

CULTIVATION OF THE BACILLUS OF WHIPPLE'S DISEASE

DIDIER RAOULT, M.D., PH.D., MARIE L. BIRG, BERNARD LA SCOLA, M.D., PH.D., PIERRE E. FOURNIER, M.D., PH.D., MARYSE ENEA, HUBERT LEPIDI, M.D., PH.D., VERONIQUE ROUX, PH.D., JEAN-CHARLES PIETTE, M.D., FRANÇOIS VANDENESCH, M.D., PH.D., DENIS VITAL-DURAND, M.D., AND TOM J. MARRIE, M.D.

ABSTRACT

Background Whipple's disease is a systemic bacterial infection, but to date no isolate of the bacterium has been established in subculture, and no strain of this bacterium has been available for study.

Methods Using specimens from the mitral valve of a patient with endocarditis due to Whipple's disease, we isolated and propagated a bacterium by inoculation in a human fibroblast cell line (HEL) with the use of a shell-vial assay. We tested serum samples from our patient, other patients with Whipple's disease, and control subjects for the presence of antibodies to this bacterium.

Results The bacterium of Whipple's disease was grown successfully in HEL cells, and we established subcultures of the isolate. Indirect immunofluorescence assays showed that the patient's serum reacted specifically against the bacterium. Seven of 9 serum samples from patients with Whipple's disease had IgM antibody titers of 1:50 or more, as compared with 3 of 40 samples from the control subjects ($P < 0.001$). Polyclonal antibodies against the bacterium were generated by inoculation of the microorganism into mice and were used to detect bacteria in the excised cardiac tissue from our patient on immunohistochemical analysis. The 16S ribosomal RNA gene of the cultured bacterium was identical to the sequence for *Tropheryma whippelii* identified previously in tissue samples from patients with Whipple's disease. The strain we have grown is available in the French National Collection.

Conclusions We cultivated the bacterium of Whipple's disease, detected specific antibodies in tissue from the source patient, and generated specific antibodies in mice to be used in the immunodetection of the microorganism in tissues. The development of a serologic test for Whipple's disease may now be possible. (N Engl J Med 2000;342:620-5.)

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WHIPPLE'S disease is a systemic bacterial infection characterized by fever, weight loss, diarrhea, lymphadenopathy, and polyarthritides and, occasionally, by cardiac manifestations such as myocarditis, pericarditis, and endocarditis^{1,2} or by central nervous system involvement.³ George Whipple described the disease in 1907,⁴ and its bacterial origins were confirmed by electron microscopy in 1961.⁵ The diagnosis is usually established on microscopy by the identification in a duodenal-biopsy specimen of infiltration by large

macrophages with bacteria positive for the periodic acid-Schiff (PAS) stain.⁶ In 1991 Wilson et al.⁷ used broad-range primers to amplify and sequence a portion of the 16S ribosomal RNA gene of the bacterium for Whipple's disease, allowing classification of the bacterium within the Actinomycetes clade. These findings have been confirmed and extended.⁸ Since then the polymerase chain reaction (PCR) has been reported to be a useful tool for the diagnosis of Whipple's disease.^{9,10}

Culture of the bacillus has been an elusive goal for many generations of microbiologists.¹¹ In 1997 the bacterium was isolated and grown in human macrophages inactivated with interleukin-4.¹² However, that isolate could not be subcultured, and no isolate is currently available.¹³

We report the successful isolation and establishment of a strain of the bacterium for Whipple's disease obtained from the mitral valve of a patient with blood-culture-negative endocarditis, the generation of specific antibodies against the bacterium in mice, the detection of the bacterium in the patient's mitral valve by immunochemistry with these antibodies, and the detection of specific antibodies against the bacterium in the patient's serum.

METHODS

The Index Patient

A 42-year-old man with mental retardation owing to encephalitis as a child was noted to have clubbing and a heart murmur in the autumn of 1997. Apparently, he had also had rheumatic fever as a child, although this could not be confirmed. An echocardiogram showed a thickened aortic valve with severe insufficiency and normal left ventricular function. During the winter, worsening congestive heart failure developed, and the patient was hospitalized twice with weight loss and pneumonia. By May 1998, he had lost 15 kg in weight and was admitted to the hospital with nausea and vomiting. There was no history of diarrhea, no evidence of organomegaly on abdominal examination, and no lymphadenopathy. He was transferred to a hospital in Halifax, Nova Scotia, Canada, in May 1998. An echocardiogram demonstrated a vegetation on the anterior leaflet of the mitral valve and a small

From the Unité des Rickettsies, Université de la Méditerranée, Faculté de Médecine, Marseille, France (D.R., M.L.B., B.L., P.E.F., M.E., H.L., V.R.); the Service de Médecine Interne, Groupe Hospitalier Pitié-Salpêtrière, Paris (J.-C.P.); the Laboratoire de Bactériologie, Hôpital Louis Pradel, Lyons, France (F.V.); the Service de Médecine Interne, Centre Hospitalier Lyon Sud, Lyons, France (D.V.-D.); and the Department of Medicine, University of Alberta, Edmonton, Canada (T.J.M.). Address reprint requests to Dr. Raoult at the Unité des Rickettsies, Université de la Méditerranée, Faculté de Médecine, CNRS UPRESA 6020, 27 Blvd. Jean Moulin, 13385 Marseille, France, or at didier.raoult@medecine.univ-mrs.fr.

vegetation on the chordae of the anterior leaflet. There was associated mitral insufficiency with a flail anterior leaflet, severe aortic regurgitation, vegetations visible on the leaflets, and a small abscess of the annulus next to the septum. During surgery no aortic-valve tissue could be identified on gross examination, but there were masses of vegetations in the location of the aortic valve that were completely excised and replaced with a homograft. The patient recovered uneventfully and was sent home 14 days later while taking antibiotics. At a follow-up visit nine months later the patient remained well.

The surgically resected tissues were fixed in formalin or frozen at -80°C . Slices of paraffin-embedded tissue samples were cut $5\ \mu\text{m}$ thick and stained with hematoxylin and eosin. The PAS stain and other stains were used to detect bacteria.¹⁴

Primary Isolation by Cell Culture

Culture was performed by the centrifugation-shell-vial technique with a human fibroblast cell line (HEL) that is used in our laboratory to detect intracellular bacteria, as previously described.^{15,16} All cell lines and culture reagents are checked weekly for bacterial contamination. Frozen cardiac-valve tissue was placed in minimal essential medium and crushed, and the suspension was used to inoculate three shell vials (Table 1). The inoculated vials were processed as previously described.^{15,16} The cultures were analyzed for bacteria by cytocentrifugation of $100\ \mu\text{l}$ of the shell-vial supernatant followed by Gimenez staining¹⁷ on days 10, 20, and 30. On day 30, the shell-vial supernatant and inoculated cells were harvested, inoculated into 25-cm^2 cell-culture flasks (flask 1) with 5 ml of medium, and incubated at 37°C in an atmosphere of 5 percent carbon dioxide. Every week for six weeks (until day 72), the cells were examined with an inverted microscope for cytopathic effects, and the incubation medium was replaced. Before the medium was replaced, $200\ \mu\text{l}$ of the supernatant was obtained for cytocentrifugation and staining with Gimenez, Gram's acridine orange, Ziehl-Neelsen, and PAS stains.

Propagation of the Isolate

The isolate was propagated in HEL cells grown under previously described conditions (Table 1).^{15,16} On day 75, 3 ml of supernatant from flask 1 was used to inoculate 10 shell vials, and 2 ml was used to inoculate a confluent monolayer of cells in a 25-cm^2 cell-culture flask (flask A) with 5 ml of medium. One of the shell vials was used to study generation time. The cells were harvested with the remaining supernatant and resuspended in fresh medium in order to obtain 10 ml of cell suspension, which was divided into five 2-ml aliquots. The cells of one aliquot were lysed by four cycles of freezing and thawing in liquid nitrogen and hot water (55°C) and inoculated onto confluent monolayers of cells in a 25-cm^2 cell-culture flask (flask C) with 5 ml of medium. Two aliquots were inoculated onto a confluent monolayer of cells in two 25-cm^2 cell-culture flasks (flasks B and D) with 5 ml of medium. On day 85, the medium in all flasks and shell vials was replaced by fresh medium. The cells in flask D were harvested and inoculated into a cell-free 75-cm^2 cell-culture flask (flask D2) with 15 ml of medium. Before the medium was replaced, $200\ \mu\text{l}$ of each supernatant was obtained for cytocentrifugation and PAS staining. On days 95 and 105, the medium in all flasks and shell vials was again replaced. Small portions of the cell monolayers were scraped to obtain cell smears for PAS staining. The efficacy of propagation was evaluated by semiquantitative counts of these cell smears. Each smear was analyzed microscopically at a magnification of 1000 for PAS-positive bacilli. A score of 0 was assigned if no PAS-positive bacilli were found; a score of + indicated that bacilli were present but hard to find, a score of ++ indicated that bacilli were easily detected but were not present in all fields, and a score of +++ indicated that bacilli were present in all fields. All smears were evaluated in a blinded fashion by two investigators. To ensure continued production of the isolate, as soon as a flask was given a score of +++, the cells were harvested and inoculated into three 150-cm^2 cell-free cell-culture flasks, with the vol-

TABLE 1. SUMMARY OF THE ISOLATION PROCEDURE.

STUDY DAY	PROCEDURE
1	Inoculation of 3 shell vials
30	Passage of 1 shell vial in a 25-cm^2 cell-culture flask
65	Evidence of cytopathic effect and microorganisms
72	Detection of growth (PCR-positive)
75	Inoculation of 1 shell vial ($1\ \text{cm}^2$) for the study of generation time, 9 shell vials for stock preparation (frozen), and 5 25-cm^2 flasks for the study of growth conditions
95	Passage of the shell vial on a 25-cm^2 flask
285	Generation of $3750\ \text{cm}^2$ of infected tissue from the original shell vial passaged on day 30 (calculated doubling time, 18 days)

ume adjusted to 35 ml by the addition of fresh medium. We also attempted to propagate the isolate by inoculating the cells onto monolayers of MRC 5 cells cultured in the same way as were HEL cells and in axenic medium (chocolate agar and Columbia sheep's-blood agars, BioMérieux, Marcy l'Etoile, France) and incubated at 32° and 37°C in the presence of 5 percent carbon dioxide and in microaerophilic and anaerobic conditions. We also incubated the isolates with cell-culture medium alone and with cell-culture medium containing a lysate of HEL cells at 32° and 37°C in the presence of 5 percent carbon dioxide.

Transmission Electron Microscopy

On day 105, about 1000 infected cells from a second-passage flask were prepared for examination with a transmission electron microscope (model 1220, Jeol, Croissy sur Seine, France) as described previously.¹⁸

Immunofluorescence Staining

On day 105 the monolayer from one shell vial was examined by direct immunofluorescence as previously described,^{16,19} with the use of the patient's serum as the primary antibody. Coverslips were examined with a laser scanning confocal fluorescence microscope (model DMIRBE, Leica, Wetzlar, Germany) equipped with an oil-immersion lens ($100\times$). To evaluate our serologic methods, we also analyzed nine serum samples from patients with proved Whipple's disease. The serum sample from our patient, one from a patient with endocarditis due to Whipple's disease as proved by PCR testing,²⁰ and seven from patients with histologically proved Whipple's disease (in two of whom *Tropheryma whippelii* DNA was detected in duodenal tissue by PCR assay)¹ were tested. The group of seven patients were considered to have classic Whipple's disease, to distinguish them from the two patients with endocarditis due to Whipple's disease. Forty-one serum samples were used as negative controls: 11 were obtained from patients with autoimmune diseases, 10 were obtained from patients with endocarditis due to *Coxiella burnetii* (5 patients) and to *Bartonella quintana* and *B. henselae* (5 patients), and 20 were obtained from healthy blood donors.

We devised a procedure that uses eight-well Lab-Tek chamber slides (Nunc, Naperville, Ill.) and allows 12 slides to be prepared at a time. The supernatant was removed from a 150-cm^2 cell-culture flask containing approximately 10,000 HEL cells and in which infection was present in all fields (+++) by semiquantitative count.

Four milliliters of 0.25 percent trypsin (GIBCO, Grand Island, N.Y.) was added to the cell monolayer, and the culture was incubated at 37°C for about 10 minutes until the cells became separated from the bottom of the flask. The cells were then resuspended in 35 ml of fresh medium, and 350 μ l of suspension was added to each well of the Lab-Tek chamber slides. The slides were incubated for 12 hours at 37°C in the presence of 5 percent carbon dioxide so that the cells could adhere to the glass slide. The serum samples were diluted in phosphate-buffered saline (1:25, 1:50, and 1:100) that contained 3 percent nonfat dry milk, and the titers of IgG and IgM were determined. For patients with IgM titers of 1:25, the serum samples were diluted from 1:50 to 1:400. To remove IgG, rheumatoid factor adsorbant (RF-adsorbant, Behringwerke, Marburg, Germany) was added before the determination of IgM, according to the manufacturer's instructions. The culture medium was removed, the cells were fixed with methanol, and then the wells were washed twice with phosphate-buffered saline. Next, 100- μ l samples of each serum dilution were added to the wells, and the chamber slides were incubated in a moist chamber at 37°C for 30 minutes. The slides were washed three times with phosphate-buffered saline and then incubated for 30 minutes at 37°C with 100 μ l of goat antihuman IgG (Fluoline G, BioMérieux) or IgM (Fluoline M, BioMérieux) at a dilution of 1:300 in phosphate-buffered saline. The slides were washed three times with phosphate-buffered saline, the plastic upper structures mounted on the slides were removed, and the slides were mounted in phosphate-buffered glycerol medium (pH 8) and examined at a magnification of 400 with an epifluorescence microscope (Zeiss, Thornwood, N.Y.).

Production and Characterization of Mouse Polyclonal Antibodies

Immunocompetent BALB/c mice that were six to eight weeks of age were inoculated subcutaneously with 0.5 ml of a solution containing 10^6 of the bacteria obtained from the supernatant of infected cells mixed with 0.5 ml of Freund's complete adjuvant. The mice were inoculated on days 0, 10, 20, and 30. On day 40, the mice were killed and the antibody titers were measured by microimmunofluorescence testing. Before further use, the serum samples were diluted 1:50 and adsorbed on HEL cells to remove non-specific anti-cell antibodies.

Amplification and Sequencing of the 16S Ribosomal RNA Gene

Bacterial DNA was extracted from 500 μ l of supernatant from a cell-culture flask by Qiagen columns (QIAmp tissue kit, Qiagen, Hilden, Germany) according to the manufacturer's instructions. PCR amplification with the broad-range 16S ribosomal RNA gene primers fD1 and rP2 and sequencing and purification of PCR products were performed as previously described.²¹

Statistical Analysis

We used Fisher's exact test for all statistical analyses. All P values are one-sided.

RESULTS

Gross pathological examination of the excised aortic valve revealed that the cusps were thickened, distorted, and fibrotic, with a large friable vegetation. Histologically, the aortic valve had organizing superficial platelet-fibrin thrombi on the cusps with focal calcific deposits and necrotic cellular debris. These vegetations were associated with extensive fibrosis and with acute and chronic inflammation. The granulation tissue beneath the surface of the cusp included a chronic inflammatory infiltrate with numerous foamy macrophages. PAS-positive bacilli were identified in coarse masses of rod-shaped bodies within the foamy

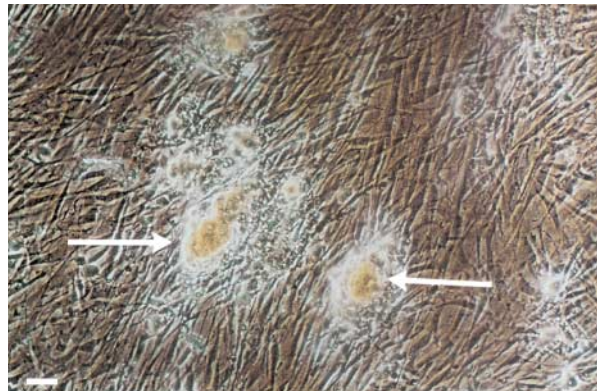


Figure 1. Large, Coarse, Round Structures (Arrows) within HEL Cells in a Six-Week-Old Culture of the Whipple's Disease Bacterium.

An inverted microscope was used. The bar represents 30 μ m.

macrophages, the hallmark of Whipple's disease.^{22,23} However, no microorganisms were detected on staining with Giemsa, Brown and Hopps, Gomori-Grocott, or Warthin-Starry stains. The chronology of isolation of the bacterium for Whipple's disease is summarized in Table 1. A cytopathic effect and microorganisms were not detected until day 65 after inoculation. Using an inverted microscope, we identified small, coarse, dark inclusions and large, coarse, round structures within cells on day 72 (Fig. 1). Gimenez staining of the supernatant after centrifugation revealed several slender pink bacilli. The majority were intracellular, and the intracellular bacilli were shorter than those outside the cells. However, most of these bacilli were poorly stained or not stained by the Gimenez stain and appeared pale blue. Numerous bacilli were also revealed by Gram's staining. Most were gram-positive, but several were only partially purple or gram-negative. On Ziehl-Neelsen staining, these bacilli were not acid-fast. More bacilli were PAS-positive than were positive for the Gimenez or Gram's stains. The cells were filled with coarse, PAS-positive conglomerates and short, slender, PAS-positive rods.

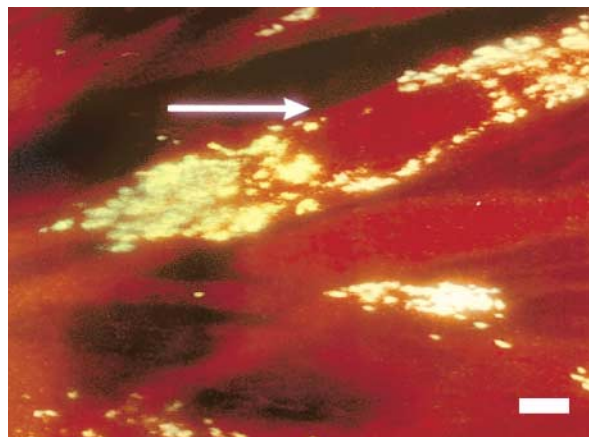
Amplification and sequencing of the 16S ribosomal RNA gene of the isolate produced a segment of 1450 bp. We compared the sequence with DNA-sequence data bases (Blast, version 2.0, National Center for Biotechnology and Information, Bethesda, Md.) and found that it was 99.9 percent homologous to the 16S ribosomal RNA sequence of *T. whippelii* (European Molecular Biology Laboratory accession number, X99636).

All subculture procedures used for the propagation of the isolate were effective, since in all cases, the isolate was recovered after 30 days of subculture. However, the most effective procedures were inoculation of supernatant onto fresh cell monolayers, as

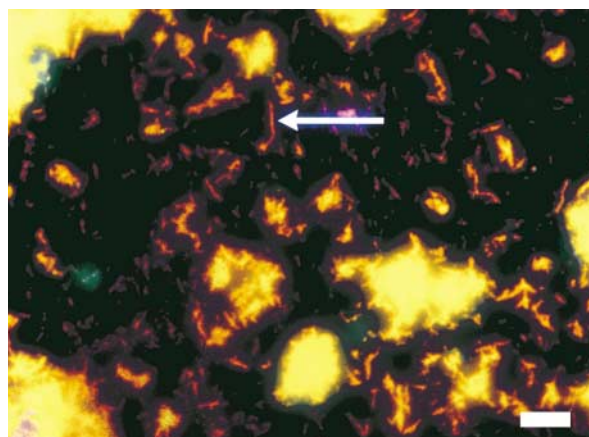
was done in the case of flask A, or the duplication of infected cells, as was done in the case of flask D2. In those cases, the cultures were evaluated semiquantitatively after 30 days of subculture, and bacilli were easily detected, but not in all fields. The results of attempts to subculture the bacilli on MRC 5 cells were similar. All attempts at subculture on axenic medium were unsuccessful. At each stage of the propagation procedure, 500- μ l samples of the cultures were tested by PCR and the bacterium of Whipple's disease was confirmed to be present in the culture.

Immunofluorescence staining demonstrated that staining with PAS and other stains undervalued the extent of cell infection. Immunofluorescence examination of shell-vial coverslips after 30 days of subculture showed that all cells contained large amounts of the isolated antigen. The intracellular location of the bacilli was confirmed by confocal microscopy (Fig. 2A). Several bacteria were seen that resembled the short, slender rods observed on PAS staining. Nevertheless, most immunopositive material was found in larger inclusions, where individual bacteria were not seen. No immunopositive material was detected within the nuclei. Large numbers of bacteria were found by acridine orange staining of the cell-culture supernatant (Fig. 2B). Transmission electron microscopy confirmed that the PAS-positive inclusions and immunopositive material corresponded to intact and degenerating bacteria. Dividing cells were observed. The cell wall included a structure whose presence was consistent with previous descriptions of the bacterium of Whipple's disease.²⁴ The plasma membrane was surrounded by a thin, homogeneous wall, which was itself surrounded by a plasma-membrane-like structure, giving a trilamellar appearance (Fig. 3).

IgG antibodies against the bacillus were detected in most serum samples, including those from the control subjects. Cutoff values were selected after the results were known. When a cutoff value of 1:100 was selected, samples from all nine patients with confirmed Whipple's disease (endocarditis or classic) were positive, as compared with samples from 29 of 40 controls ($P=0.08$) (Table 2). The presence of IgM antibodies was more specific to patients with Whipple's disease. When a cutoff value of 1:50 was selected, 7 of 9 patients with Whipple's disease had positive results, as compared with 3 of 40 control subjects ($P<0.001$). Both patients with Whipple's disease endocarditis had a positive IgM antibody titer, as compared with none of the 10 control subjects with endocarditis from other causes ($P=0.015$). Five of 7 patients with classic Whipple's disease had a positive IgM antibody titer, as compared with 2 of 10 control subjects with autoimmune diseases ($P=0.052$). The IgM antibody titer was 1:400 or more in three of seven patients with classic Whipple's disease and in both patients with Whipple's disease endocarditis, but in none of the patients without Whipple's disease. The serologic re-



A



B

Figure 2. Whipple's Disease Bacterium.

For immunofluorescence staining of the bacteria (arrow) in infected HEL cells, the patient's serum was used as the primary antibody (Panel A). Specimens were examined with a confocal microscope. In Panel B, acridine orange staining reveals the numerous bacteria (arrow) in the supernatant of infected HEL cells. The bar in Panel A represents 6 μ m, and the bar in panel B represents 15 μ m.

sults for one control subject with autoimmune disease who had antineutrophil cytoplasmic autoantibodies were uninterpretable because of the presence of diffuse immunofluorescence.

Antibodies were produced in mice at high titers (1:1000) and were successfully used to detect the bacteria in the patient's excised tissue by immunohistochemistry.

DISCUSSION

Although Whipple's disease was identified nearly a century ago, the causative agent of this bacterial infection has not been successfully established in vitro. Even though no type strain of the bacterium is available, it is usually referred to by its provisional

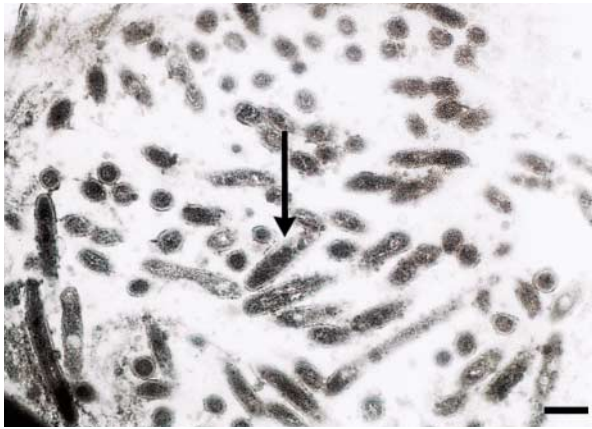


Figure 3. Transmission Electron Micrograph Showing the Bacterium (Arrow) in Infected HEL Cells. The bar represents 500 nm.

name, *T. whippelii*.^{8,25} The molecular identification of this bacterium in biopsy specimens or peripheral-blood samples is the basis for the diagnosis.⁹ However, this technique remains to be validated, and the diagnosis of Whipple's disease requires a biopsy specimen for either microscopical study or PCR analysis. Therefore, a serologic test could be highly useful, since the diagnosis could be made on the basis of a single blood sample. This is particularly important in patients with life-threatening complications such as endocarditis.

Unlike previous investigators who completed only two passages of the bacterium cultures of human macrophages,¹² we used a human fibroblast cell line with no specific culture conditions. We completed seven passages of our isolate and believe that the culture is now definitely established. By day 285 we had 120 heavily infected 150-cm² cell-culture flasks. The strain has been deposited in the French National Collection at the Pasteur Institute in Paris and is available. Since it requires 210 days to obtain 25 well-infected 150-cm² cell-culture flasks from a 1-cm² shell vial, the generation time (or doubling time) of the bacterium is about 18 days, which is similar to that for *Mycobacterium leprae* in animal models (12 days).²⁶ Patience has been a key to culturing new pathogens, as in the case of bartonella species,²⁷ which can take as long as 45 days to isolate, and *Helicobacter pylori*, for which a prolonged incubation is also necessary.²⁸ One other difficulty is the staining of bacteria in cells. PAS is useful but fails to detect all bacteria, unlike immunofluorescence of the tested strain. Acridine orange was the most useful stain. However, mouse-specific antibodies may be very useful for this purpose.

The successful isolation of intracellular bacteria is partly based on two critical points. First, the ratio of bacteria to cells should be as high as possible. Sec-

TABLE 2. RESULTS OF INDIRECT IMMUNOFLUORESCENCE ASSAY OF SERUM SAMPLES FROM PATIENTS WITH WHIPPLE'S DISEASE AND CONTROL SUBJECTS.

GROUP	NO. OF SUBJECTS	IgG ANTIBODY TITER ≥1:100	IgM ANTIBODY TITER ≥1:50	no. of subjects	
Patients with Whipple's disease	9	9*	7†		
Whipple's disease endocarditis	2	2	2‡		
Classic Whipple's disease	7	7	5§		
Control subjects¶	40	29	3		
Endocarditis	10	9	0		
Autoimmune disease	10	9	2		

*P=0.08 for the comparison with all control subjects.

†P<0.001 for the comparison with all control subjects.

‡P=0.015 for the comparison with control subjects with endocarditis.

§P=0.052 for the comparison with control subjects with autoimmune disease.

¶Twenty control subjects were healthy blood donors. The assay results for one control subject with autoimmune disease were uninterpretable and were therefore excluded from the analysis.

ond, centrifugation, which was shown to enhance the adhesion of other intracellular bacteria to the cells,²⁹ probably favored isolation of the bacillus.

We used our serologic method in different groups of patients. IgG antibodies were detected more frequently in patients with Whipple's disease, but they were present at a titer of 1:100 or more in 29 of 40 control subjects and therefore are not suitable for diagnostic purposes. We do not know whether this high level of IgG results from previous contact with the bacterium or from a cross-reaction with other bacteria. However, the fact that all patients with Whipple's disease had antibodies to the isolated bacterium provides support for the idea that it has a causative role in the disease.

The presence of IgM antibodies at a titer of at least 1:50 was significantly associated with Whipple's disease overall and with Whipple's disease endocarditis. For the comparison with patients with classic Whipple's disease, we chose patients with autoimmune disease as the control group, a controversial choice, because these patients frequently have false positive serologic reactions. The fact that we removed rheumatoid factor from the serum samples may have controlled for the rare false positive result. In fact, in one patient, the presence of antineutrophil cytoplasmic autoantibodies made it impossible to interpret the results. However, the difference between the two groups was of borderline significance (P=0.052). The serologic data are preliminary, and it would be interesting to learn whether patients have higher titers of IgM antibodies early in the course of the disease.

Our results are encouraging, and the establishment

of the strain will enable researchers to define the role of this microorganism in several clinical syndromes. It may also be useful in developing an animal model of Whipple's disease as well as for antibiotic-susceptibility testing. Finally, the purification of the strain will clear the way for genetic studies, so that specific gene sequences can be obtained, in contrast to the current ones, which are based on universal primers.

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CORRECTION

Cultivation of the Bacillus of Whipple's Disease

Cultivation of the Bacillus of Whipple's Disease . On page 620, on line 5 of the abstract and line 19 of the right-hand column, specimens from the *aortic* valve were obtained, not the *mitral* valve, as printed.