeled nanocolloids. The first animal received initially nanocolloid and 1 month later the nanobacteria. The order was reversed in the other animal.

2.4 SPECT imaging

Dynamic planar imaging (90 frames each 1 min) was performed in the AP-position immediately after injection of tracer using a gamma camera. Serial SPECT scans were acquired up to 24h and one planar image was taken at 45h. Regions of interest (ROIs) were drawn for brain, lung, heart, liver, spleen, kidney, urinary bladder and bone. Time activity curves of the ROIs were printed out. Regional nanobacteria-to-nanocolloid ratios were calculated with respect to time.

After rabbits were sacrificed 45h after injection, tissues were removed, analyzed for radioactivity using well-counter and stained for nanobacteria. Pieces of wet tissue were separated and weighed. The radioactivity of the samples was measured in a well-counter.

2.5 Nanobacterial culture from rabbit urine and immunodetection of their cell adherence with a 3T6 cell model

After nanobacterial or nanocolloid injection, rabbit urine samples were collected and sterile filtered (0.2 µm). These samples were cultured for three weeks as described above. As a control, gamma-irradiated serum was cultured in the same culture medium alone. These cultures were thoroughly mixed and 100 µl samples were taken and added to 3T6 cells in chlamydia tubes and incubated for 24 h. The primary antibody used for indirect immunostaining technique was monoclonal antibody Nb 8/0, prepared by us, against nanobacteria. Coverslips were washed with PBS and the cells were fixed in 4% formaldehyde, permeabilized with 1% Triton X-100, and stained with the monoclonal antibody and FITC-conjugated anti-mouse IgG. Mounted coverslips were viewed under a Nikon Microphot-FXA microscope with fluorescence and differential interference contrast optics.

2.6 Detection of nanobacteria from rabbit kidney

Rabbit kidneys were fixed in 4% paraformaldehyde, dehydrated, embedded in paraffin and cut using standard techniques. Deparaffinized sections were stained with standard methenamine-silver staining.

3. RESULTS

3.1 Radiolabeling

Nanobacteria could be labeled with the method used. The radiolabeling yield was 30%. The radiolabel concentration was stable in nanobacteria when stored in 0.9% saline. When stored for 24h in 0.9% saline at +4°C, 10% of label was lost in the subsequent washing steps.

3.2 Tissue distribution

Radiolabeled nanobacteria remained intact and showed a tissue specific distribution (Fig. 1) with a high accumulation to the kidneys and also in urine (nanobacteria-to-nanocolloid ratio in the kidney was 6 and in the urine 9, Fig. 2). Spleen, stomach, heart and intestine had also increased uptake of nanobacteria. Excretion into urine started 10-15 min after injection. The nanobacteria detected in urine were still viable.

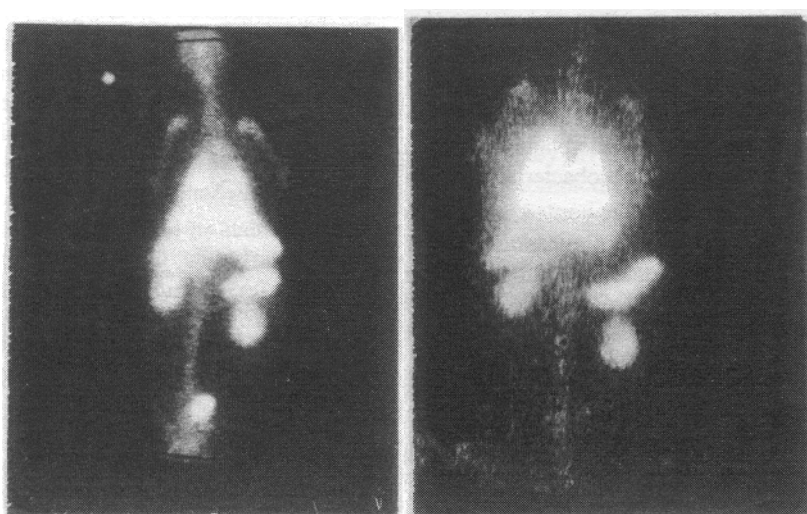


Fig.1. Planar anterior images of a rabbit 10-12 minutes after injection of [^{99m}Tc]nanobacteria (left) and [^{99m}Tc]nanocolloids (right).

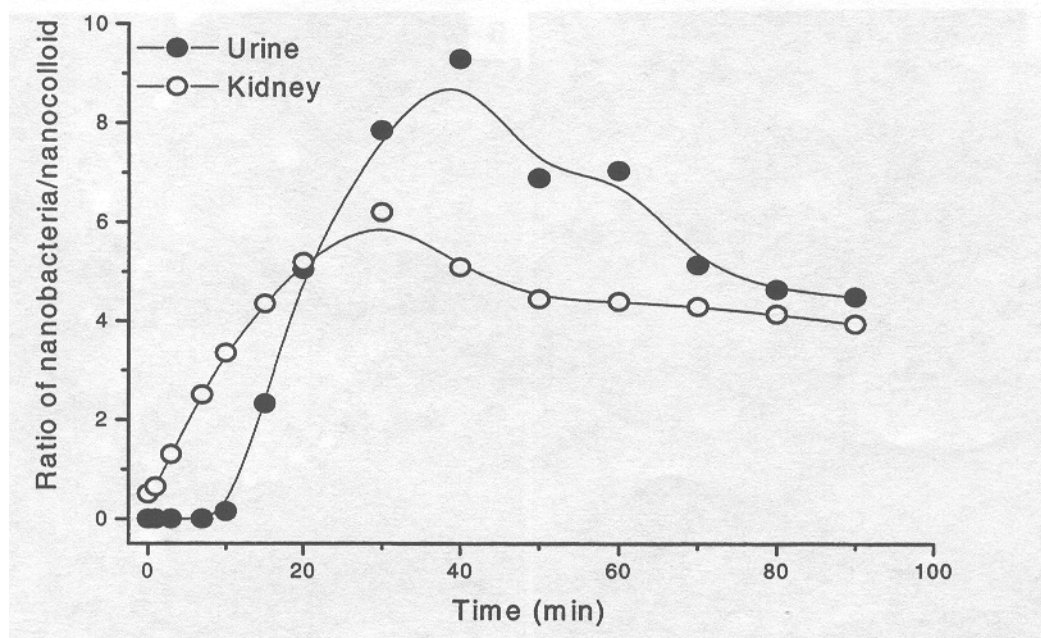


Fig.2. The ratio [^{99m}Tc] nanobacteria over [^{99m}Tc] nanocolloids in kidney and urine at 0-90 minutes after injection of the labeled nanobacteria or nanocolloids. The plasma ratio for the ^{99m}Tc labeled substances remained throughout this period at about 1.

Table.1. Percentage of injected dose per gram (%ID/g x 1000) of wet tissue in [^{99m}Tc] nanobacteria injected rabbit and nanocolloid injected rabbit. The radioactivity concentration in red blood cells, urine, kidney and spleen were higher in tissues of that rabbit which received nanobacteria. In the rabbit injected with nanocolloid, plasma, liver and bone showed higher activities.

| Tissue | Nanocolloid injected rabbit radioactivity in tissue (%ID/g x 1000) | Nanobacteria injected rabbit radioactivity in tissue (%ID/g x 1000) | Ratio of nanobacteria/ nanocolloid |
|-----------------|--|---|---------------------------------------|
| Blood | 0.133 | 0.145 | 1.09 |
| Plasma | 0.178 | 0.112 | 0.68 |
| Red blood cells | 0.011 | 0.034 | 3.09 |
| Urine | 4.03 | 4.78 | 1.18 |
| Kidney | 4.83 | 5.65 | 1.17 |
| Liver | 6.67 | 1.66 | 0.25 |
| Spleen | 17.96 | 18.46 | 1.03 |
| Bone | 0.954 | 0.176 | 0.18 |

Table 1 shows the tissue distribution ratios of radioactivity in nanobacteria and nanocolloids at 45h after injection. The radioactivity of [^{99m}Tc] nanobacteria was highest in kidney and spleen, while high radioactivity of [^{99m}Tc] nanocolloid was observed in kidney, liver and spleen. The kidney/liver ratio (per g of tissue) with [^{99m}Tc] nanobacteria was 3.4, and for [^{99m}Tc] nanocolloid it was 0.8, the spleen/liver ratios were 11.1 and 2.7, respectively.

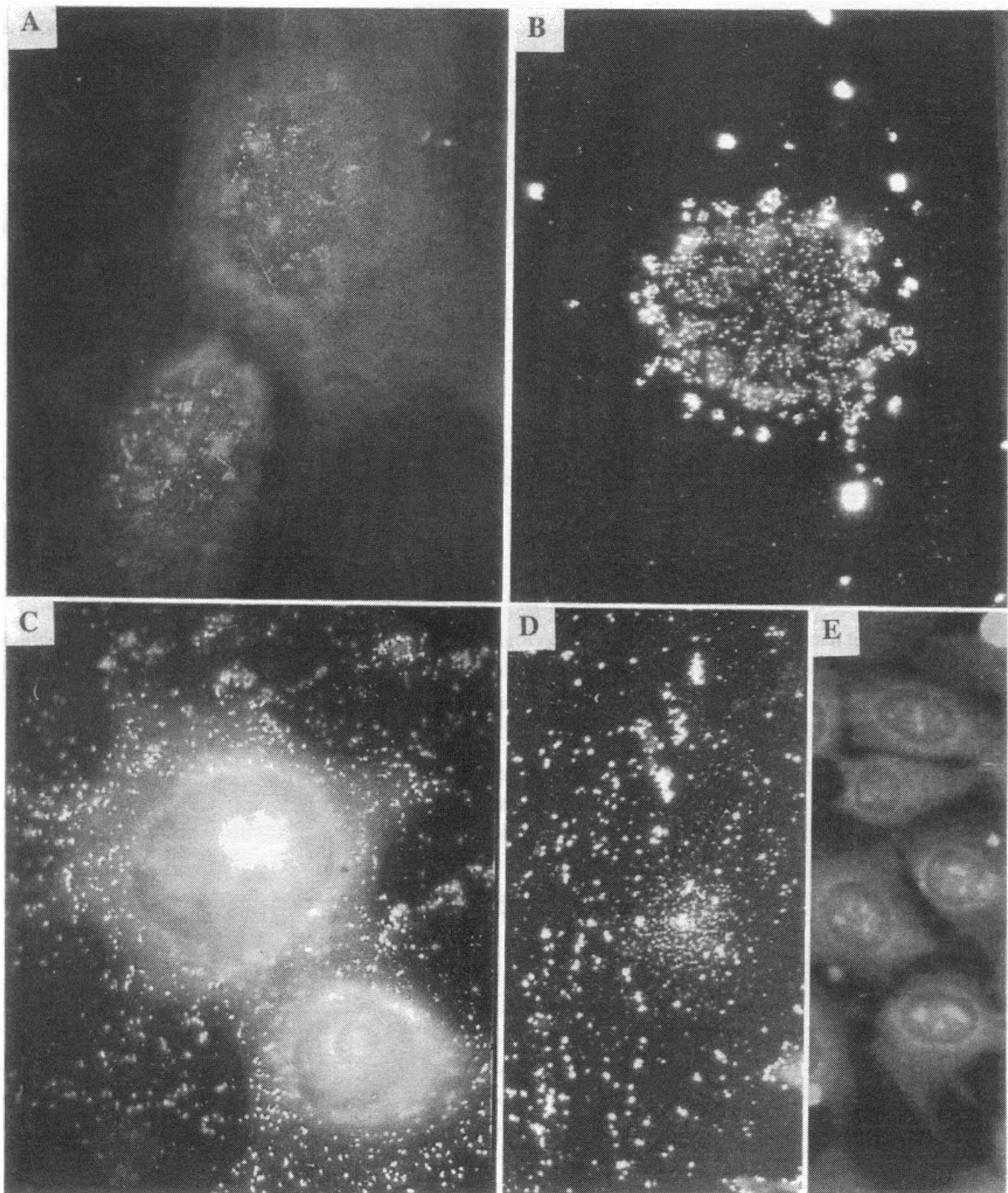


Fig.3 Immunostaining results in a 3T6 cell model with nanobacteria from the rabbit urine and, for comparison, from the original culture injected into the rabbit. A: nanobacteria isolated from urine invading the nuclei of 3T6 cells, B: their appearance in culture, C: nanobacteria from original culture adhere to 3T6 cells but do not invade effectively, D: their appearance in culture and E: 3T6 cells not exposed to nanobacteria.



Fig.4. Structure of nanobacteria in TEM and silver staining of rabbit kidney 48h after animal was injected with nanobacteria. A: thickly walled nanobacteria, bar 100 nm. Silver stained kidney section: B 190x, C 380x and D 760x. Silver is present as black dots.

3.3 Culture and immunostaining of nanobacteria

Nanobacteria could be cultured from only the urine of the nanobacteria injected rabbit (see Fig.3). The culture result was specific, no other bacteria were present as evidenced by microscopy, culture tests, and lack of cloudiness or pH change in medium. The shape and morphology were typical of nanobacteria and the organisms reacted characteristically as assessed with the special modification of Hoechst 33258 DNA staining method and in immunostaining. The nanobacteria isolated from urine of rabbit showed increased penetration into 3T6 cells in culture compared to the original culture (see Fig.3).

3.4 The detection of nanobacteria from rabbit kidney and penetration into urine

Fig.4 shows silver staining of rabbit kidney. Small coccoid particles are clearly seen in the cytoplasm of tubular cells and on the tubular surface. We have shown that nanobacteria stain strongly positive with silver. No positive signs were seen in control kidneys not exposed to nanobacteria. It seems likely that the thick cell envelope of nanobacteria shown in Fig. 4A stains strongly with silver but is impermeable to standard staining techniques with common stains.

4. DISCUSSION

Nanobacteria could be labeled with ^{99m}Tc , adopting a method previously used to label living cells like red blood cells⁵. This suggests that pertechnetate ($^{99m}\text{TcO}_4$) goes through nanobacterial membrane and is retained inside the cell. The main finding was that the tissue distribution of nanobacteria differs clearly from that of nanocolloid. The main difference is that the highest accumulation of nanobacteria was seen in spleen and kidneys, while nanocolloid accumulated in liver and spleen. Accumulation in spleen in both cases may be secondary to accumulation in erythrocytes. The high accumulation in kidneys and to subsequently urine is a most surprising finding since nanobacteria are generally over 200 nm in size. Thus, they cannot be ultrafiltrated into urine via glomeruli unless the glomeruli are damaged. The intense efflux to urine may be due to active transport from tubular cells. This is supported by direct microscopic evidence of the presence of nanobacteria inside the cells. To our knowledge, there are no previously known bacteria have such a strong preference for kidney cells and transport to urine.

About 30% of dose of radiolabeled nanobacteria was incorporated to lungs only in one of the studied animals, which may be due to aggregation of particles or erythrocyte aggregation with nanobacteria. This may have some confusing effect to the tissue distribution, but it cannot be considered to have any major effect on the subsequent metabolism and distribution.

In conclusion, nanobacteria can be radiolabeled with ^{99m}Tc . In rabbits SPECT detection revealed that [^{99m}Tc] nanobacteria showed in vivo a specific distribution, compared to that of ^{99m}Tc -nanocolloids. The results suggest that nanobacteria utilize the endocytic transport of tubular cells and might have a role in the pathogenesis of mineral formation also in mammals, e.g. in the creation of kidney stones.

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