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Are There Naturally Occurring Pleomorphic Bacteria in the Blood of Healthy Humans?

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ABSTRACT

Dark-field microscopy of blood from healthy individuals revealed the existence of pleomorphic microorganisms. These bacteria exhibited limited growth and susceptibility to antibiotics and could be detected by fluorescent in situ hybridization and flow cytometry. They were further characterized by analysis of their 16S rRNA and *gyrB* genes.

In our search for spirochetes involved in Alzheimer's disease (13), we observed pleomorphic bacteria in the blood of healthy human subjects by dark-field microscopy. This was a surprising finding since it is generally acknowledged that the bloodstream in healthy humans is a sterile environment (7) except when there is a breach in the integrity of the tissue membranes (6). However, the concept of the occurrence of bacteria in the blood of healthy humans is now more plausible because of cultivation-independent laboratory approaches. The main techniques employed in such studies include PCR amplification and sequencing of the 16S ribosomal DNA (rDNA). These methods have revealed the presence of a wide diversity of microorganisms in the environment, and indeed within the human body (12). In this report we present evidence based on molecular phylogenetic techniques and light and electron microscopy, as well as other conventional microbiological methods, for the existence of a population of bacteria in healthy human blood. In view of the apparent controversial nature of our findings, it was encouraging to note the recent report of Nikkari et al. (14), who detected blood-associated bacterial rDNA sequences by using real-time PCR methods and a probe targeting conserved regions of bacterial 16S rDNA, and an earlier report by Tedeshi et al. (16) on the presence of pleomorphic bacteria as intraerythrocytic parasites in clinically healthy human subjects.

For light microscopic examination, blood samples from 25 healthy volunteers were drawn in a Vacutainer tube with no anticoagulants (Becton Dickinson, Franklin Lakes, N.J.); blood was drawn in the conventional manner involving antisepsis of the skin and avoidance of any introduction of external microorganisms by contamination. (Since external contamination was always a possibility, particular care and precaution were exercised at all times to avoid this. The specific procedures, as well as appropriate controls, are specified throughout the text.) A wet mount of the serum from the clotted blood of each sample, fresh or incubated at 30°C for between 5 to 7 days, was examined by dark-field microscopy (Leitz Dialux 20) for pleomorphic bacteria.

For PCR amplification, a 0.5-ml aliquot of incubated blood containing pleomorphic bacteria was used for conventional extraction of DNA by the method of Higuchi (11). Three microliters of the extract was used for DNA amplification by the method of Edwards et al. (8) using the forward primer BSF8/20 (5'-AGAGTTTGATCCTGGCTCAG-3') and the reverse primer BSR1541/20 (5'-AAGGAGGTGATCCAGCCGCA-3'). Thirty cycles of PCR were performed, with 1 cycle consisting of the following steps: (i) denaturation (1 min at 94°C), (ii) annealing (30 s at 70°C), and (iii) extension (90 s at 72°C). Vent DNA polymerase (New England BioLabs, Mississauga, Ontario, Canada) was used due to its proofreading ability.

Amplicons were resolved by standard gel electrophoresis and detected by ethidium bromide staining. DNA was purified using the GeneClean II kit (Bio 101, Inc., La Jolla, Calif.), and the DNA was cloned into the *Sma*I site of the vector pBluescript II (Stratagene, La Jolla, Calif.). Nucleotide sequence analysis of two randomly picked clones was performed using the ABI automated DNA sequencer (model 373A) and the ABI Prism cycle sequencing kits with the polymerase *AmpliTaq* FS (Perkin-Elmer Corp., Boston, Mass.).

The *gyrB* gene was PCR amplified by the protocol outlined by Yamamoto and Harayama (21). The amplicons were cloned using the TOPO TA cloning kit (Invitrogen, Carlsbad, Calif.).

Sequence data were analyzed using the BLASTN program of the National Center for Biotechnology Information (Bethesda, Md.) (1). The candidate sequences with the highest scores were retrieved accordingly and aligned by using the NALIGN program of PC/Gene (Intelligenetics, Inc.).

To perform fluorescent in situ hybridization (FISH), blood was drawn under the usual identical aseptic conditions (to avoid contamination) from three healthy subjects and incubated for 6 days at 30°C in Vacutainer brand sterile interior glass tubes (16 by 100 mm). The blood sample was diluted 1:10 with a phosphate-buffered saline (PBS) solution supplemented with 10 mM pyrophosphate. The sample was centrifuged for 5 s and then filtered through a 0.8- μ m-pore-size sterile Acrodisc syringe filter to remove red blood cells and debris. One-milliliter aliquots of the sample were centrifuged for 10 min at 14,000 \times g. The subsequent fixation and hybridization procedures were done by the protocol described by Thomas et al. (17). Briefly, the pelleted bacterial cells were fixed with 1 ml of 3% (wt/vol) freshly prepared paraformaldehyde at 4°C for 24 h. The cells were then pelleted for 10 min at 14,000 \times g and permeabilized by incubating the pellet with 1 ml of a cold mixture of ethanol and PBS (1:1, vol/vol) for 5 min at 25°C. The bacteria were washed with PBS and resuspended in 50 μ l of hybridization buffer (0.9 M NaCl, 20 mM Tris [pH 7.2]) that was prewarmed to 50°C and supplemented with 10 ng of fluorescent probes per μ l. The probes used were all rRNA-targeted fluorescein-labeled oligonucleotides: the eubacterial probe (EUB) BSR1541/20 (8); non-EUB, which is complementary to the universal bacterial probe EUB used as a negative control for nonspecificity (3); and eukaryotic probe EUK (18). The suspensions were incubated for 24 h at 50°C, and hybridization was stopped by centrifuging the samples for 2 min at 8,000 \times g, discarding the supernatant, and adding 1 ml of PBS. Ethidium bromide (0.5 μ g/ml) was added just prior to flow cytometry analysis.

For electron microscopy, bacterial cells in incubated blood samples from healthy subjects were separated from blood elements by the following method. A 1:10 dilution of blood in PBS was centrifuged in a microcentrifuge at $14,000 \times g$ for 5 s. The supernatant was removed and centrifuged as described above for 10 min. It was decanted, and the pellet was washed twice in PBS. The pellet containing a high concentration of bacteria from blood was resuspended in a final small volume of PBS. Alternatively, blood diluted 1:10 in PBS was filtered through a 0.45- μm -pore-size membrane filter. The filtrate was centrifuged at $14,000 \times g$ for 10 min, and the pellet was washed twice with PBS. This pellet was also resuspended in a small volume of PBS for electron microscopy. Negative staining was performed with 2% phosphotungstic acid. To prepare for ultrathin sectioning, the cells were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer for 2 h. The cells were subsequently dehydrated, embedded, and sectioned. The sections were stained with uranyl acetate and lead citrate and examined using the JEOL 2000 FX transmission electron microscope (TEM).

Prompted by our observation of what appeared to be an unusual microbe in a blood sample from a healthy individual, we screened several blood samples for the presence of bacteria by PCR amplification of 16S rDNA using universal primers. No product from day 0 blood was detected (by ethidium bromide staining), but PCR product was detectable after the blood had been incubated at 30°C for 5 days. Inoculation of aliquots from blood samples of either day 0 or day 5 into nutrient broth resulted in no growth of bacteria. Evidently the PCR signal obtained was not a result of saprophytic bacterial contamination.

PCR-amplified DNA fragments with sizes of 1.2 and 1.5 kb from these bacteria were cloned. Two recombinant plasmids, B16 and T5, were used for DNA sequencing. The two sequences were identical except for a truncated 5' end in B16. The near-complete 16S rRNA gene of T5 (1,257 bp) showed 99.6% identity with the 1,500-bp sequence of *Stenotrophomonas maltophilia* LMG958T, a type strain of Laboratorium voor Microbiologie Gent Culture Collection, Ghent, Belgium. The sequence of T5 determined on both strands showed four transitions and two transversions compared to the sequence of LM958T (GenBank accession number [X95923](#)). This finding has been confirmed with a second sample from another subject. (The phylogenetic position of *S. maltophilia* has been under intensive investigation [[10](#), [15](#)]. Originally called *Pseudomonas maltophilia*, it was later called *Xanthomonas maltophilia*. *S. maltophilia* is clustered in the gamma subclass of the *Proteobacteria* and represents a distinct taxon.)

Independent amplification of 16S rDNA from blood samples from three additional subjects was achieved by one of us (M. Sirois) at the University of Quebec at Trois-Rivieres in Canada. Analysis of the 16S ribosomal gene sequence from the blood samples from these three subjects showed that the bacterial genus of closest homology was *Pseudomonas*. This supported our findings at McGill University, since *Stenotrophomonas*, as indicated above, was formerly part of the genus *Pseudomonas*.

In addition to the 16S rRNA results obtained at McGill University, we have also PCR amplified the *gyrB* gene of the bacteria from blood samples. The *gyrB* gene encodes the subunit B protein of DNA gyrase. PCR amplification of the *gyrB* gene is a new molecular method for detecting and identifying bacterial strains ([21](#)). The sequence of the 475-bp PCR fragment, which we obtained in our laboratory, was found to be different from that of the partial *gyrB* gene (1,252 bp) of the *S. maltophilia* type strain ATCC 13637. Thus, the isolate that we isolated from blood is not identical to *S. maltophilia* ATCC 13637 but instead is another strain.

FISH of the bacteria from blood was also performed ([2](#), [4](#)). We found that conventional methods of whole-cell FISH using the fluorescein-labeled oligonucleotide probe BSR1541/20 (complementary to a region at the 3' end of the 16S rRNA conserved for all eubacteria) were unsuitable because of lysis of

the fragile bacteria. To circumvent this problem, the blood bacterial suspension was studied by a FISH protocol and laser flow cytometry (LFC) (3, 17). This technique has the potential of rapidly analyzing viable but nonculturable bacteria.

Figure 1A shows the light-scattering profile of the bacterial suspension recorded by LFC that reflects cells and possible debris and background noise. We gated a region (region a) of this profile in which fluorescent events were recorded. Fluorescent signals emitted from cells that hybridized with the fluorescein-labeled nonspecific (non-EUB) probe (mean fluorescent intensity [MFI] of 3.9) are shown in Fig. 1B. The MFIs recorded in region e on blood samples taken from three different individuals on 2 different days were 2.5- to 3.7-fold higher with the BSR1541/20 probe than those obtained with the non-EUB probe (Table 1). Fluorescent signals from the fluorescein-labeled EUB probe (17), specific for an 18S rRNA region conserved in all eukaryotes, were not different from those of the non-EUB probe.

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FIG. 1.

FISH on blood bacterial suspension. (A) Light-scattering profile of the bacterial suspension. Acquisition region a included, beside bacterial cells, debris and background noise. FS, forward scatter; SS, side scatter. (B) Histogram of the fluorescein (FL1) signals recorded in region a for the fluorescein-labeled NON probe (nonspecific, peak n) or the BSR154/20 probe (eubacterial, peak e).

TABLE 1.

MFIs of fluorescent blood bacterial suspensions from three human subjects as recorded by LFC^a

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^aAssays for subjects 1 and 2 (1st assay) and subjects 2 (2nd assay) and 3 were done at 1-month intervals.

^bRatio of BSR1541/20 probe to non-EUB probe.

To further characterize this group of bacteria, morphological studies were performed. Serum samples from the blood samples that were collected aseptically from 25 volunteers and then allowed to clot revealed the presence of bacteria examined by dark-field microscopy. Figure 2 shows the morphology of a group of cells prepared from a serum sample.

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FIG. 2.

TEM image of a negatively stained specimen showing a group of blood-derived bacteria exhibiting pleomorphic morphology.

In addition to their cellular movement, such as flexing, the viable nature of these pleomorphic bacteria was reinforced by their ability to increase in modest numbers in serum upon aerobic incubation of a clotted blood sample at 30°C for 7 days. Table 2 shows the increase in cell numbers of seven blood samples. The organisms were not saprophytic bacterial contaminants, since no bacterial growth was observed when aliquots of serum were streaked on enriched agar media. Unfortunately, attempts to grow the bacteria on various enriched laboratory media, such as blood nutrient agar, spirochete agar, mycoplasma agar, and leptospira medium, were not successful. It was concluded that the pleomorphic bacteria in blood were viable but could not be cultivated in vitro by conventional techniques. It should be noted that washes from the blood collection tubes yielded no pleomorphic bacteria when examined with the dark-field microscope, indicating that the tubes were not the sources of these bacteria.

TABLE 2.

Bacterial counts in blood samples after incubation

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^aTotal counts of the number of bacilli in 50 fields.

^bAerobic incubation at 30°C after 7 days.

The bacteria isolated from the blood samples were selectively inhibited by antibiotics. Table 3 shows the effects of two antibiotics on the growth of the bacteria in sera. Polymyxin B was the most inhibitory, while bacitracin had limited effects.

TABLE 3.

Selective effect of antibiotics on the growth of bacteria isolated from blood

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^aBacitracin and polymyxin B were both used at a concentration of 100 µg/ml.

^bAll counts were the numbers of bacilli in 50 fields after aerobic incubation at 30°C.

A micrograph of a thin section of bacteria isolated from blood samples is shown in Fig. 3. The cells were enclosed by a double membrane with no evidence of a dense layer of peptidoglycan; no discrete nuclei or membrane-bound intracellular organelles typical of eukaryotes were found. It is evident that the pleomorphic bacteria from blood are highly organized entities rather than random protein debris resulting from degradation of blood cellular elements.

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FIG. 3.

TEM image of an ultrathin section of bacteria isolated from blood. Bounding membranes of the cells are indicated by arrows.

Through the use of PCR and FISH techniques, we were able to demonstrate that the blood from clinically healthy human subjects contains bacterial DNA. PCR of the 16S rRNA gene has been widely used to identify uncultivable bacteria in the environment (5, 9); such identification has also been performed with unknown bacteria within the human host (12) and recently in the blood from clinically healthy human subjects (14). FISH has been successfully used for the detection of specific intact single microorganisms within environmental samples, such as lake water (18), soil (17), and activated sludge (20), and noncultured anaerobic bacteria in human fecal flora (19).

Our report of naturally occurring viable pleomorphic bacteria in the blood of healthy humans should not be controversial. G. Tedeshi et al. at the University of Camerino in Italy had reported the presence of similar bacteria as intraerythrocytic parasites of clinically healthy human subjects in 1969 in the journal *Nature* (16). They showed that red blood cells increased the uptake or incorporation of radioactive thymine, uridine, and glycine because of the presence of these bacteria. Incorporation of these compounds is not part of the normal metabolic activity of erythrocytes. The recent finding of bacterial 16S rDNA in the blood of healthy human subjects by Nikkari et al. (14) adds credibility to our findings. Using contemporary molecular biology techniques, we have demonstrated the presence of discrete bacteria in the serum of healthy human subjects that share morphological similarity to those documented by Tedeshi et al. (16). Our findings are that there are pleomorphic bacteria in the blood of healthy humans.

NUCLEOTIDE SEQUENCE ACCESSION NUMBERS.

The 1,257-bp 16S rDNA sequence of the recombinant clone T5 has been deposited in GenBank and assigned accession number [AF098637](#).

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