Purported nanobacteria in human blood as calcium carbonate nanoparticles

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Recent evidence suggests a role for nanobacteria in a growing number of human diseases, including renal stone formation, cardiovascular diseases, and cancer. This large body of research studies promotes the view that nanobacteria are not only alive but that they are associated with disease pathogenesis. However, it is still unclear whether they represent novel life forms, overlooked nanometer-size bacteria, or some other primitive self-replicating microorganisms. Here, we report that CaCO₃ precipitates prepared in vitro are remarkably similar to purported nanobacteria in terms of their uniformly sized, membrane-delineated vesicular shapes, with cellular division-like formations and aggregations in the form of colonies. The gradual appearance of nanobacteria-like particles in incubated human serum as well as the changes seen with their size and shape can be influenced and explained by introducing varying levels of CO₂ and NaHCO₃ as well as other conditions known to influence the precipitation of CaCO₃. Western blotting reveals that the monoclonal antibodies, claimed to be specific for nanobacteria, react in fact with serum albumin. Furthermore, nanobacteria-like particles obtained from human blood are able to withstand high doses of γ-irradiation up to 30 kGy, and no bacterial DNA is found by performing broad-range PCR amplifications. Collectively, our results provide a more plausible abiotic explanation for the unusual properties of purported nanobacteria.

Results and Discussion

Morphology of Purported Nanobacteria Cultured from Human Blood.

We first repeated the culture of purported nanobacteria from healthy human serum. To distinguish our findings from those in the literature, we refer to our own material as nanobacteria-like particles (NLP). The criteria used to confirm that we reproduced the phenomenology of putative nanobacteria were based on earlier publications (2, 20). They included the typical bacteria-like morphology seen by electron microscopy, slow doubling, bacterial size compatible with life (19) and the National Aeronautics Space Association (NASA) to determine the relevance of nanobacteria in exobiology and astronaut infections (20). As a result, researchers at NASA have found that human nanobacteria multiply faster in a low-gravity environment, an observation deemed to be linked to the high risk of developing kidney stones for astronauts (20). Clinical treatments commercialized by Nanobac Life Sciences, a company owned in part by the discoverers of nanobacteria, are already being promoted on the basis of the eradication of these potentially living and pathogenic microorganisms (9, 21).

The hypothesis that nanobacteria might represent living microorganisms is based on an unusual set of microbiological characteristics that include their slow growth in liquid cell culture media (20), expression of specific antigens (3), and typical bacterial appearance (2). However, observations of abiotic substrates would caution against assigning any life activity to unusual biological entities because certain chemical substrates may adopt the appearance of simple microorganisms (22). In fact, Cisar et al. (23) produced a HAP substrate morphologically suggestive of, but far from being identical to, nanobacteria preparations. More importantly, they showed that HAP could be propagated similarly to a bacterial subculture; they also reattributed the published nanobacteria 16S rDNA sequence to an occasional contaminant of PCR reagents. However, the nanobacteria field has largely ignored these important findings and, in recent years, has continued to host a flurry of research (10, 20) and review papers (11, 24) that purport to find a pathogenic role for nanobacteria in various disease processes.

In view of the importance of the nanobacteria concept to biology and medicine and the fact that they are the basis of several ongoing human therapy trials, we reasoned that the clarification over the nature of nanobacteria must be addressed first. Based on the data reported here, we show that the biology of purported nanobacteria can actually be explained by an abiotic mechanism involving gradual precipitation of CaCO₃ in biological specimens.
Energy-dispersive x-ray (EDX) spectra analysis of NLP revealed high peaks of calcium and phosphorus (Fig. 1G). In fact, the EDX spectrum and atomic P:Ca ratio (1.5 ± 0.1, based on three samples) associated with these NLP preparations (Fig. 1G) resemble those seen with control HAP (P:Ca ratio of 1.62 ± 0.08, based on three samples; Fig. 1H), indicating that HAP accumulates around these entities in accordance with earlier reports (2, 20). However, the morphology of pure HAP crystals (Fig. 1F) was markedly different from that of NLP (Fig. 1A–D); that is, no distinct bacteria-like shapes were seen in control HAP specimens. These observations indicated that the presence of HAP may not be necessary for the formation of NLP. In fact, we noticed that some types of serum fractions used (Materials and Methods) did not yield HAP. Indeed, the EDX spectrum of NLP cultured from the liquid fraction of the clotted blood revealed a simple profile of carbon, oxygen, and sodium at atomic C:O:Na ratios of 64:32:4 (Fig. 1J). No phosphorus was detected that might indicate the presence of HAP. It appears that the formation of HAP around NLP can be inhibited by blood proteins once NLP have taken their round morphology. Therefore, HAP may not be a necessary component of nanobacteria because it appears to deposit onto NLP only under particular circumstances.

Calcium Carbonate Nanoparticles Morphologically Similar to Nanobacteria-Like Particles. As shown above, it became clear that HAP could not account per se for the morphological features of NLP. We therefore sought to study other simple calcium compounds that might assume forms comparable with NLP. In particular, we focused on calcium compounds that are likely to precipitate in the presence of biological fluids like serum. Preliminary experiments showed that, of the compounds examined (CaCO3, CaCl2, and various calcium phosphate combinations), only CaCO3 gave morphologies similar to nanobacteria.

To induce CaCO3 precipitation and the formation of CaCO3 nanoparticles that could in turn be compared with NLP preparations, we used a number of simple chemical reactions known to yield CaCO3. Shown here are the results of one such reaction, involving the addition of (NH4)2CO3 crystals to a calcium salt solution or, simply, the mixing of equal quantities of (NH4)2CO3 and CaCl2. Common CaCO3 crystal polymorphs resembling vaterite, aragonite, and calcite were observed in the precipitate. We verified by EDX that the CaCO3 crystals prepared did not have impurities >0.5% of the crystal weight (Fig. 2H). When CaCO3 was prepared in DMEM, round nanostructures completely different from calcite crystals were obtained (Fig. 2A and B). These CaCO3 structures had a round morphology and appeared to have a membrane-like contour when observed under optical microscopy (Fig. 2C; compare with calcite crystals seen in Fig. 2G). They were remarkably similar to the structures described as nanobacteria observed in human and geological samples (see refs. 2 and 15; compare also Fig. 2A and B with Fig. 1A and B). These round structures of CaCO3 are probably caused by the presence of an amorphous phase of CaCO3 that surrounds the crystals, as described in ref. 25. In the absence of culture medium like DMEM, round CaCO3 particles were apparent within 10 min after mixing together (NH4)2CO3 and CaCl2, and with time, these forms steadily converted into the more common CaCO3 crystals mentioned earlier. However, in the presence of DMEM, these round nanostructures were preserved; they did not convert into calcite even after prolonged incubation in DMEM overnight.

Surprisingly, some of the CaCO3 structures were remarkably similar to cells undergoing binary fission and budding (Fig. 2C–F). Symmetrical division forms involving the addition of (NH4)2CO3 crystals to a calcium salt solution or, simply, the mixing of equal quantities of (NH4)2CO3 and CaCl2, and various calcium phosphate combinations, only CaCO3 gave morphologies similar to nanobacteria.

Materials and Methods

Bacterial Growth. L. acidophilus was actively growing nanobacteria (2), they were seen in our own studies as being porous structures with partial crystallization inside. The smooth continuity between the larger forms and the colonies of NLP suggests that they may have the same composition (Fig. 1C, arrow).

The EDX of NLP obtained by incubating healthy human serum diluted to 10% or, simply, the mixing of equal quantities of (NH4)2CO3 and CaCl2. Common CaCO3 crystal polymorphs resembling vaterite, aragonite, and calcite were observed in the precipitate. We verified by EDX that the CaCO3 crystals prepared did not have impurities >0.5% of the crystal weight (Fig. 2H). When CaCO3 was prepared in DMEM, round nanostructures completely different from calcite crystals were obtained (Fig. 2A and B). These CaCO3 structures had a round morphology and appeared to have a membrane-like contour when observed under optical microscopy (Fig. 2C; compare with calcite crystals seen in Fig. 2G). They were remarkably similar to the structures described as nanobacteria observed in human and geological samples (see refs. 2 and 15; compare also Fig. 2A and B with Fig. 1A and B). These round structures of CaCO3 are probably caused by the presence of an amorphous phase of CaCO3 that surrounds the crystals, as described in ref. 25. In the absence of culture medium like DMEM, round CaCO3 particles were apparent within 10 min after mixing together (NH4)2CO3 and CaCl2, and with time, these forms steadily converted into the more common CaCO3 crystals mentioned earlier. However, in the presence of DMEM, these round nanostructures were preserved; they did not convert into calcite even after prolonged incubation in DMEM overnight.

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budding can be understood as the juxtaposition of aggregating amorphous forms. Although cellular division formations had been used to support the notion that nanobacteria are living cells able to divide (14), these forms can be actually seen here with CaCO₃ nanoparticles prepared in vitro. These results suggested that the growth medium may contain factor(s) that slow down or inhibit the kinetics of formation of a wide range of dilutions (see Materials and Methods; data not shown). These results suggested that complex biological solutions or fluids may contain components that generate amorphous CaCO₃ aggregates that bear striking resemblance to the purported nanobacteria presented in the literature (2, 3).

To explore this possibility further, we tested whether either proteins or divalent cations like Mg²⁺ might be responsible for the inhibition of CaCO₃ crystal formation. We tested solutions of purified albumin or Mg²⁺ compounds in the presence of (NH₄)₂CO₃ and CaCl₂ (see Materials and Methods). In both cases, we were able to obtain stable amorphous CaCO₃ nanostructures as seen in Fig. 2A that are similar to NLP obtained in the presence of serum (Fig. 1A and B). It is unclear at this time how either albumin or Mg²⁺ inhibits the CaCO₃ crystal formation, giving in turn rise to the amorphous NLP. When no CaCO₃ crystal inhibitors (e.g., proteins and Mg²⁺) were present in the CaCO₃ solutions tested here, round structures were seen to grow to diameters of several micrometers (Fig. 2 C–E) and eventually to convert to calcite crystals (data not shown). Thus, the rare larger shelter-like forms described in NLP preparations (Fig. 1 C and D and ref. 2) and seen in our CaCO₃ preparations (Fig. 2 C and D) are probably the result of a similar process.

Influence of Atmosphere Composition and Sodium Bicarbonate Concentration on Nanoparticle Formation. We next explored the physiological relevance of these findings in the context of human biology. We focused on the following common biological reactions, known to produce CaCO₃ in blood as well as in all biological tissues:

\[
\text{CO}_2 + \text{H}_2\text{O} \leftrightarrow \text{H}_2\text{CO}_3 \quad [1]
\]

\[
\text{H}_2\text{CO}_3 + \text{H}_2\text{O} \leftrightarrow \text{H}_3\text{O}^+ + \text{HCO}_3^- \quad [2]
\]

\[
\text{HCO}_3^- + \text{H}_2\text{O} \leftrightarrow \text{H}_3\text{O}^+ + \text{CO}_3^{2-} \quad [3]
\]

\[
\text{Ca}^{2+} + \text{CO}_3^{2-} \leftrightarrow \text{CaCO}_3 \quad [4]
\]

In the case of human serum culture, for example, CaCO₃ can precipitate by the reaction of Ca²⁺ and CO₃²⁻ (Reaction 4) derived from the dissociation of H₂CO₃ in water as shown in Reactions 2 and 3.

The growth of NLP in culture had been monitored through an increase in optical density at 650 nm during incubation (20). We used the same method coupled to SEM observations (as described in ref. 26) to study the impact of various treatments on their growth. We first confirmed that DMEM alone did not produce any increase of optical density after prolonged incubation up to 1 month, in line with published results (20), indicating that there is the need for some seeding factor in the medium to generate the published optical density change. This change in optical density could be induced by adding DMEM with a 10% human serum inoculum (Fig. 3A). When the DMEM/serum mixture was incubated with 5% CO₂ (see Materials and Methods), we obtained the characteristic slow increase in optical density, which in the past had been used to support the notion that nanobacteria reproduce slowly, with a doubling time of 3 days (Fig. 3A; see also ref. 20). By replacing this same cell culture atmosphere of 5% CO₂ with 100% inert gas, such as argon, the increase in optical density was markedly increased (Fig. 3A), concordant with the expected shift in the equilibrium of Reactions 1–4 shown above, which in this situation would favor the precipitation of CaCO₃. In contrast, by incubating blood samples in 100% CO₂, the formation of CaCO₃ nanoparticles was markedly inhibited compared with standard cell culture conditions (Fig. 3A). SEM observations done on comparable NLP samples confirmed that these optical density measurements were representative of the number of nanoparticles (data not shown).
NaHCO3 (either 500 mM or no NaHCO3. In all experiments, 5% CO2 was shown), indicating that the NLP seen in the various experiments precipitates collected from the various treatments (data not compared the changes in optical density seen with this concentration of NaHCO3 against incubation in culture medium containing either 500 mM or no NaHCO3. In all experiments, 5% CO2 was used. As shown in Fig. 3B, a decrease in the number of nanoparticles was observed when NaHCO3 was removed from the culture medium. However, 500 mM NaHCO3 produced a slight increase in optical density (Fig. 3B); this initial increase in optical density leveled off after day 12, and by day 16, all three conditions produced comparable optical densities (Fig. 3B), indicating that HCO3− might have reached comparable concentration levels in all three experimental conditions through the gradual equilibration of CO2 in the medium.

The pH of the culture was shown to increase up to a maximum of 1.5 pH units with the argon and 500 mM NaHCO3 treatments and, likewise, to decrease by a maximum of 1.5 pH units with the CO2 and no NaHCO3 treatments (experiments depicted in Fig. 3A and B). This pH variation is consistent with the maintenance of equilibrium seen with Reactions 1 and 4 shown above to explain the formation of calcium carbonate in our system. For instance, an increase in carbon dioxide leads to the accumulation of carbonic acid in Reaction 1, which in turn increases the amount of H2O2 in Reactions 2 and 3, thereby decreasing the pH and favoring the dissolution of CaCO3 (Reaction 4). An increase in inert gas such as argon reverses this process by decreasing the concentration of CO2 (Reaction 1).

Together, these experiments point to a chemical model for the formation of the NLP. We were able to control the formation of precipitating NLP in culture based on our hypothesis that the core of these entities represents CaCO3. It is apparent that such a marked increase in growth in an atmosphere of inert gas or after the addition of NaHCO3 cannot be attributed to the stimulation of a living microorganism.

**Materials and Methods**

No difference was noted in EDX surface composition spectra on precipitates collected from the various treatments (data not shown), indicating that the NLP seen in the various experiments were chemically similar.

The formation of CaCO3 nanoparticles in DMEM, after serum inoculation, was also influenced by adding or removing NaHCO3 buffer in the culture medium (Fig. 3B). The DMEM used in our experiments contained 44 mM NaHCO3; we compared the changes in optical density seen with this concentration of NaHCO3 against incubation in culture medium containing either 500 mM or no NaHCO3. In all experiments, 5% CO2 was used. As shown in Fig. 3B, a decrease in the number of nanoparticles was observed when NaHCO3 was removed from the culture medium. However, 500 mM NaHCO3 produced a slight increase in optical density (Fig. 3B); this initial increase in optical density leveled off after day 12, and by day 16, all three conditions produced comparable optical densities (Fig. 3B), indicating that HCO3− might have reached comparable concentration levels in all three experimental conditions through the gradual equilibration of CO2 in the medium.

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To address further the issue of specificity, we reacted antibody 8D10 against the supernatant of the same 1-month-old NLP used for Fig. 4A, lane 1. This same pellet revealed an abundance of NLP when observed under SEM (data not shown). The molecular mass of the single band detected was 68 kDa (lane 1). It should be noted that most studies using 8D10 and 5/3 antibodies have actually focused on ELISA (5) and tissue or cell culture immunostaining (7). Miller et al. (10) showed that the antibody 8D10 reacts against one single band of 50 kDa from cell pellets prepared under reducing and denaturing conditions comparable with our experiments.

To address further the issue of specificity, we reacted antibody 8D10 against the supernatant of the same 1-month-old NLP used for Fig. 4A, lane 1. Because this supernatant, when concentrated and observed by SEM, did not reveal NLP, we expected to find little or no reactivity on the immunoblot. To our surprise, we observed the same reactive band of 68 kDa (Fig. 4A, lane 2). These results suggested to us that 8D10 might react to other control specimens as well. In fact, a control sample of whole blood, aged for 1 month without any further inoculation into medium, treatment, or extraction, and yielding no SEM detec-
tion of NLP, revealed the same band when reacted on the immunoblot with 8D10 (Fig. 4A, lane 3). Moreover, even fresh blood samples reacted in the same way (Fig. 4A, lane 4); here, too, there was no detection of NLP when observed under SEM (data not shown). We noticed that only protein samples derived from serum-free cell extracts did not react with 8D10 (exemplified by Fig. 4A, lane 5).

These results prompted us to speculate whether the reactive proteins might not in fact represent common human proteins that associate with CaCO₃ nanoparticles. Our earlier results suggested that, given its molecular mass, serum albumin, the most abundant protein in the plasma, might serve as a good candidate. 8D10 and 5/3 monoclonal antibodies were shown to react with both pure HSA and BSA in a stoichiometric fashion (Fig. 4 B and C). It appears that the high degree of similarity between HSA and BSA (27) makes it possible for the antibodies to react with both proteins. It is possible that these monoclonal antibodies may react with minor bands not detected in our immunoblot experiments. However, the prominence of the albumin reactivity makes it unlikely that it would not interfere with the antibody staining under any foreseeable circumstances.

The presence of albumin and possibly of other binding proteins in the NLP seems to make sense because they may serve as modulators or inhibitors of CaCO₃ crystallization, which in turn may be responsible for generating NLP. It is not clear how tightly albumin binds to the nanoparticle complex and what role it may have in the assembly of the nanoparticle, but already some hints can be obtained through preliminary experiments. NLP that had been pelleted, washed, and incubated with 3 M EDTA overnight retained their amorphous nanobacteria-like morphology as well as a dominant albumin band that reacted strongly to the two monoclonal antibodies (data not shown). These observations suggest that the presence of proteins early in the CaCO₃ nucleation process may result in the formation of an insoluble matrix that resists decalcifying treatments. On the basis of these results, we must conclude that these antibodies do not specifically recognize antigens produced by nanobacteria as reported earlier (3). They were instead found to react with serum albumin, and although this protein may be associated with NLP, it certainly cannot be used as a specific biomarker for a living microorganism.

Characteristics of NLP Irreconcilable with Those of Living Organisms. We observed NLP in the culture of every healthy human serum tested (n = 20), an observation in line with another study that reported that 90–100% of animal sera from cattle, goats, cats, and dogs gave similar nanoparticles (28). Thus, NLP formation would appear to be unrelated to pathogenesis. Besides, γ-irradiation of human serum samples up to 30 kGy before culture did not prevent the growth of NLP in our hands, contrary to earlier reports (2). Furthermore, we found no bacterial 16S rDNA from human serum using PCR amplifications conducted on NLP preparations, despite numerous attempts (data not shown). Other groups also failed to show the presence of nucleic acids within putative nanobacteria using PCR (28, 29) as well as spectroscopy and staining methods (23). These results rule out the possibility that these entities rely on the duplication of DNA or RNA for their propagation.

We also obtained NLP from serum diluted in DMEM and filtered through 0.1-μm pore size membranes (see Materials and Methods; data not shown). This result implies that the replicative unit or nucleating agent responsible for such a growth is smaller than 100 nm in diameter. Because an earlier workshop commissioned by the NAS had suggested that the minimal cellular size of life on Earth must exceed 200 nm in diameter to harbor the cellular machinery based on DNA replication (19), it is unlikely that nanobacteria represent living entities unless they contain some other type of replicating mechanism. The amorphous CaCO₃ nucleation hypothesis described in this article provides an alternative abiotic view that can now be used to reconcile the phenomenology of NLP with accepted definitions of life.

In summary, we reproduced the earlier nanobacteria-related observations by incubating human serum in cell culture conditions. However, we observed that HAP cannot by itself explain the dynamic process responsible for the formation of these nanoeentities. By searching for simple chemicals likely to precipitate from biological fluids, we found that CaCO₃ can adopt morphologies remarkably similar to NLP. In fact, these amorphous CaCO₃ complexes can easily form cellular division-like structures reminiscent of living microorganisms. We were also able to influence the speed with which NLP could be formed by varying the substrates needed for CaCO₃ precipitation in culture. Besides, the lack of specificity of nanobacteria antibodies casts doubts on the validity of previous results and warrants against their use as a diagnostic, and perhaps therapeutic, tool in medicine. Our results also point toward the possibility that formations such as those reported in meteorites (18) and geological samples (15, 16) are the result of a similar process.

Materials and Methods

Culture of NLP. NLP were cultured from human serum as described in ref. 2. Briefly, healthy human blood samples (n = 20) were aseptically drawn into sterile Vacutainer tubes without anticoagulant (Becton Dickinson) by a conventional venipuncture technique. The clear serum was prepared by centrifuging the clotted blood at 1,500 × g for 5 min and by diluting the supernatant 1:10 in DMEM (Invitrogen) which, when indicated, contained different amount of NaHCO₃. When the mixtures of serum and DMEM were incubated with different gases or various amounts of NaHCO₃, the pH was adjusted to 7.4 before incubation. The solution was filtered through a 0.2- or 0.1-μm pore size sterilizing membrane before incubation in conventional cell culture conditions (37°C; 5% CO₂, 95% air) for 1 month. The culture of NLP was also performed by using the cloudy liquid fraction of the clotted blood (without centrifugation); this fraction was diluted 1:10 in DMEM and processed as described above. This culture method was used to obtain NLP from every blood sample, to show that HAP is not a necessary component of NLP as well as for spectroscopy measurements. Spectroscopy measurements were performed as described in ref. 20. To verify the influence of inert gas and CO₂ on NLP formation, culture flasks and 96-well culture plates inoculated with human serum diluted 10% in DMEM were incubated in a hypoxic chamber (Billups-Rothenberg). Sterile argon or CO₂ was flushed inside the jar at a flow rate of 20 liters/min for two periods of 10 min separated by a 1-h interval. To verify the impact of γ-irradiation on the growth of NLP, 10% filtered human serum in DMEM was γ-irradiated up to 30 kGy (Institute of Nuclear Energy Research, Atomic Energy Council, Taiwan) before culture.

Preparation of CaCO₃ Nanoparticles. CaCO₃ nanoparticles were prepared by diffusion of (NH₄)₂CO₃ crystal vapors into a 1 M CaCl₂ solution as described in ref. 30. To prepare small uniform CaCO₃ nanoparticles, 1 M CaCl₂ was first mixed with MgCl₂ to obtain a Ca:Mg ratio of 1:1 to 1:10. Small CaCO₃ nanoparticles were also obtained by using DMEM to dilute 1 M (NH₄)₂CO₃ and 1 M CaCl₂ in ratios ranging from 1:50 to 1:500. The formation of larger amorphous CaCO₃ particles and common crystals of CaCO₃ was studied by mixing (NH₄)₂CO₃ 1 M with CaCl₂ 1 M in a 1:1 ratio. We also examined CaCl₂, CaHPO₄, Ca(OPO₄)₂, Ca₃(PO₄)₂, Ca₃(PO₄)₂(OH)₂, Na₂CO₃, and NaPO₃ by dissolving them in water or DMEM at concentrations varying from 1 to 100 mM and incubating them for 1 month. To study the influence of proteins and serum on the formation of CaCO₃ nanoparticles, whole serum or 10% serum in DMEM was used to dilute equal amounts of 1 M (NH₄)₂CO₃ with 1 M CaCl₂ in ratios ranging from 1:10 to 1:100. The role of proteins in the formation of CaCO₃ nanoparticles was studied by adding HSA at final concentrations varying from 0.1 to 1 mg/ml.

Optical Dark-Field Microscopy, TEM, and SEM. SEM and TEM sample preparation was described earlier (26). A 1230 TEM (JEOL) and S3000N SEM (Hitachi) were used. EDX spectra were acquired with an EMAX Energy EX-400 EDX device (Horiba). EDX spectra of NLP from human serum were obtained directly from pelleted, 1-month-old, culture material. For optical microscopy, homogenized CaCO₃ nanoparticles were observed without fixation or staining with a BX-51 optical microscope (Olympus) and dark-field condenser (CHERE). Images were acquired by using a KY-F55 color camera (JVC).
Western Blotting. NLP from human serum were prepared as for electron microscopy from 1-month-old culture. After two washing steps in water with centrifugation at 16,000 × g for 10 min, the following samples were solubilized in reducing Laemmli buffer: pelleted NLP, supernatant of the same 1-month NLP culture, 1-month-old whole-blood sample, and fresh human blood. BSA and HSA were purchased from Sigma; the negative control for nonspecific binding consisted of a protein extract from 239T human cells. Primary antibodies BD10 and 5/3 (Nanobac Oy) were dissolved 1:1,000, and anti-mouse horseradish peroxidase-coupled secondary antibody (Amersham Biosciences) was diluted 1:2,000; both were diluted in blocking solution. An ECL Western blot detection kit (Amersham Biosciences) was used according to the manufacturer’s recommendations.

DNA Extraction and PCR Amplifications. DNA extraction was performed from NLP pellet and supernatant by using the method of Higuchi (31). The broad-range 16S rDNA PCR amplification protocol used was described in ref. 32. Positive, negative, and spiked controls containing both the NLP sample and positive-control template were used to rule out the presence of PCR inhibitors in blood preparations.

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