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Interaction of nanobacteria with cultured mammalian cells

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Abstract

Nanobacteria were recently isolated from human blood and commercial fetal bovine serum (FBS) and were located in the α-2 subgroup of proteobacteria based upon their 16S rRNA gene sequence. They can be cultured even in the absence of mammalian cells, and have extraordinary properties, like very slow growth rate and an impermeable cell wall, making their detection difficult by standard microbiological techniques. Since they are present in FBS, and thus in cell cultures, it is essential to clarify their effects on cultured mammalian cells. In this study, we show that four out of six nanobacterial isolates from different sera exerted a cytotoxic effect on 3T6 fibroblasts verified by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] viability assay, lactate dehygrogenase (LDH) release and by direct microscopy. The cytotoxic effect of nanobacteria was attenuated after they had been subcultured several times. The cytotoxic effect was similar with all tested murine and human fibroblastoid cell lines. Differential interference contrast and electron microscopy, and FITC staining with specific monoclonal antibodies indicated selective, possibly receptor-mediated adherence, followed by internalization and cytotoxicity in the 3T6 fibroblasts used as a model in these interaction studies. Thus, nanobacteria have a special way of invading mammalian cells: they trigger cells that are not normally phagocytic to engulf them. These organisms seem to be an important cause for cell vacuolization, poor thriving and unexpected cell lysis, problems frequently encountered in mammalian cell culture. © 1998 Elsevier Science B.V.

Keywords: Nanobacteria; Adhesion; Serum; Cytotoxicity; Cell culture; MTT; LDH

1. Introduction

Cell culture contaminants are a serious problem, since they can undermine experiments. Fungi, yeasts, bacteria and their L-forms, and mycoplasmas easily invade cultures, because cultured cells do not possess immunological defence mechanisms. Fastidious organisms growing slowly without producing gross changes in the culture medium are a serious problem, because they are not easily detected [1]. The source of contamination can be external, e.g. air, worker, or internal, e.g. cells, medium or its supplements. Serum, commonly used as a supplement in cell cultures, often causes problems such as poor growth and/or cytotoxicity for reasons which are not fully understood. Serum is a biological product sterilised by using filtration procedures. Ultrafilterable organisms, e.g. viruses, cannot be eliminated using this method. We have found a novel ultrafilterable bacterium in blood and serum. The new organism was named as *Nanobacterium sanguineum* referring to its small size and blood habitat [2]. These bacteria are catalase negative, coccoidal microorganisms, 0.08–0.5 μm in size. Nanobacteria are very thickly capsulated and covered with hydroxyl apatite making them difficult to fix, stain and break, and resistant to antibiotics used in cell culture. Nanobacteria replicate with a doubling time of 1–5 days in cell culture media, and appear either on their own or in small and big clusters in a biofilm [3–5]. In addition to FBS, they have been detected from blood and blood products of some horses and from commercial human blood products pooled from Finnish blood donors [6]. Nanobacteria cannot be detected with standard sterility

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testing methods, since they are not growing in commonly used microbiological media, and standard DNA staining methods do not show them. For that reason, we had to develop a new culture, DNA staining and immunoassay methods for their detection [6].

Nanobacteria have been reposited in the German Collection of Microorganisms (DSM No: 5819–5821). Their phylogenetic position has been located in the α-2 subgroup of proteobacteria based upon their 16S rRNA gene sequences [EMBL Entries X98418 and X98419]. The α-2 subgroup of proteobacteria contains bacteria which are able to penetrate into eukaryotic cells. Bartonella and Brucella, close relatives of nanobacteria, are known mammalian pathogens that invade cells, even fetuses, causing cytopathic effects. The aim of the present study was therefore to investigate the interaction of nanobacteria with cultured mammalian cells.

2. Materials and methods

2.1. Reagents and cell culture

All culture media and supplements were from Gibco (Paisley, Scotland) and cell culture vials from Nunc (Roskilde, Denmark). Chlamydia test tubes (129AX/1) were from Bibby Sterilin (Staffordshire, UK). Hoechst 33258 and MTT stains, and Triton X-100 were obtained from Sigma (St. Louis, MO). LDH cytotoxicity detection kit was from Boehringer Mannheim (Mannheim, Germany). 3T6 cells (ATCC CCL 96) were cultured on coverslips in chlamydia test tubes in 0.5 ml RPMI-1640 medium supplemented with 2 mM L-glutamine and 10% FBS (Gibco, batch No. 30F0484F) in a humidified atmosphere of 5% CO_2–95% air. Each culture was initiated with approximately 5000 cells and was incubated at 37°C for 24 h before the tests were started. Cell culture in 96-well plates was carried out similarly but the medium contained penicillin (100 U/ml) and streptomycin (100 μg/ml). Sterility of cell culture was tested by Hoechst 33258 staining (DNA stainings were carried out with the Hoechst fluorochrome following the instructions given in Hoechst Stain Kit, Flow Laboratories, Ayshire, Scotland), with Giemsa stain and by culture in blood agar and in improved mycoplasma media [7]. Mycoplasma-free cells were used in this study. Methylcholanthrene-transformed mouse fibroblasts (B_6MCA), BHK, CHO, and human fibroblasts were obtained and cultured in MEM medium as described previously [8,9].

2.2. Nanobacterial culture

Nanobacteria were cultured in RPMI-1640 medium supplemented with L-glutamine and 10% FBS as a source of nanobacteria and their supplement, at 37°C in an atmosphere of 5% CO_2–95% air typically for 3–4 weeks. In light, immunofluorescence, and electron microscopic observations the most cytotoxic nanobacterial isolate from Sera Lab, lot 901045 (Sussex, UK) was used. In screening of cytotoxic effects of various nanobacterial isolates, the indicated sera, or their subcultures, were used as the source of nanobacterial culture. These were FBS batches Sera Lab 901045 and 301110, Biological Industries (Kibbutz Beth Haemek, Israel) 085011, Integro (Zaandam, The Netherlands) 5-40902, Atlanta Biologicals (Norcross, GA) 5001D, and human nanobacteria subcultured for 12 months from serum of a 29 year old Finnish male found positive for presence of nanobacteria. Nanobacteria after passage into rabbit were from Seralab 901045 culture injected intravenously, and thereafter they were recovered by culture from urine obtained by bladder puncture and filtered through 0.2 μm pores. Subculturing of nanobacteria was carried out by using γ-irradiated serum as the supplement (dose 30 kiloGrays, ^{60}Co), except for serum-free subculture which was carried out without any supplement. γ-irradiated serum, at this dose, has been proven to be sterile and can support the growth of nanobacteria [4]. After the culture periods, purity of the cultures was controlled by microscopy, Hoechst staining, and culture tests as described above. The cultures were thoroughly mixed, and samples were taken to infect fibroblasts. In indicated experiments, nanobacteria were first harvested by centrifugation for 30 min at 14 000 $\times$ g, washed in PBS (116 mM NaCl, 20.8 mM Na_2HPO_4, 2.9 mM KH_2PO_4, pH 7.4) and resuspended by vigorous mixing in fresh medium before being applied to infection tests.

2.3. Screening of cytotoxic effect of various nanobacterial isolates

The cultured nanobacteria were harvested and resuspended as described above into a density of McFarland 0.5 standard unit. Twenty-five millilitre aliquots were added to 3T6 cell cultures (4000 cells/well in 100 μl volume) in 96-well plates. After 48 and 72 h, the medium was aspirated out and each well washed with 200 μl of sterile PBS. Then 50 μl of MTT solution (0.8 mg/ml) in medium without serum was added into each well and incubated for 4 h. After that, the medium was removed and 150 μl dimethyl sulfoxide was added to the wells to dissolve the formed formazan crystals. Absorbances were recorded at 550 nm. Eight parallel tests were done for each experiment. For LDH release tests, a parallel plate was used: medium was first removed at 48 or 72 h and replaced by fresh medium supplemented with 1% FBS and the plate incubated for 16 h. The medium was used in LDH assay according to the instructions of the kit. The results were calculated

Table 1
Effect of various nanobacterial isolates on the growth and survival of 3T6 cells in 48 and 72 h (MTT viability-measurement test results)

Added nanobacteria[a]	% Of control ± SD 48 h	% Of control ± SD 72 h	Significance 48 h	72 h
No addition (control)	100.0 ± 16.4	100.0 ± 9.9		
Nanobacteria 1	35.0 ± 4.7	27.5 ± 4.6	$P<0.001$	$P<0.001$
Nanobacteria 2	65.0 ± 13.5	59.9 ± 5.4	$P<0.001$	$P<0.001$
Nanobacteria 3	112.8 ± 16.9	103.4 ± 8.4	N.S.	N.S.
Nanobacteria 4	123.2 ± 18.6	88.4 ± 5.9	N.S.	$P<0.05$
Nanobacteria 5	89.4 ± 8.6	87.0 ± 9.8	N.S.	$P<0.05$
Nanobacteria 6	104.7 ± 21.3	103.9 ± 8.5	N.S.	N.S.
Nanobacteria 7	81.4 ± 9.3	80.8 ± 9.2	$P<0.05$	$P<0.01$
Nanobacteria 8	92.1 ± 13.0	79.5 ± 7.4	N.S.	$P<0.001$
Nanobacteria 9	91.1 ± 24.6	98.2 ± 11.8	N.S.	N.S.

N.S. = $P>0.05$.
[a]Origin of the nanobacterial isolates: nanobacteria 1, Sera Lab 901045; nanobacteria 2, Biological Industries 085011; nanobacteria 3, Sera Lab 901045 (long term subculture); nanobacteria 4, Sera Lab 901045 (culture in serum-free condition); nanobacteria 5, Integro 540902; nanobacteria 6, Atlanta Biologicals 5001 D; nanobacteria 7, Sera Lab 301110; nanobacteria 8, Sera Lab 901045 (after passage into rabbit, isolation from urine and subculture); nanobacteria 9, an isolate from human serum.

as percentage of the control and statistical significances compared with unpaired Student's t-test. Differences were considered significant at $P < 0.05$ level.

2.4. Nanobacterial infection of fibroblasts

Samples of 100 μl from nanobacterial cultures were added to 3T6 cells in chlamydia tubes. This volume contained about 0.01 μl wet pellet (approximately one million particles with microscopic counting, about 0.03 McFarland units). Only RPMI medium was added to control experiments. A timetable for the interaction was designed with incubation times of 0.08, 0.17, 0.5, 1, 2, 4, 8, 24, 48 and 72 h for both controls and infected samples. For each incubation time, at least two tubes were used, and the tests were repeated six times. The other cell lines were similarly inoculated with nanobacteria and incubated for 24 h. For testing possible cytotoxicity of nanobacteria on various fibroblastoid cell lines, fibroblast cultures were infected with 2–1000 nl wet pellet/ml culture volume. Only the medium was added to the control cells. After incubation for 3 days, micrographs were taken from representative areas. In some experiments, γ-irradiated nanobacteria (dose 30 kiloGrays, ^{60}Co), were used for infecting the fibroblasts. Heat inactivated FBS was used in some infection tests for determining the role of the complement system in the cytotoxicity event. The consumption of the medium components was followed by standard amino acid and glucose analyses.

2.5. Immunological techniques

The primary antibodies used for indirect-FITC staining technique were monoclonal antibodies Nb 5/2 and Nb 8/0 produced against nanobacteria by us. The antibody producing hybridoma clones were obtained from mouse splenocytes immunized with cultured nanobacteria of bovine origin, fused with myeloma strain P3X63-Ag8.653 (ATCC CRL 1580). The hybridomas were cultured in the above-mentioned cell culture conditions or in the peritoneal cavity of Balb/c mice as ascites. Either cell culture supernatants (undiluted) or mouse ascites (diluted 1/5000–1/80 000) were used. While evaluating the antibody amount necessary for positive staining, also purified monoclonal antibodies (Prosep-A column chromatography, Bioprocessing, Durham, Consett, UK) were used. The secondary antibody was FITC-conjugated rabbit anti-mouse IgG (Dakopatts, Glostrup, Denmark) at a dilution of 1/50 in PBS containing 10% FBS.

In indirect-FITC staining, coverslips were washed twice with PBS, and the cells were fixed in 4% formaldehyde in PBS for 7 min at room temperature. After washing twice with PBS, the cells were permeabilized with 1% Triton X-100 in PBS for 3 min. The coverslips were washed twice with PBS and blocked with 10% FBS (Gibco) in PBS for 10 min, and then covered with 70 μl of monoclonal antibody solution. After a 30 min incubation at room temperature, antibody solution was drained off and the coverslips were washed twice (5 min each) with PBS and covered with 50 μl of FITC-conjugated anti-mouse IgG. Both primary and secondary antibodies were centrifuged at 14 000 × g for 2 min before application. After incubation for 30 min at room temperature, the antiserum was drained off, the coverslips were washed three times (3 min each) in PBS, mounted with a medium containing 50% glycerin and 0.5% n-propyl gallate in PBS, and viewed under a Nikon Microphot-FXA microscope with fluorescence and differential interference contrast optics.

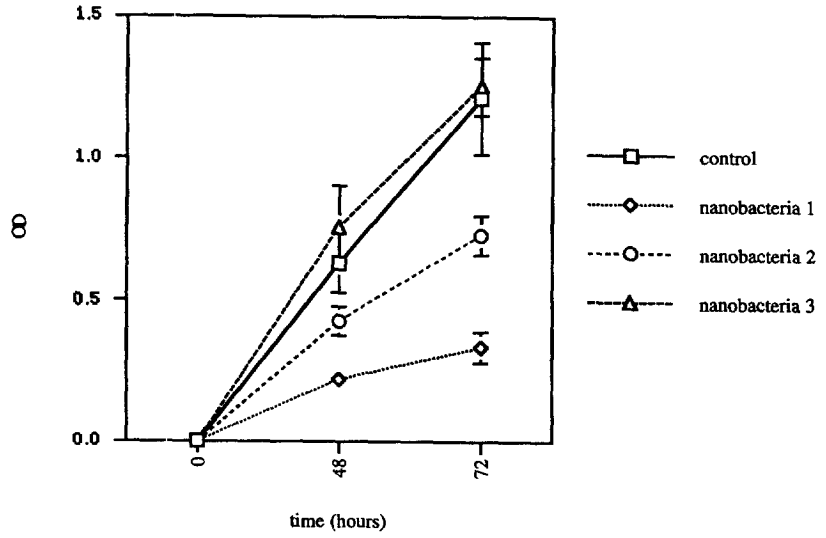

Fig. 1. Effect of nanobacteria on the growth of 3T6 cells as measured with MTT test. The nanobacterial isolates and assay conditions have been described in Table 1. Each value is the mean of optical densities of formazan dye measured from eight experiments ± SD.

2.6. Electron microscopy

For scanning electron microscopy, 3T6 fibroblasts in a logarithmic growth phase were allowed to attach to coverslips in four-well plates followed by inoculation with nanobacteria. Approximately 1 μl wet pellet was added per ml 3T6 culture and incubated for 2.5 h. Coverslips were washed with PBS and fixed with 2% glutaraldehyde in PBS for 16 h. Fixed cultures were washed with PBS, dehydrated with increasing ethanol concentration, and dried with a critical point dryer. The coverslips were coated with a 20–40 nm gold layer before being examined with JEOL JSM-35. For transmission electron microscopy, horizontal section preparations were made in situ during growth in culture [10]. For this, cultures of BHK cells (5000 cells/cm^2) were inoculated with about one million nanobacteria/ml and cultured for 2 days. Thereafter, the medium was removed and the cells were fixed with 3% glutaraldehyde as described above followed by incubation with 1% OsO_4 for 1 h, stained in uranyl acetate, dehydrated through graded ethanol, and embedded in Epon 812 resin. Thin sections were cut parallel to the substratum, stained with uranyl acetate and lead citrate, and examined with Jeol 1200 EX electron microscope at 80 kV.

3. Results

3.1. Screening of cytotoxic effect of various nanobacterial isolates

Possible cytotoxic effect of nanobacteria isolated from FBS, human serum and rabbit urine was followed by MTT test which measures cell viability. MTT can be reduced in mitochondria of living cells to purple formazan crystals. Control cells multiplied logarithmically during the test period whereas cells affected with nanobacteria demonstrated non-logarithmic growth. Nanobacteria from Sera Lab 901045 and from Biological Industries 085011 reduced significantly ($P < 0.001$) the number of living cells. The effect increased with longer exposure times to nanobacteria. Four out of five primary isolates exerted significant reductions in the amount of the living cells after 72 h with the used nanobacterial dose, standardized (see Section 2) by McFarland units (Table 1). Interestingly, subcultures from the isolate Sera Lab 901045 (including its rabbit passage) in media supplemented with γ-irradiated serum or without the supplement showed a reduced cytotoxic effect on the cells (Fig. 1). The human isolate subcultured with γ-irradiated supplement did not affect the cells.

LDH tests were carried out at 48 and 72 h for evaluating the lysis of the cells at these time points. The culture medium was assayed using the reaction of the Cytotoxicity Detection Kit. At 48 h, all cultures produced approximately the same results as untreated controls. At 72 h, nanobacteria 1–9 (see Table 1) infected cells gave the following results: 0.454 ± 0.009, 0.665 ± 0.128, 1.353 ± 0.123, 1.324 ± 0.067, 1.259 ± 0.077, 1.225 ± 0.087, 1.192 ± 0.094, 1.167 ± 0.133, 1.420 ± 0.052 (optical densities of formazan dye formed), respectively. The untreated control gave 1.191 ± 0.154. Obviously the major cell death time-point (with the dose used) was between 48 and 72 h. The reduced LDH levels observed at 72 h were due to the reduced number of living cells present (after effects of nanobacteria), also observed with microscopy and the MTT assay, see Fig. 1.

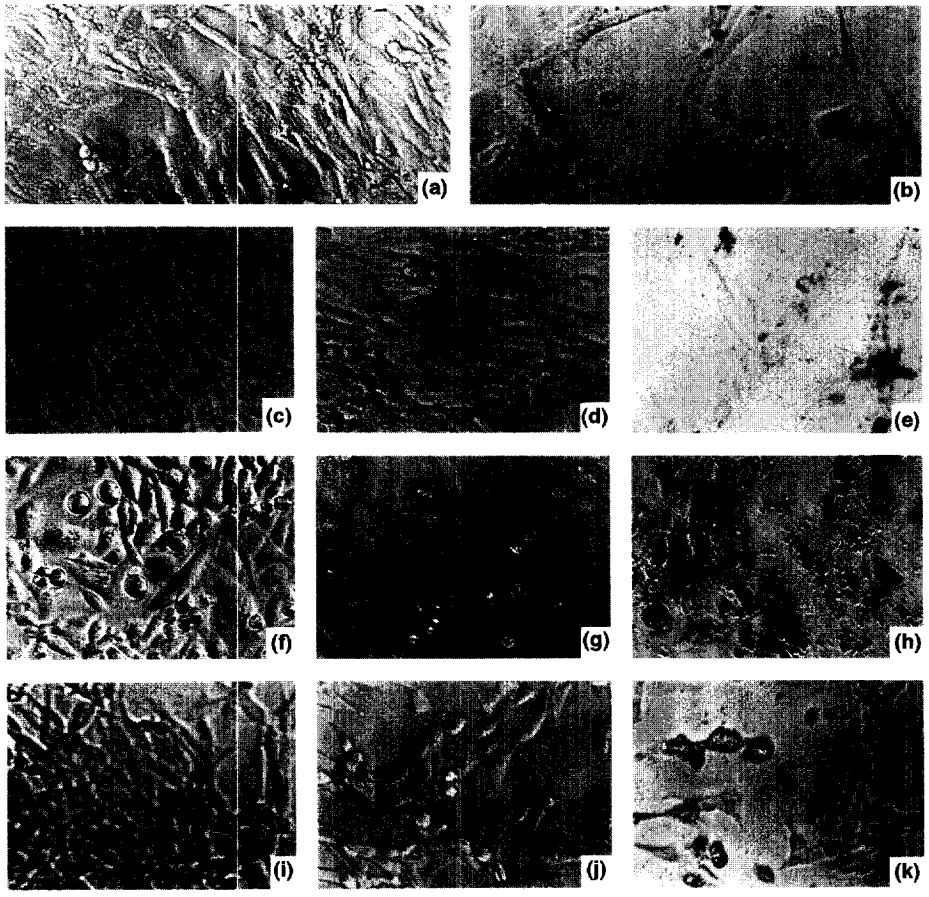

Fig. 2. Cytotoxicity of added nanobacteria (per ml culture volume) towards fibroblasts. (a) Mouse transformed embryonal fibroblast B_6MCA, control; (b) B_6MC + 8 nl wet pellet of nanobacteria; (c) BHK control; (d) BHK + 8 nl; (e) + 16 nl wet pellet of nanobacteria; (f) CHO control; (g) CHO + 8 nl; (h) + 32 nl wet pellet of nanobacteria; (i) 3T6 control; (j) 3T6 + 8 nl; (k) + 16 nl wet pellet of nanobacteria. After incubation for 3 days, the micrographs were taken from representative areas. The pictures show dramatically decreased cell amounts and deteriorated morphology of fibroblasts infected with nanobacteria. Noticable vacuolization can be seen especially in (g).

3.2. Screening of cytotoxic effect of nanobacteria towards fibroblastoid cells

Cytotoxic effect of nanobacteria on various fibroblastoid cell lines was screened by infecting the cells with the most cytotoxic isolate of nanobacteria (nanobacteria 1, Table 1). Phase contrast microscopic observations showed that nanobacteria were cytotoxic towards growing human and murine fibroblasts, starting at a dose of 1–8 nl wet pellet added per milliliter culture medium (Fig. 2). There was only a slight variation in susceptibility, B_6MCA fibroblasts being the most (Fig. 2b) and CHO cells the least sensitive lines (Fig. 2g). Doses of 16 nl wet pellet added per milliliter culture medium caused profound, and doses over 30 nl/ml massive, cytotoxity for all fibroblasts (human primary and murine transformed cell lines). Depending on the dosages used, cytotoxicity took place 1–3 days after addition of nanobacteria to the cell culture, but if nanobacteria were added at 1000 nl/ml culture medium, all cells were lysed after 2 h. Thus cytotoxicity depended on nanobacterial concentration and exposure time.

The cause of the observed cytotoxicity was briefly evaluated by measuring amino acid and glucose concentrations from the culture media surrounding the cells. No gross imbalances in these were found using the doses of nanobacteria described. Similar adherence and cytotoxicty were obtained when γ-irradiated nanobacteria were used for the infection of the fibroblasts. Heat inactivation of the serum used for cell culture did not affect the process. The presence or absence of penicillin–streptomycin in the medium did not influence the cytotoxicity results.

3.3. Immunostaining of nanobacteria

Two monoclonal antibodies with different binding sites (no competition for binding to nanobacteria) were obtained against nanobacteria isolated from FBS. Monoclonal antibody Nb 8/0 detected a 30-kDa protein band from nanobacteria but no bands from serum. Monoclonal antibody Nb 5/2 showed no immunoreactivity in Western blotting (data not shown).

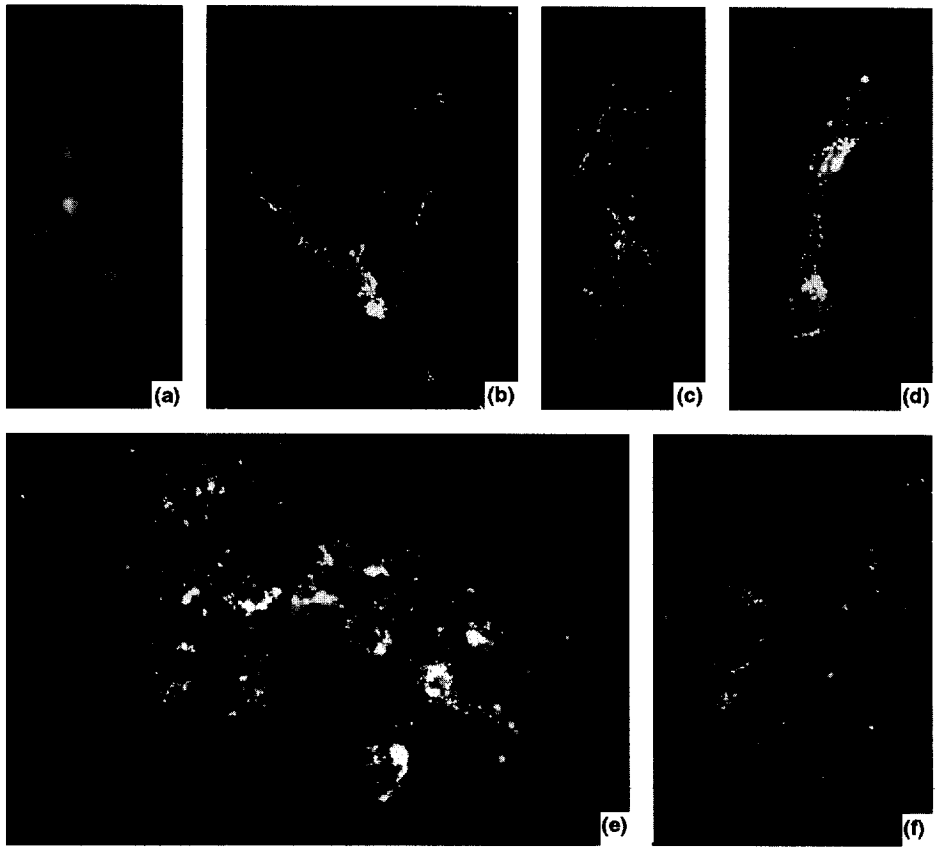

Fig. 3. Immunostaining of mammalian fibroblasts infected with nanobacteria (b–f) and the control (a); Cultures of logarithmically growing human fibroblasts (b); B_6MCA (c); BHK (d); 3T6 cells (e); and CHO (f) were inoculated with cultured nanobacteria 1 day before indirect immunostaining was carried out. For all cell lines, uninfected controls were made, and these remained negative as shown in (a) for the human primary fibroblasts. Nanobacteria can be seen as bright small dots, mostly attached onto cell surfaces.

In two-site ELISA utilizing Nb 5/2 for capture and peroxidase labelled Nb 8/0 for detection (Kuronen et al., submitted), all cultured nanobacteria gave a positive result. Uncultured sera (resulting in positive culture results) gave lower positive signals. Thus the antibodies were specific for nanobacteria in ELISA and Western blotting. In indirect immunofluorescence staining, both antibodies were specifically bound to the surface of cultured nanobacteria. The antibodies could be used to detect nanobacteria attached on various fibroblastoid cells as demonstrated in Fig. 3. If either the primary or the secondary antibody was omitted, no fluorescence could be seen. Results shown in Fig. 3 indicate that nanobacteria attached on murine and human fibroblasts.

As shown in Fig. 4a, the control 3T6 cells were negative with a minimal background. It was difficult to visualize the control cells in the stainings, e.g. the control cells depicted were photographed using an exposure time of 12 min, whereas the positively stained cells could be photographed with as short as 5 s exposure (Fig. 4g). Ascites antibody preparations were found to be devoid of nanobacteria. Using purified antibody from ascites, the nanobacteria infected 3T6 cells could be stained similarly when compared with the culture supernatant antibody. In this experiment, Nb 8/0 was used at 0.2 μg/ml. A positive reaction could still be obtained using antibody at 0.004 μg/ml. Thus, non-specific binding was not found. In further experiments, Nb 8/0 hybridoma culture supernatant was used as the antibody.

In previous experiments, 1-month cultured nanobacteria in their own medium were added into 3T6 cell cultures. As a control, we also tested the effect of harvested and PBS-washed nanobacteria on 3T6 cells. There was no difference in binding to the cell surface or in the appearance of cytotoxicity. In further experiments, we used cultured nanobacteria in their own medium.

3.4. Non-random adherence of nanobacteria to 3T6 cells

The nanobacterial interaction with the fibroblasts requires that the added nanobacteria are free, and not aggregated, in order to be bound to the cell surface and

Fig. 4. Follow-up of adherence and internalization of nanobacteria to 3T6 cells with indirect FITC staining. (a) Control cells without infection at the start of the experiment. This image showing no stained nanobacteria did not change in the control preparations at other time points. (b–h) Typical images of infected 3T6 cells after the following time points: (b) 10 min, (c) 15 min, (d) 30 min, (e) 4 h, (f) 24 h, (g) 48 h; and (h) 72 h. Arrowheads show nanobacteria adhered to 3T6 cell surfaces. Arrows show nanobacteria apparently inside the cells. Bar, 10 μm.

internalized. Therefore, we used a relatively short culture time to obtain nanobacteria and vigorously mixed them before the tests. Under the microscope, the nanobacterial suspensions were free of aggregates. When the suspension was added into the 3T6 cell culture, the nanobacteria attached to cell surfaces (Fig. 5b). The nanobacteria appeared to adhere to a limited surface area and formed clusters associated with many cells (Fig. 5b–f). The clusters were located preferentially either in the perinuclear area (Fig. 5c,d) or on cellular extensions (Fig. 5e,f). The attachment of nanobacteria to the cell extensions could be verified with scanning electron microscopy. In the control cells, no structures similar to nanobacteria could be seen (Fig. 5a). Microscopy revealed that nanobacteria were attached to most but not all cells as revealed by Fig. 6. In the same preparation, some cells were heavily infected with nanobacteria, but even adjacent cells could be almost devoid of them.

3.5. Timetable of adherence and internalization of nanobacteria by 3T6 cells

After the addition of nanobacteria, 3T6 cells were studied periodically with microscopy and with immunostaining. Infected 3T6 cells were covered with nanobacteria and were also more vacuolized. A typical appearance of the 3T6 cells is shown in Fig. 7 after an 18 h incubation with nanobacteria (b) or with sterile medium alone (a).

With the immunostaining technique, adherence of nanobacteria to 3T6 cells was seen within 15 min after their addition (Fig. 4c). At 0–10 min, only very few or no nanobacteria were associated with the cells (Fig. 4b). Thereafter, the number of attached nanobacteria per cell increased as shown in pictures of later time points (Fig. 4c–g). During the first hour, nanobacteria could only be seen on the surfaces of the 3T6 cells (Fig. 4, arrowheads). Thereafter, apparently intracellular nanobacteria could be seen in immunostainings (Fig. 4,

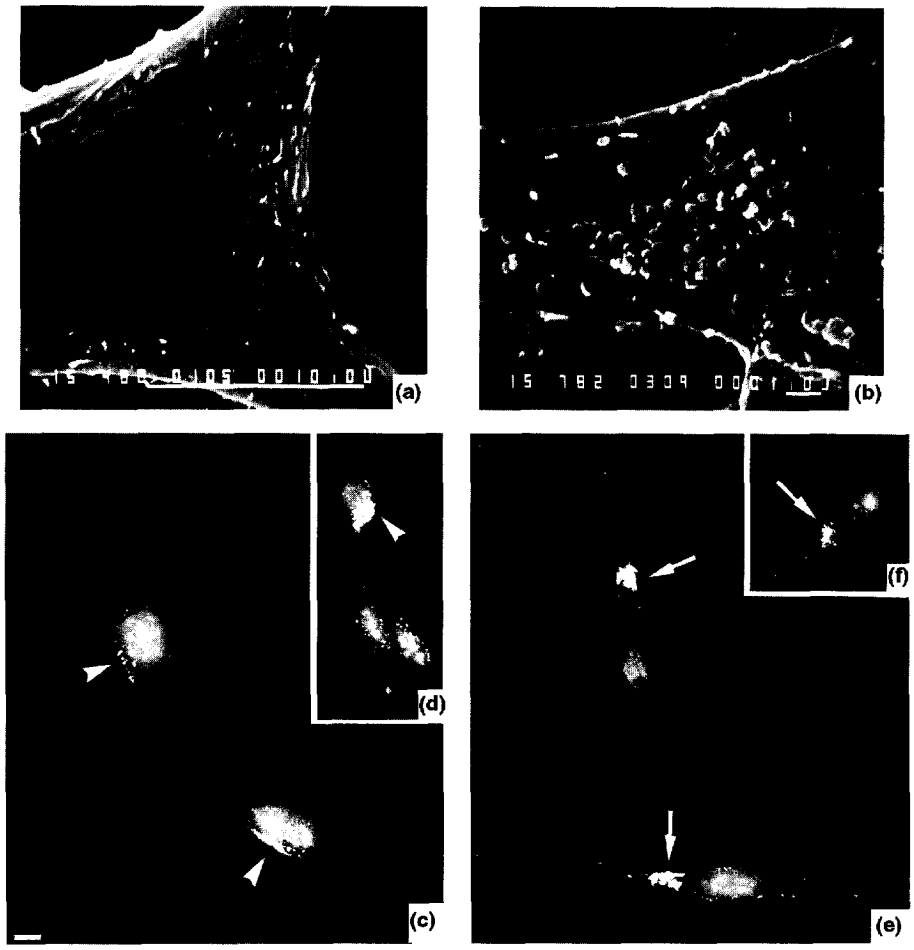

Fig. 5. Localization of nanobacteria as clusters on patchy areas on the surface of 3T6 cells. (a) Scanning electron microscopy of a control 3T6 cell; (b) a 3T6 cell infected with nanobacteria, (c–f) indirect FITC staining of 3T6 cells 24 h after the inoculation with nanobacteria. Nanobacteria in the perinuclear area of the 3T6 cells are shown in (c) and (d) (arrow heads). Arrows in (e) and (f) show attached nanobacteria as clusters on cellular extensions. Bars (a,c) 10 μm; (b) 1 μm. The scale in panel c applies also for (d–f).

arrows). Their intracellular location was evident, when the cells were viewed in different focus planes. The positively stained intracellular nanobacteria usually appeared larger than free extracellular nanobacteria and may have been in vacuoles. Subsequently, the amounts of attached and internalized nanobacteria increased, but cell morphology did not change much (Fig. 4e–g) until cell death, which took place within 72 h (Fig. 4h). Cells in control cultures reached confluency and were viable at 72 h. At this time point, there were very few unlysed cells remaining in infected cultures, and these were heavily immunostained. Nanobacteria started to attach also to the coverslips after a 1 day incubation and were seen as positively immunostained dots outside the cell area (Fig. 4f,g).

3.6. Proof for internalization of nanobacteria

Nanobacteria appeared to be in intracellular vacuoles, possibly endosomes and lysosomes. They appeared especially in the perinuclear area in immunostainings. Even without staining, such 3T6 cells could often be seen full of vacuoles as shown in Fig. 8a. Immunostaining of BHK cells gave quite similar results to 3T6 cell experiments (Fig. 3c,d). Infected BHK cells were full of vacuoles that were often filled with particles showing the typical ultrastructure of nanobacteria as previously detected [3,4,11]. In Fig. 8b, a thickly capsulated nanobacterium between two BHK cells seems to be in the process of being engulfed by the upper cell. Fig. 8c shows on the left a nanobacterium in an intracytoplasmic vacuole delimited by a single membrane, resembling an endosome. On the right, another nanobacterium can be seen that has changed its stainability, possibly because of being in a lysosome. Fig. 8d shows several vesicles with nanobacteria in various degrees of degradation. Especially noticeable is the large fusion vesicle containing two nanobacteria and a remnant of a capsule. The infected cells showed an apoptotic appearance with abnormal nucleus and gross vacuolization (Fig. 8e).

Fig. 6. Selective adherence of nanobacteria to 3T6 cells. Arrows show cells heavily infected with nanobacteria. Arrowheads show cells associated with very few nanobacteria. Bar, 10 μm.

4. Discussion

Many cell culturers have been faced with the problem of vacuolized cells and poorly thriving cultures but have been unable to detect any known contaminant by standard methods. We have shown one possible answer to this puzzle: nanobacteria can cause cellular vacuolization and lysis. Several nanobacterial isolates could reduce the numbers of living cells in the cultures of many fibroblast cell lines.

The cytotoxic effect of nanobacteria was attenuated after many subcultures in vitro. Subculturing was carried out without mammalian cells. During subculturing in vitro, there was no selection pressure for virulence. Thus, it was essential to use as short culture times as possible for obtaining nanobacteria for cell interaction studies.

Adherence of nanobacteria to 3T6 and other fibroblasts took place relatively rapidly, but cytotoxicity occurred later. In between, the cells were covered with nanobacteria clumps (Fig. 7) and became vacuolated (Fig. 8). This suggests that nanobacteria were bound and internalized by the cells. Fibroblasts were chosen as experimental models, because they are the most ubiquitous cells in the animal body and might be most accessible in wound tissue for invading pathogens with the exception of phagocytic leucocytes [12]. Numerous investigators have alluded to the phagocytic potential of fibroblasts [13–16]. Fibroblasts, although they are non-phagocytic cells, can internalize bacteria covered with invasin [17] and latex particles covered with fibronectin [18]. The internalization of microorganisms has been given many names, such as microfilament-dependent phagocytic process [19], phagocytosis [20], endocytosis [21], receptor mediated endocytosis [22], penetration [23], or engulfment [24]. The pathway of receptor-mediated endocytosis first proceeds through the binding of extracellular ligands to specific cell surface receptors followed by internalization [25]. We have shown that nanobacteria were bound as clusters on the cell surfaces. Adherence took place on cellular extensions and to the perinuclear area of the cells. Receptor-mediated adherence is a rapid process occurring within minutes [26]. We found that nanobacteria adhered to 3T6 cells within 15 min. Ligands internalized by receptor-mediated endocytosis are first routed into peripheral endosomes and appear in perinuclear endosomes some time later [27]. Our immunostaining results are in agreement with this. Electron microscopy proved that nanobacteria did indeed enter the cells (Fig. 8b–e). It is concluded that nanobacteria are internalized either by receptor-mediated endocytosis or by a closely related pathway. During the first hours of infection, nanobac-

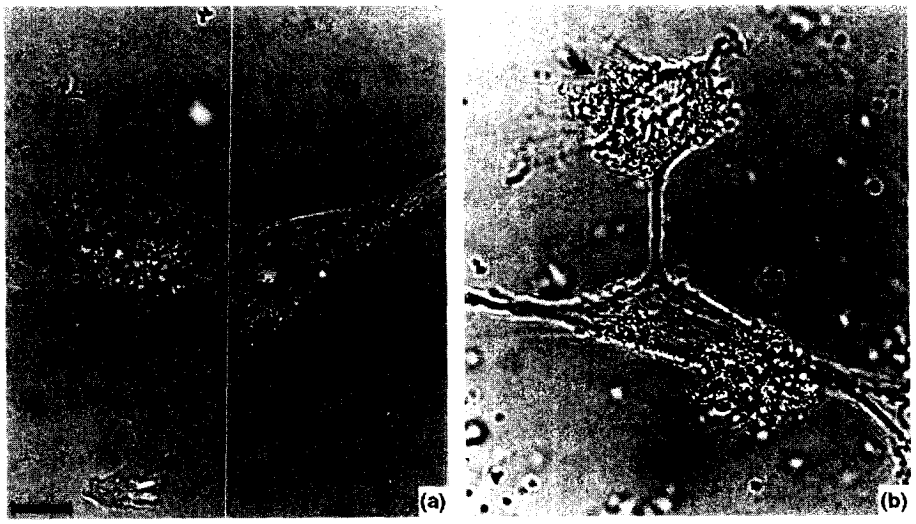

Fig. 7. Differential interference contrast image of healthy (a) and infected 3T6 cells; (b) Bar, 10 μm.

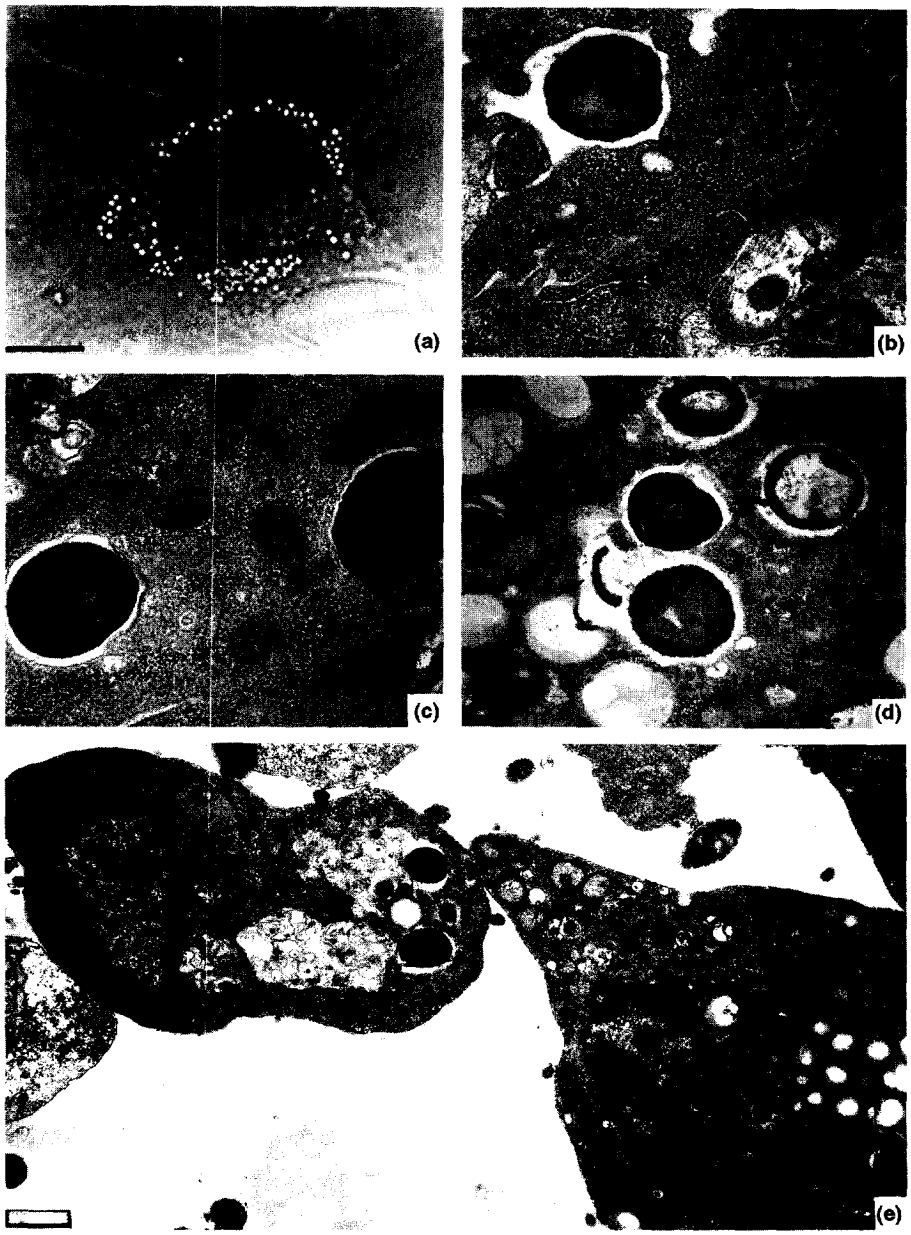

Fig. 8. Native image of an infected, vacuolized 3T6 cell and transmission electron micrographs of BHK cells inoculated with nanobacteria. (a) Perinuclear vacuolization of an infected 3T6 cell under phase-contrast microscope; (b) a nanobacterium being engulfed by a BHK cell; (c–d) nanobacteria inside vesicles in BHK cells; arrow in (d) shows a remnant of a nanobacterium in a large vesicle with two nanobacteria; (e) a typical apoptotic appearance of infected BHK cells. Bars: (a) 10 μm; (b–d) 200 nm; (e) 1 μm.

teria adhered massively only to a fraction of the 3T6 cells. This might be related to a differential expression of target receptors on 3T6 cells, possibly associated with the cell cycle because ultimately all 3T6 cells were covered with nanobacteria.

Normal bacteria, e.g. *Staphylococci* adhere and become internalized very rarely by 3T6 cells [12,16]. Nanobacteria are much more efficiently internalized. Our preliminary results indicate that nanobacteria can be internalized by all cultured mammalian fibroblasts. It is thus likely that they are coated in adherence molecules that trigger phagocytosis in cells that are not normally phagocytic. The invasin protein of *Yersinia enterocolitica* or *Yersinia pseudotuberculosis* is a known example of such a molecule [28].

Cytotoxicity among cell culture contaminants is usually not profound and cells can have good morphology even in the presence of high amounts of the contaminant. Cultures are mostly affected by competition for essential nutrients. The metabolism of nanobacteria is rather slow and changes found in the culture medium composition were minimal during similar incubations

described in the figures. Thus, consumption of medium components was not responsible for cytotoxicity. Possibly cytotoxicity was a consequence of toxic degradation products of nanobacteria in the lysosomes. Similar adherence and cytotoxicity were obtained when life nanobacteria were replaced with γ-irradiated specimens (data not shown). This indicates that nanobacteria do not have to replicate to cause cytotoxicity. The complement system was not involved, because heat-inactivation of the sera did not affect the process. Washed nanobacteria exerted similar cytotoxicity to those that were inoculated in their own culture medium, and thus they seem to carry cytotoxic component(s) in their body that may be transferred via an endocytotic mechanism into cells. Internalization seems to be necessary for cytotoxicity. Dying cells always contained numerous ingested nanobacteria. They resembled apoptotic cells (Fig. 8e). Apoptotic cells have been characterized by a series of defined morphological changes, including chromatin condensation, cytoplasmic shrinkage, separation from neighboring cells, nuclear fragmentation, and membrane blebbing [29]. Our observations on cell morphology suggest apoptotic abnormalities.

In our hands, sera with abundant nanobacteria gave poor results especially in clonings of fibroblasts. The clones died after 10–20 days, even though they had shown initially good growth, with the concomitant appearance of huge numbers of nanobacteria. Such moribund cultures could not be rescued by antibiotics (nanobacteria are highly resistant to common antibiotics used in cell cultures). Hybridomas and many lymphocytes were found to be affected by nanobacteria, but considerably higher doses were needed. These cells could often be recovered by washing and subculturing in nanobacteria-free medium sterilized with γ irradiation. Uncontaminated clones were obtained from hybridoma cultures by subcloning with nanobacteria-free media. In this, colostrum-based medium supplement (Viable HC-1, Valio Bioproducts, Turku, Finland) could successfully replace γ-irradiated serum, which poorly supported clonal growth (our unpublished observations).

Generally, only about 10% of serum batches support hybridoma cloning [30]. Our data indicates a prominent answer to this dilemma: presence of cytotoxic, multiplying nanobacteria that affect both primary and transformed cells. The ability to invade mammalian cells is an essential virulence determinant of many pathogenic bacteria and intracellular parasites [31]. The internalization process, as well as intracellular survival, is virulence-dependent [19]. These features are relevant to nanobacteria suggesting that these blood-derived organisms may be pathogenic also in vivo.

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