

## Endotoxin and nanobacteria in polycystic kidney disease

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### Endotoxin and nanobacteria in polycystic kidney disease.

**Background.** Microbes have been suspected as provocateurs of polycystic kidney disease (PKD), but attempts to isolate viable organisms have failed. Bacterial endotoxin is the most often reported microbial product found in PKD fluids. We assessed potential microbial origins of endotoxin in cyst fluids from 13 PKD patients and urines of PKD and control individuals.

**Methods.** Fluids were probed for endotoxin and nanobacteria, a new bacterium, by the differential Limulus Amebocyte Lysate assay (dLAL), genus-specific antilipopolysaccharide (LPS) antibodies, monoclonal antibodies to nanobacteria, and hyperimmune serum to *Bartonella henselae* (HS-Bh). Selected specimens were also assessed by transmission electron microscopy (TEM) and nanobacterial culture methods.

**Results.** LPS or its antigenic metabolites were found in more than 75% of cyst fluids tested. Nanobacteria were cultured from 11 of 13 PKD kidneys, visualized in 8 of 8 kidneys by TEM, and immunodetected in all 13 PKD kidneys. By immunodetection, nanobacterial antigens were found in urine from 7 of 7 PKD males, 1 of 7 PKD females, 3 of 10 normal males, and 1 of 10 normal females. “*Nanobacterium sanguineum*” was dLAL positive and cross-reactive with antichlamydial LPS and HS-Bh. Some cyst fluids were also positive for LPS antigens from *Escherichia coli*, *Bacteroides fragilis* and/or *Chlamydia*, and HS-Bh, as were liver cyst fluids from one patient. Tetracycline and citrate inhibited nanobacterial growth in vitro.

**Conclusion.** Nanobacteria or its antigens were present in PKD kidney, liver, and urine. The identification of candidate microbial pathogens is the first step in ascertaining their contribution, if any, to human disease.

New technologies are verifying the old idea that microbes or their parts cause chronic disease in humans, in whom both the genetic background of the patient and microbe(s) interact to influence disease initiation and/or progression [1]. Such interactions of genetic background

**Key words:** *Chlamydia*, *Bartonella*, *Bacteroides*, liver cysts, simple cystic disease, tetracycline, citrate.

Received for publication July 13, 1999  
and in revised form September 21, 1999  
Accepted for publication January 3, 2000

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and microbe may have added importance and nuance in diseases once viewed as being exclusively caused by genetic anomalies. In such cases, an understanding of both the human genetic mutation and resultant biology must be matched with the relevant human microbe(s) to fully understand the disease process and potential therapies.

Environmental factors are reported to influence the progression of polycystic kidney disease (PKD), the most prevalent autosomal dominant disease in humans [2–6]. In addition to the inherited mutated PKD allele, autosomal dominant PKD (ADPKD) offspring may themselves undergo multiple focal, intrarenal mutations of the remaining normal PKD allele (that is, loss of heterozygosity), resulting in rapid expansion of cysts via clonal growth of the “double-hit” cells [7, 8]. Damage or mutation of the remaining normal PKD allele has been speculated to involve local metabolic stresses, misalignment of vulnerable DNA structures, and the actions of microbes and their toxins [9–11]. In some experimental forms of PKD, germ-free conditions protect against cystogenesis, but the addition of bacterial endotoxin provokes cystogenesis [3–5]. The greater incidence of infections and resultant greater morbidity and mortality in PKD individuals than the general population have prompted speculation that PKD may involve an as yet uncharacterized defect in microbe clearance mechanisms [12–14]. In addition, the approximately 80% of PKD patients reported to have colonic anomalies described as diverticula may show greater bioavailability of microbial products derived from the diet and microflora, as observed in “leaky gut syndrome” [6, 15, 16].

Although interstitial renal inflammation occurs in PKD, it is not known whether this inflammation is due exclusively to PKD cell biology or microbial factors impacting kidney and other affected tissues, especially the gastrointestinal and cardiovascular systems. Bacterial endotoxin [lipopolysaccharide (LPS)], a potent nephrotoxic inflammatory substance, has been found in PKD

cyst fluid and urine [9, 17, 18]. LPS is composed of a lipid A moiety, core polysaccharides, and O-antigens. Antibodies to the core polysaccharides of genus-specific LPS have been used to detect microbes in tissue and fluids and provide insight into pathophysiological mechanisms. In this study of endotoxin in PKD, we used genus-specific LPS antibodies to ascertain the microbial origins of endotoxin present in human PKD cyst fluids.

In the course of this work, in PKD serum, cyst fluids, and urine we found evidence of nanobacteria, a recently discovered, novel, calcium apatite-forming bacterium that is undetectable by routine microbiological culture methods [19–22]. Nanobacteria exhibit an array of effects in animals and in vitro culture that are plausibly related to the known anomalies of PKD. For example, intravenously injected nanobacteria are renotropic and cause apoptosis of kidney tubule cells and sloughing of renal tubule cells into the urine, and they are found in 97% of kidney stones [23–27]. Nanobacteria cause mineralization in vitro under physiological concentrations of calcium and phosphate [20, 21, 24, 25]. Dysregulation of apoptosis and tubular obstruction is reported in PKD, as is a heightened occurrence of kidney calcification [28–31]. Awareness that viable nanobacteria are present in human PKD kidney is important for future tests of environmental factors that impact the genetically unique aspects of PKD biology as well as broader issues of the role of nanobacteria in health and disease.

## METHODS

Kidneys from 13 patients with ADPKD were obtained in cold University of Wisconsin solution [32] within two hours of nephrectomy at UICOM-Department of Surgery, St. Francis Medical Center (Peoria, IL, USA). Handling of the kidneys and aspirated cyst fluids was done using aseptic techniques, tools rendered pyrogen- and glucan-free by heating at 160°C for four hours, and pyrogen- and fungal glucan-free reagents, as determined by differential Limulus Amebocyte Lysate assay (dLAL) [9]. The dLAL distinguished endotoxin from fungal glucans. Cyst fluids were individually aspirated, cultured by routine microbiological methods, and stored at –60°C for later analysis of endotoxin content, immunodetection, transmission electron microscopy (TEM), and nanobacteria culture, as described later in this article. In one PKD patient (donor 2; Table 1), liver cyst fluids were individually aspirated prior to nephrectomy. In a patient with simple renal cysts (donor 13), 200 mL of fluid from a single cyst were obtained by radiologically guided needle aspiration under local anesthetic. Blood by venipuncture and midstream, clean catch urine were obtained from a 23-year-old PKD Finnish male with rapidly enlarging renal cysts but normal creatinine and urea levels. Urine from PKD and healthy control individ-

uals previously reported for endotoxin content was also analyzed for nanobacterial antigens [17].

Endotoxin in cyst fluids and urine was assayed by dLAL using the gel clot endpoint as previously described (Charles River ENDOSAFE, Charleston, SC, USA) [9]. Each specimen was assayed in duplicate with serial dilutions to assess the concentration. To expose epitopes in a few select experiments, nanobacteria were demineralized by incubation in 100 mmol/L EGTA made in sterile, pyrogen-free water for one hour at 37°C. Following demineralization, both the 15,000 × g pellet and supernate were amenable to dLAL and dot immunoblot assay; such concentrations of EGTA in the specimen did not interfere with these assays.

For the detection of LPS antigens, lyophilized cyst fluids reconstituted in commercial pyrogen-free water to one tenth of their original volume were dot blotted (2 µL) onto nitrocellulose paper, blocked with 3% gelatin, individually exposed to genus specific anti-LPS antibody prepared in mouse or rabbit, washed, probed with a horseradish peroxidase (HRP)-linked antimouse or antirabbit IgG secondary antibody, washed, and reacted with HRP-amplifying reagents to give a purple color [33]. Negative controls included pyrogen-free water as specimen; PKD and control specimens were processed as experimental, except that incubation with the primary antibody was omitted. Positive controls used genus-specific LPS as the test specimens. The intensity of HRP reaction was visually estimated and rated from 0 to 5, the greatest intensity. Monoclonal antibodies (mAbs) against “*Nanobacterium sanguineum*” antigens were used separately as the primary antibody in the dot immunoblot assays described earlier in this article. [Note: “*Nanobacterium sanguineum*” is the type culture designate for nanobacteria. It has been deposited in the German Collection of Microorganisms (DSMZ no. 5819; Braunschweig, Germany) and is described in U.S. patent no. 5,135,851,1992. The 16S rRNA gene sequence is available in GENBANK (accession No. X98418). “*N. sanguineum*” was isolated from bovine serum and is the source of antigens used in the preparation of mAbs.] Dot-immunoblot assays used the following antibodies [34]: Enteric mAb WN122.5 for rough/smooth LPS of *Escherichia coli*, *Salmonella*, *Shigella*; *Bacteroides* “C” (common LPS antigen) polyclonal antiserum; mAb B7 to *Chlamydia* spp. LPS; mAb Nb 8/0 (porin protein epitope) and Nb 5/2 (carbohydrate peptidoglycan epitope) of nanobacteria [24]; and hyperimmune antisera to *Bartonella henselae* from infected mice (gift of K. Karem) [35]. The antibody to *Bacteroides*-LPS used in this study reacted with LPS purified from *Bacteroides fragilis*, the predominant species associated with the colonic mucosa [36]; however, the antibody did not react with LPS of *B. vulgatus*, the predominant species in the colonic lumen and feces. The dot-immunoblot and immunofluorescence (IF) procedures were used to

**Table 1.** Findings of endotoxin and nanobacteria by several methods in kidney and liver samples from patients with cystic kidney disease

Donor no. Patient data <sup>a</sup>	Endotoxin by dLAL <sup>b</sup> p/t; EU/mL	Endotoxin by dot immunoblot (IB) (No. positive/tested; IB score) <sup>c</sup>			Nanobacteria by dot immunoblot, electron microscopy, and culture (p/t) <sup>c,d</sup>			
		<i>E. coli</i>	<i>B. fragilis</i>	<i>Chlamydia</i>	Nb 8/0	Nb 5/2	EM	Culture
(1) AD 46y/F HD	2/9;0.51	7/9;3.4	3/9;2.0	7/9;2.3	2/3;3.5	2/3;4.0	Yes	1/2
(2) AD 56y/F T and Liver CF	4/21;0.11 1/10;0.04	6/21;2.7 10/10;3.3	4/21;2.3 2/10;3.0	12/21;3.6 10/10;3.7	6/6;2.3 10/10;3.9	5/6;1.7 10/10;3.2	Yes	2/2 0/1
(3) AD 38y/M HD	3/23;0.38	13/22;3.4	7/22;3.0	12/22;2.9	9/9;2.8	9/9;2.7	Yes	2/2
(4) AD 60y/M T	3/20;0.19	17/22;3.6	5/22;2.6	16/22;2.9	8/8;2.9	8/8;2.6		0/2
(5) AD 47y/F T	2/14;0.12	12/16;3.1	3/16;1.3	10/16;2.8	4/6;2.5	5/6;2.0	Yes	2/2
(6) AD 47y/F HD	3/11;0.20	4/4;2.0	0/4;0.0	1/4;3.0	3/6;2.3	3/6;2.7		0/1
(7) AD 43y/F PD	4/13;0.21	8/8;3.1	5/8;1.3	6/9;3.7	7/7;3.3	7/7;1.7	Yes	2/2
(8) AD 37y/M	2/11;3.84	6/6;3.0	4/6;2.0	2/6;2.0	3/4;4.0	3/4;2.3	Yes	2/2
(9) AD 41y/F	3/16;0.88	9/9;2.2	4/8;1.7	6/9;2.4	2/4;2.5	2/4;1.5		1/1
(10) AD 52y/F	4/6;1.71	6/6;3.3	1/6;4.0	3/5;2.3	3/4;2.5	3/4;3.0		2/2
(11) AD 45y/F	3/5;0.48	5/6;3.6	4/6;2.5	6/6;2.5	3/3;2.7	3/3;3.7		2/2
(12) AD 48y/M	11/20;1.60	8/8;3.4	6/8;2.3	9/9;2.9	4/4;2.5	4/4;3.5	Yes	1/1
(13) SC 48y/F	200 mL;3.84	4.0	2.0	3.0	4.0	4.0	Yes	Yes
(14) AD 40y/F HD T					9/11;3.6		Yes	15/15

<sup>a</sup>AD, autosomal dominant PKD; SC, simple cyst fluid; age in y(ears)/F(emale), M(male); HD, hemodialysis; T, transplanted; PD, peritoneal dialysis; Liver CF, cyst fluid

<sup>b</sup>dLAL, differential Limulus Amebocyte Lysate assay; p/t, positive finding/per number of cyst fluids tested; EU/mL, endotoxin units per mL (1 EU = ~0.1 ng of standard *E. coli* LPS). Blank cells indicate not tested.

<sup>c</sup>Dot-immunoblot assays used the following Abs: Enteric mAb WN1 222.5 for rough/smooth LPS of *E. coli*, *Salmonella*, *Shigella*, *Bacteroides* "C" (common LPS antigen) polyclonal antiserum; mAb B7 to *Chlamydia* spp. LPS; mAb Nb 8/0 (porin protein epitope) and Nb 5/2 (carbohydrate peptidoglycan epitope) of *Nanobacterium*. A visual scoring scheme rated the intensity of the immunoreactions from 0 to 5, the highest positivity. Positive reaction scores were averaged.

<sup>d</sup>EM, transmission electron microscopy: Yes indicates the presence of structures (Figs. 1 and 2) compatible with type species designate, "*Nanobacterium sanguineum*."

assess any cross-reactivity of primary antibodies between the various LPSs and "*N. sanguineum*" [19]. Negative control was assayed without primary antibody.

Dissected kidney tissue and pellets of cyst fluids (15,000 × g, 45 min) were prepared for electron microscopy by initial fixation in cold 2.5% glutaraldehyde and processing as previously described [37]. For nanobacteria, uranyl acetate staining was omitted without loss or alteration of nanobacterial morphology.

For culture of nanobacteria (donors 1 through 13), frozen original cyst fluids or urines were thawed; 0.5 mL aliquots of the original and filtered (0.22 μ pore size filter) were placed in 5 mL of RPMI-1640 with L-glutamine (GIBCO, Paisley, Scotland, UK) supplemented with 10% high γ-irradiated (30 kGy), heat-inactivated fetal bovine serum (Hγ-FBS; Atlanta Biologicals, Atlanta, GA, USA) in T-25 tissue culture flasks and incubated at 37°C in a humidified 5% CO<sub>2</sub>/95% air environment for three weeks [19–21, 26]. The identification of nanobacteria used phase contrast microscopy, IF assay with Nb 8/0 as the primary antibody, differential DNA-staining using Hoechst 33258 fluorochrome (Flow Laboratories, Ayrshire, Scotland, UK) that distinguishes nanobacteria from common bacteria, and a double staining method that combined both IF and Hoechst methods [19, 20, 23, 24, 26]. The results were recorded by light and fluorescence photomicroscopy. TEM was also used to visualize nanobacteria in specimens with the type culture designate serving as the control for growth and morphologic comparison.

For ADPKD patient 14, cyst fluids were collected as mentioned previously in this article, but were cultured within two hours of nephrectomy. Aliquots (0.5 mL) of nonfiltered and filtered (0.45 μ pore size; Millipore Corp., Bedford, MA, USA) cyst fluid were placed in 5 mL of RPMI-1640 (Cellgro Mediatech Inc., Herndon, VA, USA) with and without 10% Hγ-FBS (Life Technologies, Grand Island, NY, USA) in T-25 flasks and incubated as previously in this article. After one month, the cultured cyst fluids were examined by phase light and electron microscopy. Sterile controls were media with and without Hγ-irradiated serum.

An initial determination of cytotoxic potential of nanobacteria from PKD patients used a 3T6-fibroblast (ATCC CCL 96) test system described elsewhere [26]. The loss of fibroblasts caused by cell lysis was easily observed by direct microscopic inspection.

Nanobacterial growth in response to tetracycline and citrate solutions was assessed in 96-well plates by recording the increase in optical density at 650 nm over a 14-day period [22, 25]. Serial dilutions of potassium citrate-citric acid solution described by Tanner were made in Dulbecco's modified Eagle's medium with 1 mmol/L glutamine (DMEM; Sigma, St. Louis, MO, USA) [38].

## RESULTS

Endotoxin or its remnants were found in all PKD kidneys examined in this study. Table 1 lists our findings of endotoxin by dLAL and dot-immunoblot method.

For each patient, the number of cyst fluids found to be positive by dLAL for endotoxin per the number of fluids tested (p/t) is given. For the dLAL assay, the average level of detected endotoxin expressed in endotoxin units (EUs)/mL cyst fluid is given for those cysts positive for endotoxin. In patients 1 through 7, all of whom had been on dialysis or received a kidney transplant prior to nephrectomy, the average endotoxin load (0.245 EU/mL) was sevenfold lower than for patients 8 through 12 (1.70 EU/mL) who had not received renal replacement therapy. The incidence of dLAL positivity for cyst fluids within each kidney was also lower in the renal replacement group (20.2 vs. 43.7%, respectively).

In ADPKD patients, there was a greater rate of positivity by dot-immunoblot for endotoxin than by dLAL (Table 1). The LPS antibodies recognized epitopes in the core polysaccharides of LPS and thus did not require an intact lipid A moiety for reactivity. A difference in the rates of dLAL and dot immunoblot positivity suggests the presence of LPS remnants, which was observed in all PKD kidneys examined in this study.

"*Nanobacterium sanguineum*" was positive for endotoxin, but not glucan by the dLAL assay; pretreatment of "*N. sanguineum*" and nanobacteria from donor 14 with EGTA prior to dLAL assay enhanced endotoxin positivity eightfold. By TEM, PKD kidney tissue and cyst fluids exhibited mineralized structures compatible with nanobacteria (Figs. 1 and 2); neither *Chlamydia* nor *Bartonella*-like structures nor other common microbes were observed. Antibodies Nb 8/0 and Nb 5/2 against "*Nanobacterium sanguineum*" showed a high incidence of positivity in kidney cysts from all 13 ADPKD patients (Table 1) [19, 23]. Positivity was also observed in liver cyst fluids and simple kidney cyst fluid where available. As exemplified by Figure 1H, common bacteria were not present, as determined by Hoechst staining, in the nanobacteria cultures from PKD cyst fluids.

During checks of antibody specificity, chlamydial LPS mAb was observed to react with washed, intact nanobacteria. This mAb did not cross-react with purified LPS from either *E. coli* (ATCC #25922) or *B. fragilis* (ATCC #25215), reference organisms used by the National Committee for Clinical Laboratory Standards. "*Nanobacterium sanguineum*" did not react with the other LPS antibodies. This raised the possibility that our positive findings of chlamydial LPS in cyst fluids could be due to the LPS of nanobacteria.

Of the 66 kidney and liver cyst fluids assayed using mAb to chlamydia LPS and nanobacterial porin and peptidoglycan antigens (Nb 8/0 and Nb 5/2; Table 1), 39 were positive with all three mAbs, 18 positive for only Nb 8/0 and Nb 5/2, 7 negative for all three, and 2 positive only for chlamydial LPS. Thus, nearly all of the reactivity of cyst fluids could be accounted for by nanobacterial epitopes or cross reaction with mAb to chlamydial LPS;

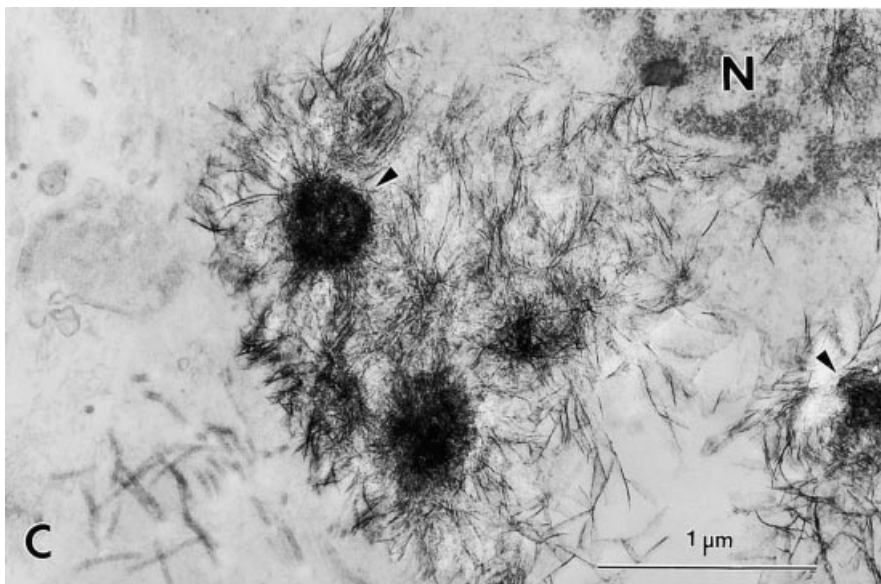
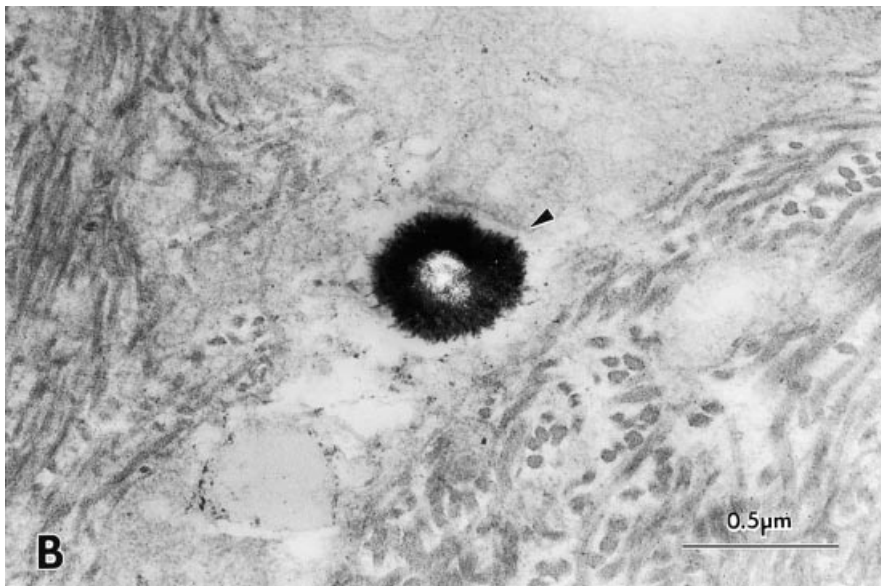
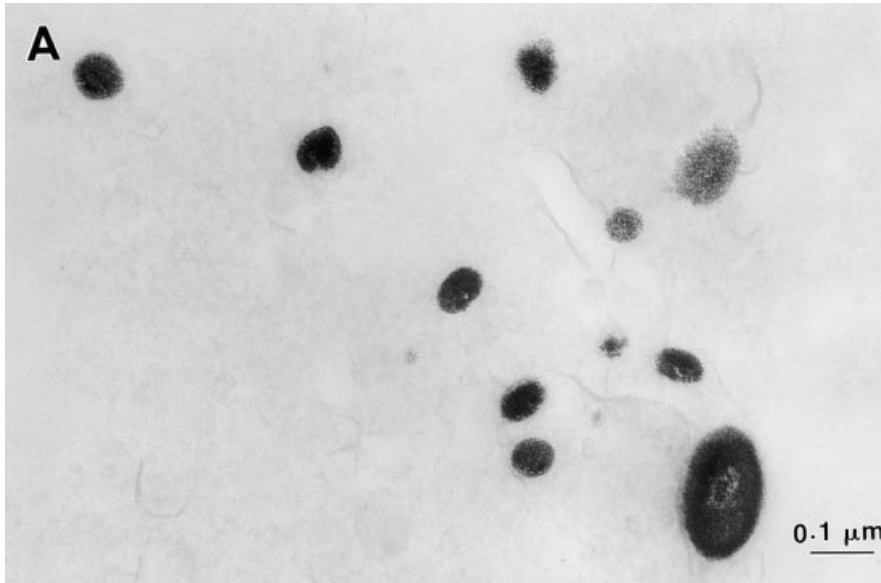
these data do not exclude the possibility that chlamydial LPS is also present. The lack of strict stoichiometry across microbial epitopes may reflect different rates of generation, release, and degradation within cyst fluid or delivery of epitopes to cysts from other sites.

By dot-immunoblot assay, 10 of 10 liver cyst fluids positive for nanobacteria were also positive for *Bartonella* (donor 2; Table 1). In 31 kidney cyst fluids selected from the ADPKD donors 1 through 12, a very similar but not identical distribution of antigens for these organisms was observed. Twenty-one fluids were positive for both *Bartonella* and nanobacteria (Nb 8/0 and 5/2). Seven were negative for both organisms. One was weakly positive only for nanobacteria (Nb 8/0 and 5/2), and two were weakly positive only for *Bartonella*. Washed, intact nanobacteria were negative by dot-immunoblot assay to hyperimmune serum to *B. henselae* (HS-Bh). However, incubation of nanobacteria at 37°C for one hour in 100 mmol/L EGTA rendered the decalcified nanobacteria reactive with HS-Bh; no reaction was observed when HS-Bh was omitted from the assay. Simple kidney cyst fluid from donor 13 also reacted with these antibodies (Table 1). HS-Bh did not react with ATCC reference strains of *E. coli* or *B. fragilis*.

In an earlier study, we found endotoxin in the urine of a majority of PKD patients, but only comparatively low levels in control females and none in control males [17]. Here, we assayed archived urine from the previous study by dot-immunoblot using Nb 8/0 and WN1 222.5 (Table 2). Nb 8/0 gave the highest positivity in PKD males with normal males showing greater positivity than normal females. In one PKD male, four sequential urine samples collected over a two year period exhibited Nb8/0 positivity (scores 3, 1, 2, 4), while three urines from each of two PKD females remained negative over this same period. The antibodies against *E. coli* LPS was reactive with all PKD male urines; 4 of 10 of the normal females were also WN1 222.5 positive.

Structures consistent with nanobacteria were observed by TEM in PKD kidney tissue (Table 1 and Fig. 1). Nanobacteria were cultured from frozen kidney cyst fluids from donors 1, 2, 3, 5, 7 through 13 and identified by IF methods (Table 1 and Fig. 1). They were also found by culture and IF in blood and urine samples from a 23-year-old Finnish male PKD patient. Multiple kidney cyst fluids were cultured without prior freezing from donor 14 (Table 1), who had received both hemodialysis for 10 months and a kidney transplant 4 months prior to nephrectomy. All 15 nonfiltered cyst fluids grown in serum for four weeks were nanobacteria positive by light microscopy. In selected cultures, TEM (Fig. 2 c, g) and dot-immunoblot with Nb 8/0 confirmed the growth to be nanobacteria. Growth media plus serum alone were negative by dot-blot, light, and electron microscopy (Fig. 2 a, e). Of the 13 filtered cyst fluids, 11 were nanobacteria





**Fig. 1. Transmission electron micrographs (TEM) and photomicrographs of human polycystic kidney disease (PKD) kidney and organisms cultured from cyst fluids showing structures typical of *Nanobacterium*.** (A) TEM of a PKD kidney section from donor 14 (not stained with uranyl acetate) showing electron-dense structures of sizes typical of nanobacteria (80 to 500 nm) [23]. (B–D) TEM of kidney sections stained with uranyl acetate showing nanobacteria (arrow; B; patient 1) with hairy apatite layer surrounding a central cavity adjacent to a tubule cell (C; patient 7; N, cell nucleus) and with multiple apatite spicules in kidney cell cytoplasm, and (D; patient 8) embedded in basal lamina adjacent to tubule cell. (E) TEM of pelleted kidney cyst fluid showing nanobacteria (arrows) surrounded by cellular debris (patient 13). (F and G) IF micrographs of nanobacteria (arrows) cultured from kidney cyst fluid from patients 7 (F; in suspension) and 8 (G; bound to surface of 3T6 cells), fixed, and probed with Nb 8/0 followed by FITC-conjugated rabbit antimouse IgG. (H and I) IF micrograph of Nb8/0-positive bacterium cultured from patient 13 that has been internalized by 3T6 fibroblasts (H) and same field stained with Hoechst 33258 (0.5 μg/mL) to visualize 3T6 cell nuclei (I) [26]. The absence of bacterial staining in (I) indicates the absence of classic bacteria that might have been carried along during culture and testing; nanobacteria are not stained by these conditions, but will stain at 5 μg/mL and longer incubation times.

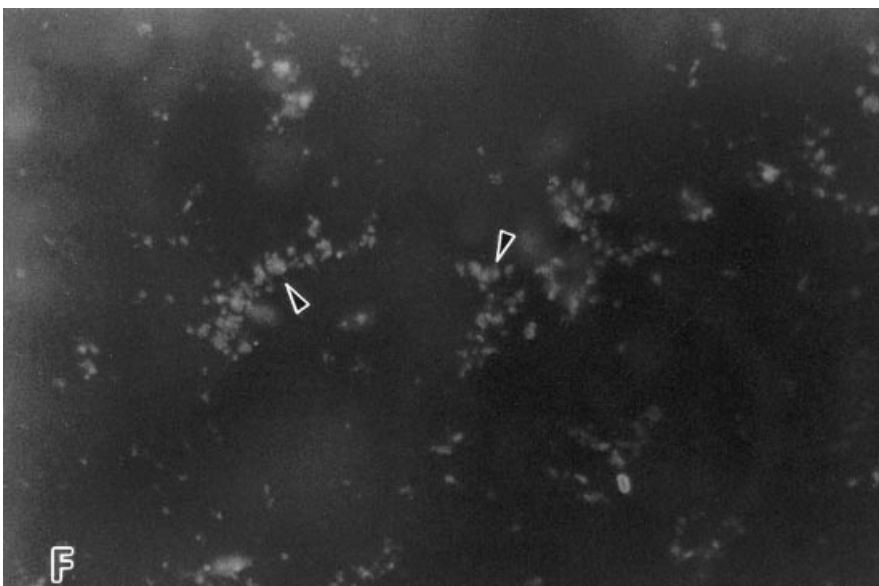
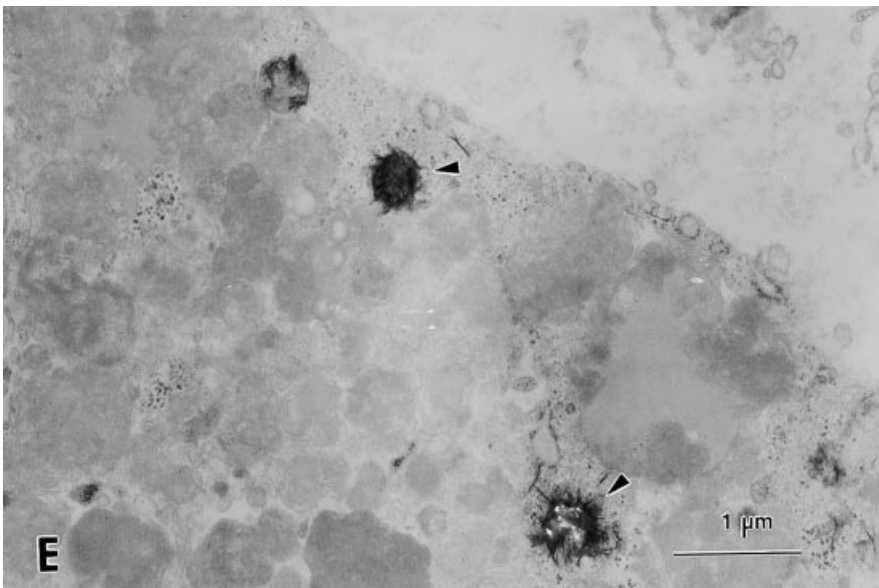
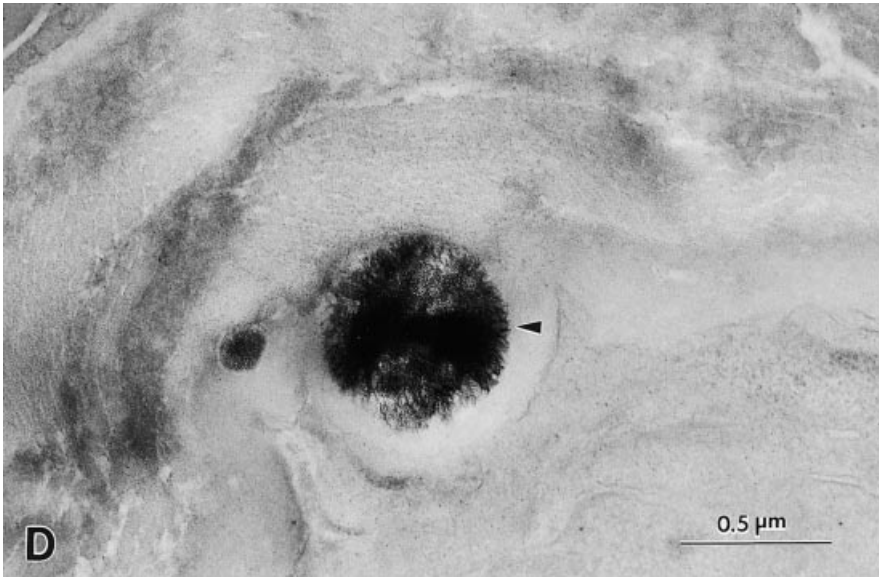


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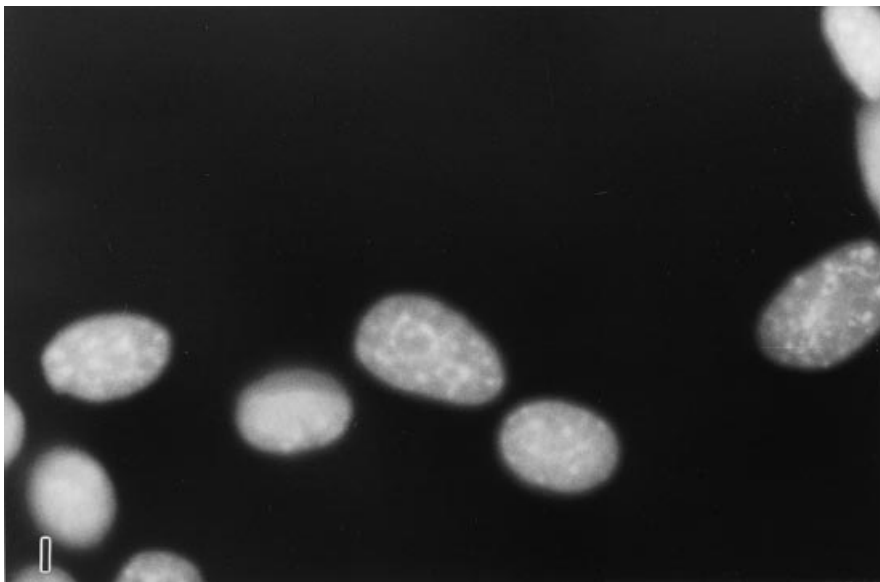
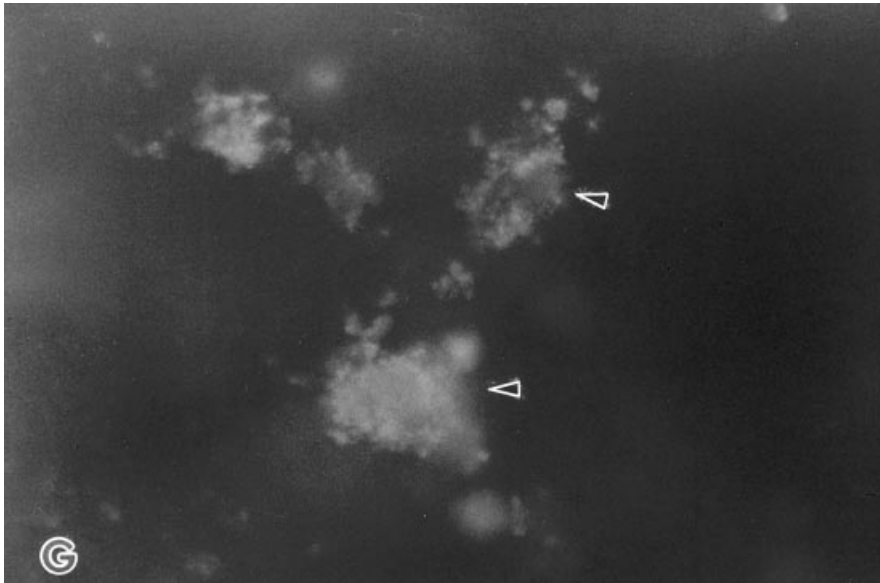
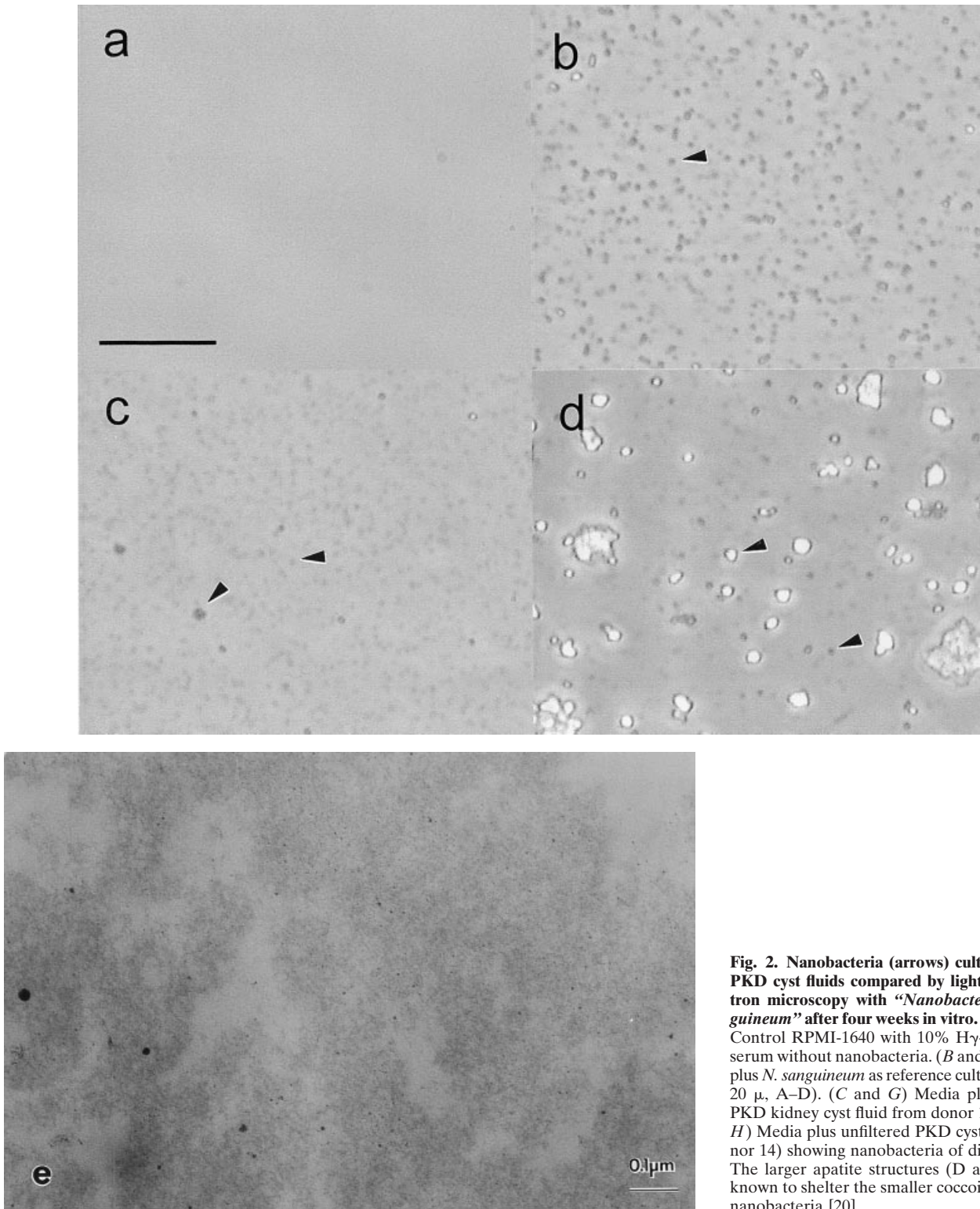


Fig. 1. (Continued).





**Fig. 2. Nanobacteria (arrows) cultured from PKD cyst fluids compared by light and electron microscopy with “*Nanobacterium sanguineum*” after four weeks in vitro.** (A and E) Control RPMI-1640 with 10% H $\gamma$ -irradiated serum without nanobacteria. (B and F) Media plus *N. sanguineum* as reference culture (bar = 20  $\mu$ , A–D). (C and G) Media plus filtered PKD kidney cyst fluid from donor 14. (D and H) Media plus unfiltered PKD cyst fluid (donor 14) showing nanobacteria of diverse size. The larger apatite structures (D and H) are known to shelter the smaller coccoid forms of nanobacteria [20].



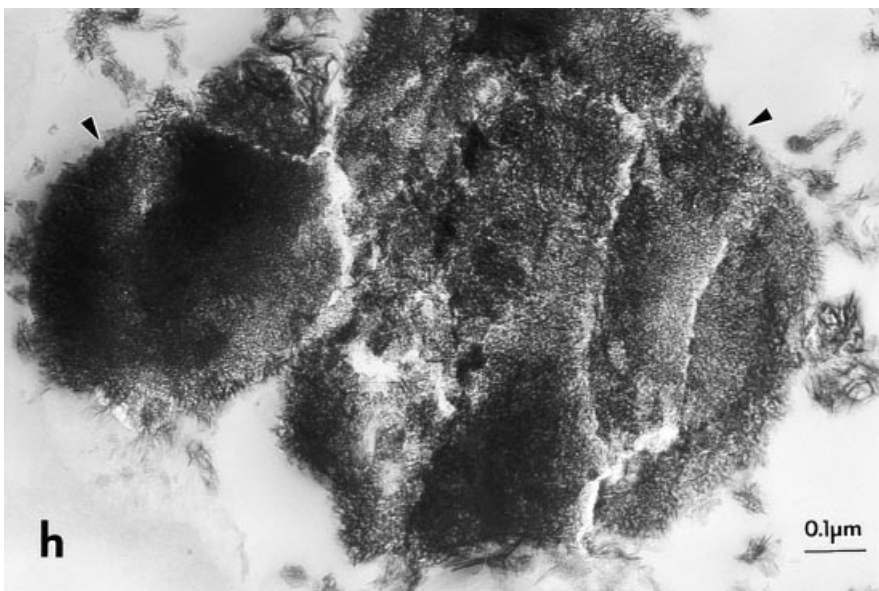
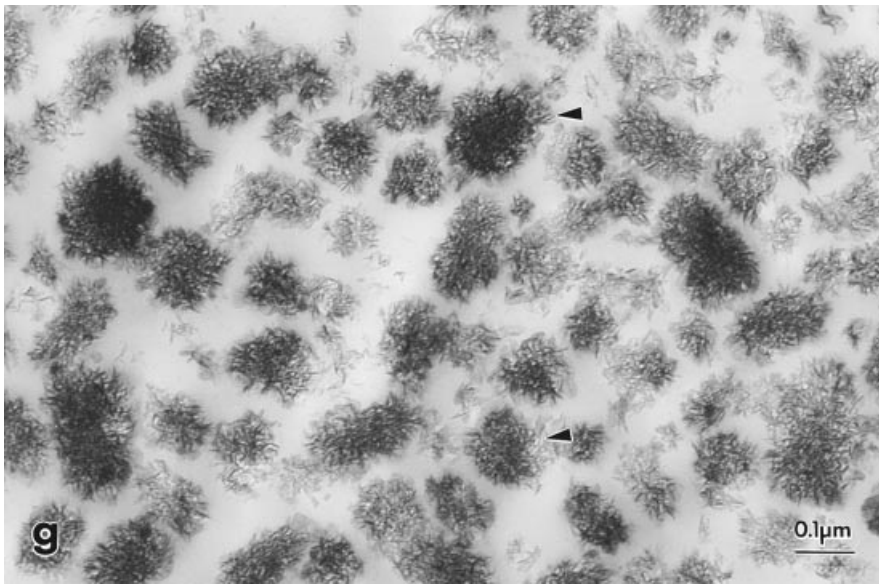
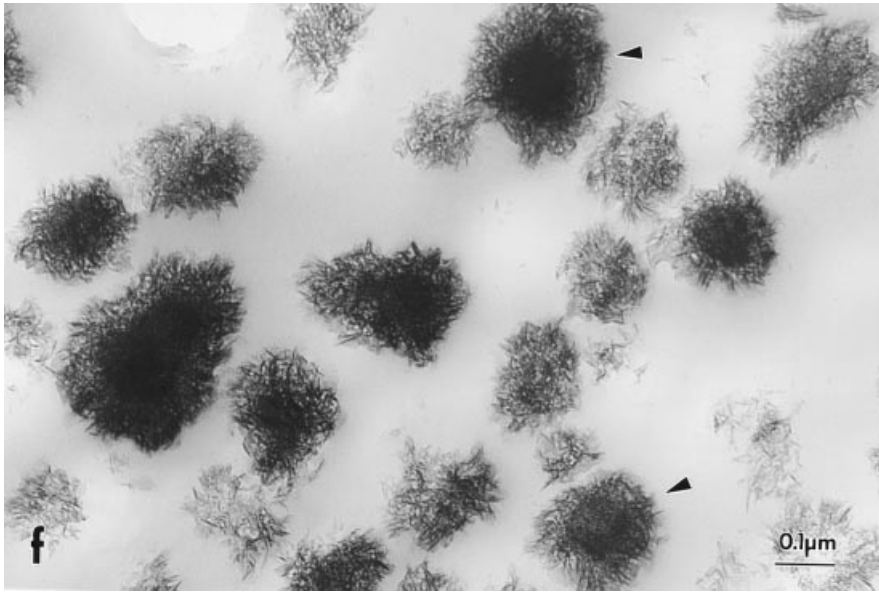


Fig. 2. (Continued).

**Table 2.** Assay of urines from control and PKD patients for endotoxin and nanobacterial antigen

Urine donor groups	Dot immunoblot <sup>a</sup>		LAL assay <sup>b</sup>
	Nb 8/0	WN1 222.5	
PKD males	7/7; 3.1	7/7; 2.2	5/7; 1.24 EU/mL
PKD females	1/7; 1.0	0/7	5/7; 1.36 EU/mL
Control males	3/10; 2.0	0/10	0/10
Control females	1/10; 1.0	4/10; 1.5	5/10; 0.26 EU/mL

<sup>a</sup>Dot immunoblot results given as number positive of number tested; the scoring of HRP-reaction intensity is from 0 to 5. Positive reaction scores were averaged. Nb 8/0 (nanobacterial porin protein epitope); WN1 222.5 (*E. coli* LPS epitope)

<sup>b</sup>Classic LAL assay detects the lipid A portion of endotoxin, but also reacts with fungal glucans [17]; positive findings averaged with estimates of EU/mL based on serial dilutions

positive when grown in serum-fortified media, and in serum-free media only 6 showed growth by light microscopy. For nonfiltered cyst fluids cultured in serum-free media, 10 of 15 were culture positive by light microscopy. By TEM and light microscopy, nanobacteria isolates from donor 14 were indistinguishable from “*Nanobacterium sanguineum*” incubated in media with and without serum for four weeks (Fig. 2 b, f).

In two flasks of nonfiltered cyst fluid incubated in serum-free media, nanobacteria were observed to be bound to and present within PKD renal epithelial cells that were present in the aspirated cyst fluid (Fig. 3). By direct-phase microscopy, nanobacteria were observed to be actively endocytosed by the PKD cells in vitro (data not shown). The epithelial cells exhibited intracellular structures typical of highly mineralized forms of nanobacteria (Fig. 3B) and other pleomorphic forms of nanobacteria in vitro (Fig. 3C) [19, 20, 23]. Conditioned media from this cell-containing culture of cyst fluid were positive for endotoxin by the dLAL assay; both the filtered and unfiltered cyst fluids grew nanobacteria.

Using a 3T6-fibroblast test system, nanobacteria isolates from donors 1, 7, 8, 12, and 13 were screened for cytotoxicity. These isolates caused cytotoxicity that ranged from mild to severe (20 to 100% cell loss compared to control cultures) during the first 24 hours of exposure [26]. A loss of cells was estimated by microscopic inspection. Within 15 minutes of exposure to 3T6 cells, nanobacteria demonstrated binding to the cell surface (Fig. 1 F, G). When examined after 18 hours of incubation, sequestration of IF signal within the 3T6 cells was apparent (Fig. 1H).

The initial test of PKD-derived nanobacteria susceptibility to an antimicrobial agent used a serum-derived nanobacteria isolate from a 23-year-old Finnish PKD patient and tetracycline at 4 µg/mL, which completely inhibited growth (Fig. 4). In the absence of inhibitors, absorbance increased, indicating growth of nanobacteria typical of a bacterial growth curve. Serial dilutions of potassium citrate-citric acid (55 mmol/L/67 mmol/L) in DMEM

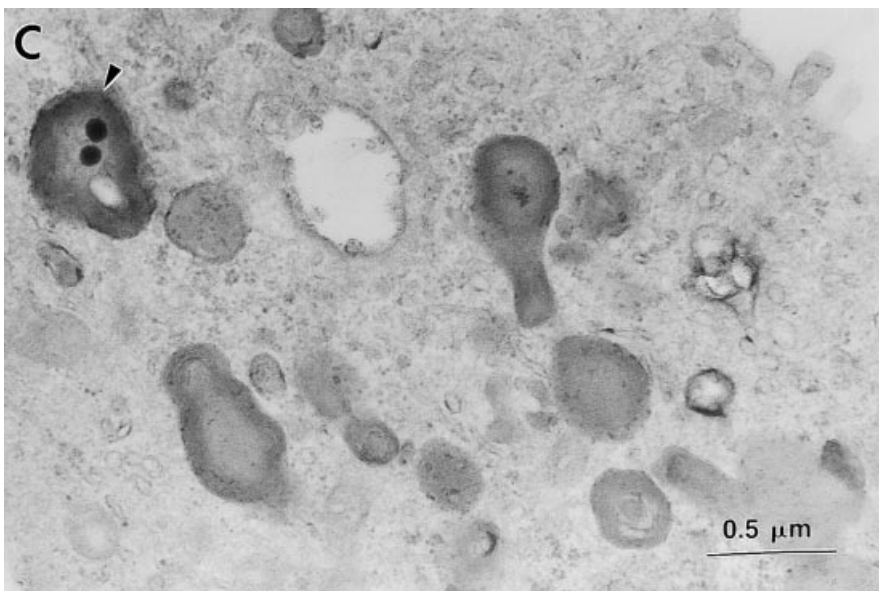
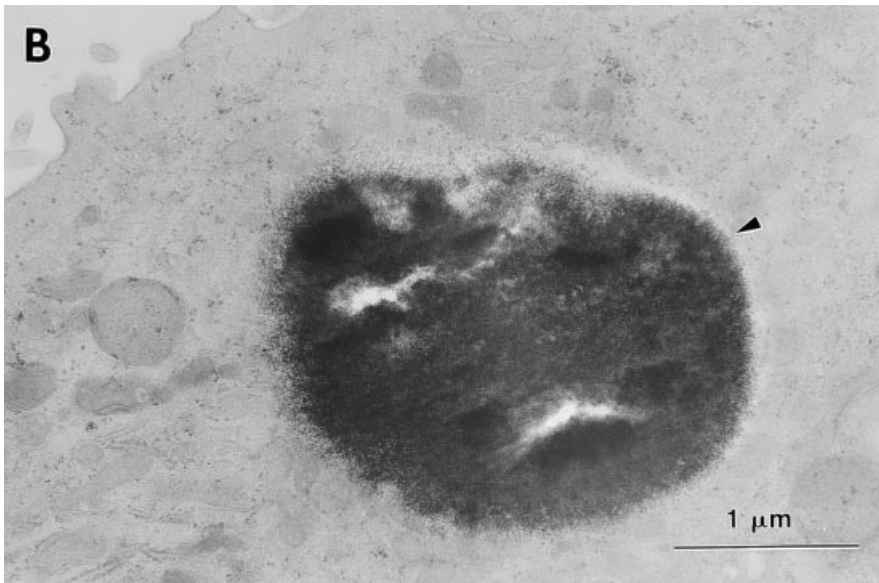
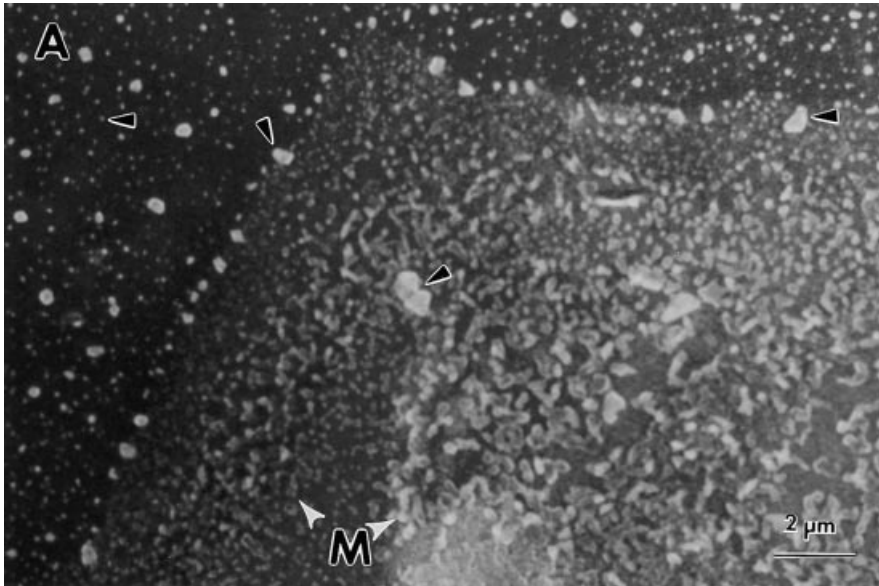
tested against “*Nanobacterium sanguineum*” revealed growth inhibition over the entire range of dilutions (0.05/0.06 to 28 mmol/L/33 mmol/L) tested.

## DISCUSSION

We have proposed that PKD is an emerging infectious disease in a genetically vulnerable population [9]. To establish the presence of nanobacteria in PKD kidneys, we used three criteria: nanobacterial morphology by EM, reactivity with mAb against nanobacteria, and growth (that is, multiplication) in culture [19–25]. Small size (80 to 500 nm) and calcification are two essential characteristics of nanobacteria morphology. These criteria make a compelling case for the presence of nanobacteria in PKD. The immunologic cross reactivity of nanobacteria with two other known pathogens (*Chlamydia* and *Bartonella*) requires the use of multiple criteria to establish identity. Sequencing of novel nanobacterial proteins for use as antigens may yield additional immunologic probes for nanobacteria in human specimens (Kajander, unpublished data). Poor penetration of the calcium coat by DNA probes may limit a DNA-based approach to clinical specimens (Kajander, unpublished data).

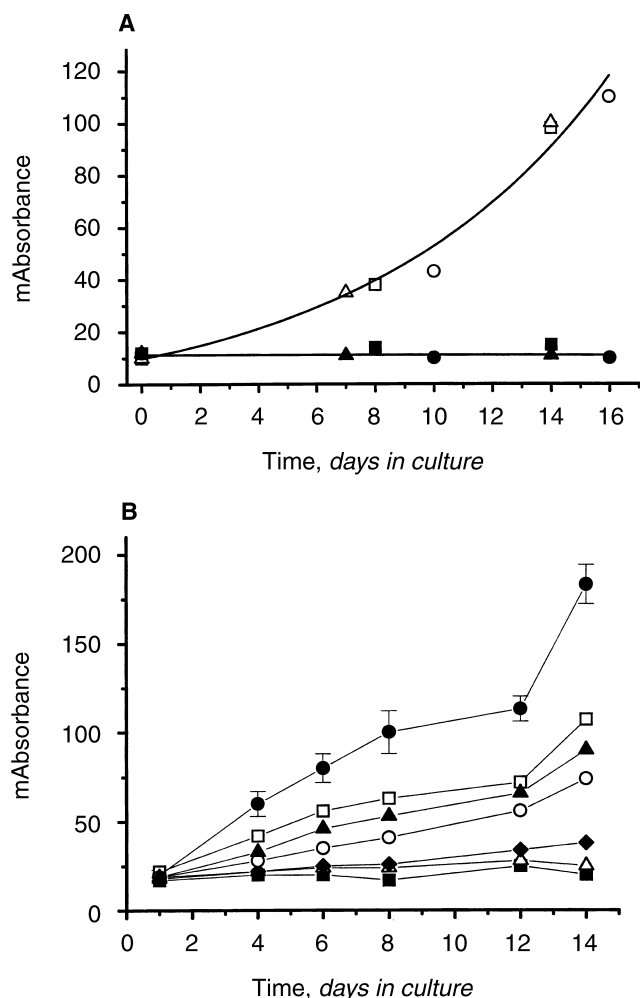
It is possible that humans are routinely exposed to nanobacteria and clear them as a matter of normal physiology. Five percent of 1000 Finnish volunteers exhibited nanobacteremia [23]. In this United States-based study, control males (30%) and females (10%) exhibited nanobacterial antigen in their urine (Table 2). In an unpublished work (Hjelle), 20 of 50 consecutive non-PKD human kidney biopsies examined thoroughly by EM for nanobacteria showed structures compatible with nanobacteria. Only patients with compromised kidney function (nondialyzed) were biopsied. As 60% of human kidney biopsies did not show evidence of nanobacteria, they might serve as a surrogate for control human kidney tissue. At this time, it is not known whether these findings represent episodic/continuous infections or colonizations. Theoretically, disease would occur if (1) nanobacteria could not be cleared, (2) there was infection by virulent species/strains of nanobacteria, or (3) genetic/environmental factors resulted in enhanced vulnerability to this microbe. All of these are possible in PKD.

Regarding these three general points, (1) the kidney may play an important role in the clearance for this tiny, calcium-coated cytotoxic organism. Akerman et al have reported that radiolabeled, viable nanobacteria appeared in urine within 15 minutes of their intravenous injection into rabbits [27]. By TEM and silver staining, nanobacteria were associated with the tubule epithelium and fluid. Nanobacteria were not observed in the control animals. Apoptosis of tubule cells at the site of nanobacterial localization was observed. Dysregulation of apoptosis and tubular obstruction are frequently observed in PKD



**Fig. 3. Electron micrographs of PKD kidney epithelial cells and nanobacteria present in aspirated cyst fluid that was cultured five weeks in vitro. (A)** SEM revealed nanobacteria (arrows) bound to the plastic flask, accumulated at the cell margin, and on the surface of the cell (M, microvilli; white arrows). **(B)** TEM shows highly mineralized form of nanobacteria (arrow) within the epithelial cell cytoplasm. **(C)** Also prominent were less mineralized, somewhat lamellar structures containing coccoid forms of nanobacteria (arrows) [20].





**Fig. 4. Effect of 4 µg/mL tetracycline (A) and potassium citrate/citrate concentration (B) on nanobacterial growth in vitro.** (A) Growth of nanobacteria isolated from PKD serum in the absence (open symbols) and presence (solid symbols) of drug (3 separate experiments). (B) Growth of "*Nanobacterium sanguineum*" in the presence of increasing K<sup>+</sup> citrate/citrate levels: (●) zero, mean ± SD, N = 3; (□) 0.05/0.06 mmol/L; (▲) 0.42/0.5 mmol/L; (○) 0.85/1.0 mmol/L; (◆) 1.7/2.1 mmol/L; (△) 3.5/4.1 mmol/L; and (■) 7.0/8.2 mmol/L. Means, N = 3, are shown for the citrate groups. Higher concentrations of citrate solution completely inhibited growth (data not shown).

pathophysiology [28, 29, 31]. An inability to clear obstructed tubules would lead to retention of nanobacteria, thereby providing selection pressure for the emergence of more progressively cystic phenotypes [7, 8, 10, 11]. Accumulation of nanobacteria caused by the lack of renal clearance would be consistent with their presence within poorly drained PKD cysts and simple kidney cysts and the 80% rate of nanobacteremia in hemodialysis patients [23]. Interestingly, 80% of non-PKD dialysis patients will develop acquired kidney cysts [6].

(2) Pathogenic and nonpathogenic strains of nanobacteria are possible given the range of cytotoxicities (no toxicity to 100% cell death) produced by isolates from

a variety of animal sources [26]. The five random nanobacterial isolates from separate cystic kidneys (4 PKD, 1 simple cystic disease) tested in this study exhibited cytotoxicity. The cytotoxic components of nanobacteria are yet to be identified, but candidate toxins include its putative chlamydia-like endotoxin reported here and its mineral coat, which may contribute to free-radical formation [39]. The LPS of *Chlamydia pneumoniae* is reported to be a key component in its atherogenic effect [40]. Endotoxin from at least two other genera of bacteria was identified in cyst fluid. Thus, a portion of the renal inflammation observed in PKD kidney [10] may be related to the presence of endotoxin, a potent inflammatory agent and nephrotoxin.

Related and cross-reacting pathogens may share similar virulence factors and mechanisms of toxicity. Maurin et al reported serological cross reactions between *Bartonella* and *Chlamydia* species for LPS and non-LPS epitopes [41]. We found hyperimmune sera to *B. henselae*, a close phylogenetic relative of nanobacteria, reacted with "*Nanobacterium sanguineum*" and cyst fluids from all 12 PKD patients examined. Kajander et al observed that Nb 5/2, but not Nb 8/0, reacted with intact *Bartonella* [19]. Interestingly, patients with bacillary peliosis caused by *B. henselae* develop cystic liver lesions [42]. *Bartonella* sp. are also linked to cardiac and vascular lesions [43]. In PKD, cardiac and vascular lesions are a leading cause of death, and liver cysts are found in 77% of patients by age 60 [6]. The expression of *Bartonella*-induced pathology is influenced by the patient's immunocompetency [43], a concept of potential relevance to PKD patients and their reported heightened vulnerability to infection [14].

Our finding of reactivity of antichlamydial LPS with nanobacteria and cyst fluid suggests the presence of a chlamydia-like endotoxin. This raises the possibility that certain routine immunologic tests may not distinguish nanobacteria from *Chlamydia* sp. The nanobacteria-specific mAb Nb 8/0 used in this study did not react with intact *C. pneumoniae* (Kajander, unpublished observations). *C. pneumoniae* has recently been implicated by immunologic methods in the calcifying cardiovascular disease observed in patients with renal failure [44], but *C. pneumoniae* has not been reported to cause tissue calcification. Carson has proposed that all extraskeletal calcifications are caused by nanobacteria, the only known blood-borne, calcium apatite-forming microbe [45].

(3) Four points (a through d) relate to genetic/environmental factors that impact PKD. (a) The distribution of PKD lesions in kidney, liver, vasculature, spleen, heart, and colon follows the expression of polycystin 1, one of two proteins regarded as defective in PKD [11] and that we have speculated may bind microbes or their components [9]. In this study, we found nanobacteria and/or its antigens consistently in PKD kidneys, the available multiple liver cysts from one patient and blood and urine

of another. While it is unknown whether nanobacteria bind to normal or PKD-defective polycystins, nanobacteria of PKD origin were found bound to and present in human PKD kidney cells in vivo and in vitro (Figs. 1 and 3) and rodent fibroblasts in vitro (Fig. 2). Cytotoxicity was evident. Endocytosis of nanobacteria or its remnants was required to induce apoptosis in fibroblasts [26], a cell type that expresses polycystin 1 [11]. Calvet has proposed that PKD involves a defect in cellular repair mechanisms that would make PKD individuals especially vulnerable to commonly encountered renotoxic environmental factors [11, 46].

(b) Kidney calcifications become several-fold more prevalent in PKD individuals than in the general population [30]. Nanobacteria are now strongly linked to kidney stone formation [24]. Hypocitraturia is a risk factor for kidney stones [47]. The relationship between kidney calcification and cystogenesis is unclear. However, exogenous citrate prevented loss of kidney function in genetically PKD rats [38]. Dietary flaxseed meal, which increased citrate levels in the kidney, also preserved kidney function in genetically PKD rats [48]. Based on our observation that citrate inhibits nanobacterial growth in vitro (Fig. 4B), we propose that citrate protects against nanobacteria-driven stone formation in general and loss of kidney function and reduced cyst volumes in PKD.

The mineral coat of nanobacteria may play a role in the concentration, persistence, and resultant killing action of tetracycline, a drug known to bind calcium and used in the treatment of diseases with pathological calcifications. Putative *C. pneumoniae* infections have been treated with tetracycline, a drug that also inhibited the growth in vitro of PKD and animal isolates of nanobacteria (Fig. 4A). Ciftcioglu et al reported that tetracycline was bactericidal for nanobacteria at a minimum inhibitory concentration of 0.3 µg/mL (abstract; Ciftcioglu et al, *Gen Meet Am Soc Microbiol* 24, 1999). Tetracycline is considered bacteriostatic against other susceptible bacteria. Nanobacteria may have been the unintended target of therapies used in a variety of chronic diseases with calciferous lesions.

(c) The finding of *Bacteroides* LPS antigen in PKD kidney coupled with an 80% rate of colonic anomalies in PKD patients raises the possibility that enhanced leakage of microbes and their components from the gut into blood may occur in PKD (Table 1) [6, 15]. *Bacteroides* species are the predominant bacteria of the colon and are rarely associated with urinary tract infections (UTIs). In contrast, *E. coli* is the primary etiologic agent in UTI and a gut microbe. A dietary exposure to nanobacteria is probable given the prevalence of nanobacteria in bovine serum [19, 20] and their resistant to heat and  $\gamma$  radiation [22]. Disruption of gut integrity in PKD could account for the entrance of endotoxin, nanobacteria, and other microbial antigens found in PKD cyst fluid [9].

(d) Gender differences have been reported for the oc-

currence and severity of kidney cysts, which are greater in males, and liver cysts, which are greater in females [2, 6]. These consistent, but as yet unexplained gender differences in expression of PKD pathology and pharmacology [49] are now reflected in the occurrence of nanobacterial antigens in PKD urines (Table 2: PKD males > control males > PKD females  $\approx$  control females). In contrast, the endotoxin levels found in PKD individuals were greater than those found in control females. Findings of both *E. coli* LPS antigen and fivefold lower levels of Limulus Amoebocyte Lysate positivity in control females may be due simply to introital contamination (Table 2) [17].

In summary, we demonstrate that cytotoxic nanobacteria are present in PKD patients. Based on our data, we propose that the currently known cellular toxicities, tissue distribution, and pharmacology of nanobacteria are plausibly related to the known pathology and pharmacology of PKD. The mechanism responsible for the heightened vulnerability of PKD individuals to infection and the lesions of PKD is unknown, but must ultimately involve the loss of functional polycystins [10, 11]. Awareness that nanobacteria are present in PKD, and likely other kidney diseases and hemodialysis, may lead to improved therapies and management. Additional research is required to prove that nanobacteria promote PKD. Such infectious disease research will need careful attention to the resistance of nanobacteria to most disinfectants and routine sterilizing techniques (that is, autoclaving, radiation, filtration) [19, 22, 23], and the presence of nanobacteria in serum used for tissue culture [19, 20].

#### NOTE ADDED IN PROOF

A related review, *Microbes in PKD*, has recently been published: HJELLE JT, MILLER-HJELLE MA, NOWAK DM, DOMBRINK-KURTZMUN MA, PETERSON SW: Polycystic kidney disease, fungi, and bacterial endotoxin: Shifting paradigms involving infection and diet. *Rev Med Microbiol* 11:23–35, 2000.

#### ACKNOWLEDGMENTS

This research was supported by gifts from “X-Bugs: Friends of Microbes in Chronic Disease Research.” Portions of this work were presented as abstracts at the following meetings: 18th Annual Conference on Peritoneal Dialysis (Nashville, TN, USA; March 1998); International Conference on Emerging Infectious Diseases (Atlanta, GA, USA; March 1998); Fifth International Conference of The International Endotoxin Society (Santa Fe, NM, USA; Sept 1998); and 99th General Meeting of the American Society of Microbiology (Chicago, IL, USA; May 1999). We thank the PKD tissue donors and John Nixon and associates (St. Francis Medical Center-Peoria) for enabling the timely collection of tissues. We appreciate the generous gifts of LPS antibodies from Sheila Patrick (The Queen’s University of Belfast, Belfast, Ireland) and G. Robin Barclay (Scottish National Blood Transfusion Service, Edinburgh, Scotland, UK), and we thank Kevin Karem (Centers for Disease Control and Prevention, Atlanta) for his gift of *Bartonella* hyperimmune antisera and helpful discussions. We also thank Mary Ann Dombrink-Kurtzman, Stephen W. Peterson, and James Cooper for provocative and insightful discussions.

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## APPENDIX

Abbreviations used in this article are: ADPKD, autosomal dominant polycystic kidney disease; dLAL, differential Limulus Amebocyte Ly-sate assay; DMEM, Dulbecco's modified Eagle's medium; EUs, endotoxin units; HRP, horseradish peroxidase; IF, immunofluorescence; LPS, lipopolysaccharide/aka endotoxin; mAb, monoclonal antibody; PKD, polycystic kidney disease; TEM, transmission electron microscopy; UTIs, urinary tract infections.

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