The Discovery and Characterization of Mimivirus, the Largest Known Virus and Putative Pneumonia Agent

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During recent years, the usefulness of amoebal co-cultures as an alternative means of isolating and cultivating fastidious microorganisms has been increasingly recognized. While characterizing a collection of bacteria that had been isolated using this approach, we encountered an organism that, on preliminary analysis, appeared to be a gram-positive coccus. However, additional examination revealed that it was not a bacterium but rather, surprisingly, a virus. The dimensions of the virus particle (diameter, 0.8 μm) and its genome size (1.2 Mb) are far more akin to those of bacteria than to those of previously recognized viruses. These characteristics, together with such features as the breadth and complexity of its gene content, challenge the current definition of a "virus." Furthermore, the virus, now named "Mimivirus," has been implicated as an agent of pneumonia in humans and, thus, should be considered a putative emerging pathogen.

INTRODUCTION

For a large proportion (20%–50%) of cases of pneumonia, a precise etiology is not obtained [1]—a shortfall that, in part at least, results from our failure to recognize the full diversity of microorganisms capable of inducing these symptoms. Our research group has long been interested in exploring this diversity as part of a larger program that focuses on the biology of other fastidious pathogens, such as *Rickettsia* species, *Bartonella* species, *Coxiella burnetii,* and *Tropheryma whipplei* (see the Marseille, Maladies Infectieuses Web site at http://ifr48.timone.univ-mrs.fr/portail2/index.php?option=com_content&task=view&id=78). One approach that we have increasingly employed is the use of amoebal co-cultures to isolate organisms that cannot be grown on axenic media [2–6]. Our adoption of these methods, in which amoebae essentially serve as a selective medium for the recovery of organisms able to invade and persist within phagocytic cells, has led to the identification of novel potential pathogens in hospital water systems [7] and has resulted in the description of 12 new bacterial species (table 1).

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In 1995, one of us (R.J.B.) began a postdoctoral fellowship in the laboratory, bringing along a collection of obligate intra-amoebal bacterial parasites. Most of these organisms had been recovered using amoebal co-culture by Dr. Tim Rowbotham. Although microscopic observation suggested the presence of a gram-negative, rod-shaped bacterium (figure 1), attempts to grow these organisms on axenic media repeatedly failed; thus, the organisms were referred to as "*Legionella*-like amoebal pathogens" (LLAPs) [3, 16]. In addition to cultures of LLAPs, there were 2 cultures of apparently gram-positive coccoid bacteria, which were referred to as "the Bradford coccus" and "Hall's coccus," the latter of which had been sent to Dr. Rowbotham by a colleague in the United States [11].

CHARACTERIZING INTRA-AMOEBAL LEGIONELLAE

The advent of 16S rRNA–encoding gene (16S rDNA) sequencing provided a means of obtaining reliable, universally comparable genetic identities for all bacteria, including *Legionella lyticum,* regardless of their "culturability" on axenic laboratory media [24–26]. 16S rDNA sequencing and sequence comparison of the LLAPs that were analyzed in our laboratory suggested that several of them were additional strains of *L. lyticum.* The remaining isolates could not be adequately accommodated in any of the currently described species. Additional examination of these isolates resulted in the proposal of their delineation into 4 new *Legionella* species, which were named *Le-*

Table 1. Intra-amoebal growing bacteria isolated, first isolated, characterized, or demonstrated as being human pathogens in our laboratory.

Organism	Category	Reference(s)
Proteobacterium		
Afipia massiliensis	AB	[8–10]
Afipia birgiae	AB	[8–10]
Afipia felis genospecies A	AB	[8–10]
Bosea massiliensis	ABC	[7, 9, 14]
Bosea vestrisii	AB	[7, 9, 14]
Bosea eneae	AB	[7, 9, 14]
Legionella rowbothami	AB	[15, 16]
Legionella drozanskii	AB	[15, 16]
Legionella fallonii	AB	[15, 16]
Legionella drancourtii	ABC	[21, 22]
Odyssella thessalonicensis	AB	[23]
Rhodobacter massiliensis	ABC	…
Nordella oligomobilis	AB	…
Rhizobium massiliae	ABC	…
Roseomonas massiliensis	ABC	…
Chlamydia: *Parachlamydia acanthamoeba*	A	[11–13]
Flavobacteria: *Amoebinatus massiliae*	ABC	…
Mycobacteria: *Mycobacterium massiliense*	ABC	…
Virus: *Acanthamoeba polyphaga Mimivirus*	ABC	[17–20]

NOTE. A, first isolation or description of the microorganism; B, official description; C, description of the first human case.

gionella drozanskii, Legionella rowbothamii, Legionella fallonii, and *Legionella drancourtii* (figure 1) [11, 15, 21].

In an effort to begin to determine the medical relevance of these new species, they were incorporated as antigens in serological assays used to screen a large panel of serum specimens collected from Canadian patients who experienced pneumonia. We observed seroconversions against *Legionella lytica*, *L. drozanskii,* and *L. drancourtii* in several patients with ambulatory pneumonia [22]. A study from the United States reported similar findings, with 8% of enrolled patients in this study possessing high (>512) titers to one of a panel of antigens derived from 9 LLAPs [27]. One of the original LLAPs we studied turned out not to live up to its billing, proving to be unrelated to the Legionellaceae. Instead, this organism was a novel a proteobacteria for which we proposed the name "*Odysella thessalonicensis*" [23]. Subsequent characterization of bacteria recovered from hospital water supplies in Marseille using amoebal co-culture has further demonstrated their taxonomic diversity; we have isolated *Afipia felis* [8] together with novel *Afipia* species now classified as *Afipia birgiae* and *Afipia massiliae* [14], and new *Bosea* species [9] now classified as *Bosea vestrisii*, *Bosea eneae,* and *Bosea massilensis* [14].

IDENTIFICATION OF A NEW CHLAMYDIAL TAXON

The study of Hall's coccus yielded surprising results. This organism had been isolated in 1989 from a humidifier in Vermont [28] and had been subsequently maintained in amoebal co-culture. When these co-cultures were used as antigens in indirect immunofluorescence tests to screen patients from the United States and Canada with pneumonia, specific antibodies were detected [11]. When we determined the 16S rDNA sequence of Hall's coccus, we found that it was very similar to those of the Chlamydiaceae—specifically, it was very similar to a sequence obtained during a study independent from ours in which a *Chlamydia*-like organism that had infected an *Acanthamoeba* strain recovered from human nasal mucosa had been partially characterized and proposed as *Candidatus Parachlamydia acanthamoeba* (figure 1) [29]. We have subsequently obtained additional evidence of the role of this species as an occasional agent of pneumonia [12, 13, 30].

A GIANT "GRAM-POSITIVE" VIRUS

Among all the intra-amoebal "bacteria" that constituted our original collection, one provided us with the most headaches but, ultimately, the most exciting findings. All attempts to amplify 16S rDNA from the Bradford coccus failed, despite the use of PCR assays that incorporated "universal," pan-bacteria primers [31]. Eventually, we decided that examination of the ultrastructure of Bradford coccus by electronic microscopy might provide us with a solution to our technical problems [32, 33]. To our great surprise, we observed remarkable "unbacterial" bodies within infected amoebae that consisted of very regular icosahedral forms (figure 2), much like those observed in giant iridoviruses. Our suspicion that it was a virus was supported by additional preliminary work, in which we demonstrated that the organism contained a large double-stranded DNA chromosome and underwent an eclipse-phase replication typical of viruses [33]. Furthermore, we now know that assembly of virus particles takes place in specific intracellular locations that have been termed "virus factories" when previously observed in viruses, including iridoviruses [34, 35]. We estimated that the diameter of the Bradford coccus was ~600 nm when fibrils surrounding the capsid were included in the size calculation [34], making it currently the largest known virus; its size is akin to that of small bacteria, such as *Mycoplasma* species [33], *Rickettsia* species, *C. burnetii,* or *T. whipplei* [32] (figure 3), and it can be observed using light microscopy.

We have proposed the name Mimivirus [17], partially as a reflection of its mimicry of microbes and partially as a tribute to one of our forefathers (D.R.), who was a doctor who taught tropical medicine and studied nutrition. When teaching his 10-year-old son about evolution, he referred to the last eukaryotic common ancestor as "Mimi the amoeba."

Our suspicion that Mimivirus was related to iridoviruses was confirmed by phylogenetic inference derived from alignment of ribonucleotide reductase gene sequences. This analysis indicated that the virus grouped with other nucleocytoplasmic

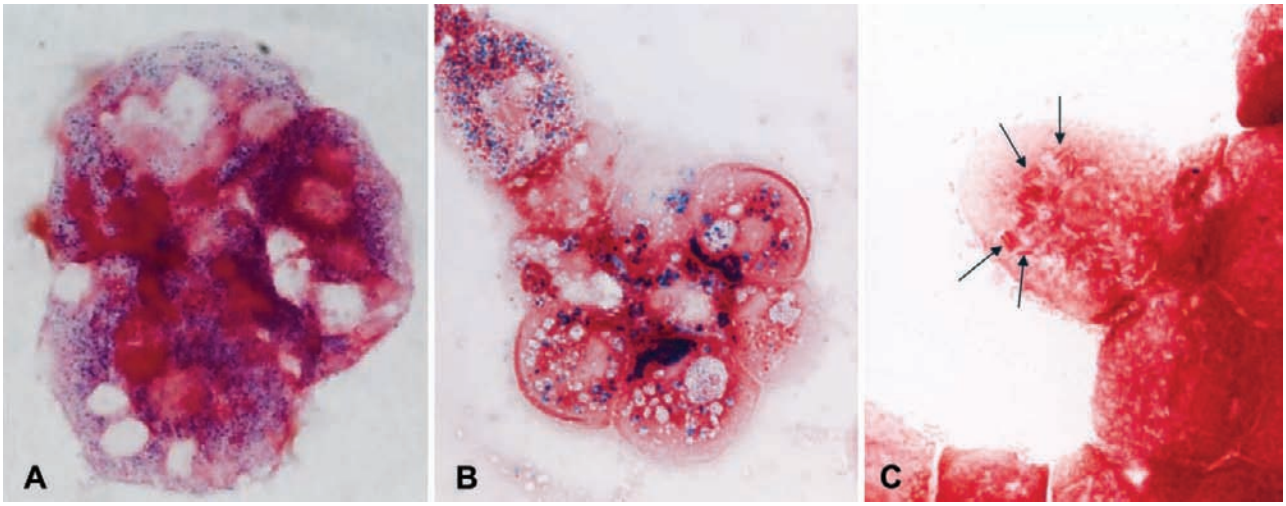

Figure 1. Strict intra-amoeba–growing microorganisms, as observed under light microscopy after Gram staining. *A, Acanthamoeba polyphaga Mimivirus* (formerly called "the Bradford coccus"). *B, Parachlamydia acanthamoeba* (formerly called "Hall's coccus"). *C, Legionella drancourtii,* which appears as thin-clustered bacilli.

large DNA viruses (NCLDVs), including viruses in the Iridoviridae, Baculoviridae, Phycodnaviridae, and Poxviridae families. However, because the Mimivirus bore no specific evolutionary relatedness to any other family in this group, we proposed that it be accommodated in its own family, the Mimiviridae. The virus type is named "*Acanthamoeba polyphaga* Mimivirus" [17].

MIMIVIRUS GENOME SEQUENCING AND ANALYSIS

Such was the novelty of Mimivirus that our initial attempts to publish our findings were unsuccessful. One of the main criticisms we faced was that we had generated insufficient DNA sequence data to reliably infer the phylogenetic position of the Mimivirus. Thus, we decided to perform complete genome sequencing with the help of J. M. Claverie and his bioinformatics team [18]. We completed this task in 2004, finding that Mimivirus possessed a genome size of 1.2 Mb. Thus, the genome of Mimivirus is larger than those of several bacteria [18] and represents the largest known coding capacity for a virus. The Mimivirus genome possessed only a very low proportion of noncoding DNA (9.5%) and ~1260 putative open-reading frames (ORFs). Many of the genes within the Mimivirus genome had not previously been found in viruses, including 8 that putatively encoded proteins with a role in protein translation (4 of which are aminoacyl-tRNA synthetases), 5 that putatively encoded proteins involved in DNA repair, and 3 that putatively encoded chaperones. Interestingly, many of the sequences that were found to be most similar to Mimivirus ORFs were obtained in a study that involved systematic sequencing of DNA extracts derived from aliquots of Sargasso seawater [36], indicating that similar viruses may exist in marine environments.

The availability of complete genome sequence data provided us with the means to more reliably infer the phylogenetic position of Mimivirus. We identified 9 genes that were shared by all NCLDV families (these encoded DNA polymerase, a capsid protein, 3 helicases, a virion packaging ATPase, a thiol oxidoreductase, a protein kinase, and a transcription factor). Phylogeny inferred from alignments of these genes from Mimivirus and representatives of the other NCLDV families indicated that, although Mimivirus was grouped with the NCLDVs, it lay at a basal position within the cluster [37]. However, this position has subsequently been contested [38], with the inference that Mimivirus and phycodnaviruses form a specific clade.

It is widely hypothesized that the majority of viral genes originated from their hosts, and an initial response to our publication of the genome sequence suggested that this would also be the case for Mimivirus. Moreira and Lopez-Garcia [39] carried out comparative analysis of the Mimivirus tyrosine aminoacyl-tRNA synthetase gene, a gene known to be commonly subject to horizontal transfer [40]. They observed that the Mimivirus gene was most closely related to that of an amoebal species, *Entamoeba histolytica.* However, subsequent comparative analysis of a number of well-conserved Mimivirus genes revealed best matches with bacterial rather than amoebal or protozoal homologues [37, 41, 20].

The detection of an increasing number of bacteria-like genes in the Mimivirus genome [32] adds to the ongoing debate surrounding the relative importance of lateral gene transfer in shaping Mimivirus gene content. In addition, in the presence of many paralogous gene families in the Mimivirus genome,

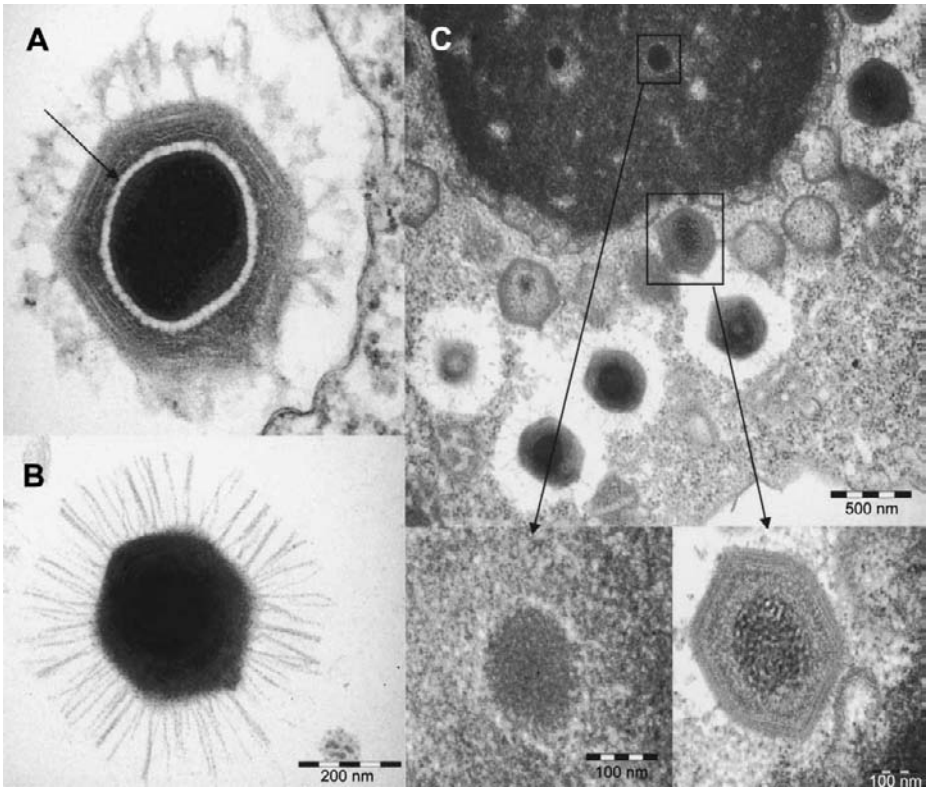

Figure 2. *A,* Mimivirus transmission electron microscopy study showing viral particles in phagocytosis vesicles 30 min after inoculation. The clear area *(arrow)* observed in these particles is not retrieved on the mature particle observed in supernatant after amoebal lysis. *B,* Assembly of viral particles occurring at the periphery of the virus factories, with apparent membrane ruffling, likely corresponding to capsid assembly. *C,* Young particles at the periphery of the nucleus that are not surrounded by fibrils *(right bottom). Left bottom,* Amoebal dense virus factory inclusions interpreted as inner replication centers.

gene duplication has undoubtedly played a role in its evolution [38, 42, 43]. Thus, the relative significance of different evolutionary processes in shaping the current Mimivirus genome remains an area of debate.

WHAT DO MIMIVIRUS GENE PRODUCTS DO?

Our discovery of an unusually high number of putative genes in the Mimivirus genome [18] raised the question "why so many?" We have now used several approaches to answer this question. Bioinformatics studies demonstrated that a unique promoter, with an unprecedented degree of conservation, was present upstream of 446 putative ORFs. This suggests synchronization of Mimivirus gene transcription and that regulation of these ORFs remains functional [38].

We further investigated the functionality of Mimivirus genes by measuring their transcription. All 70 of the genes we examined were transcribed at some point during viral multiplication in amoebae. Moreover, 34 were expressed within the viral particle (unpublished data). These genes must play a key role in viral exploitation of their protozoan host [35].

We recently characterized 109 proteins in Mimivirus particles [44], 36 of which were encoded by ORFs with no known homologues. The functionalities of some Mimivirus proteins have been reported, such as tyrosine aminoacyl tRNA synthetase (the first found in a virus) [45], DNA topoisomerase 1B [44], and NAD^+-dependent DNA ligase [41]. Moreover, some Mimivirus genes without homologues encoded proteins that were found to be antigenic when tested using serum from immunized mice [44] or from a laboratory-infected technician (see below) [19]. Taken together, these data strongly suggest that the majority of the gene repertoire of Mimivirus is functional rather than being a redundant remnant.

AN EXPERIMENTAL MODEL OF MIMIVIRUS INFECTION

To establish a possible role of Mimivirus as a human pathogen, we developed an experimental model of infection [46]. Laboratory mice were inoculated intracardially with 10^8 infecting units of Mimivirus and were subsequently humanely killed. Autopsy revealed histopathologic evidence of pneumonia, and Mimiviruses were reisolated from samples from the lung [46]. We also inoculated human macrophages with Mimiviruses and

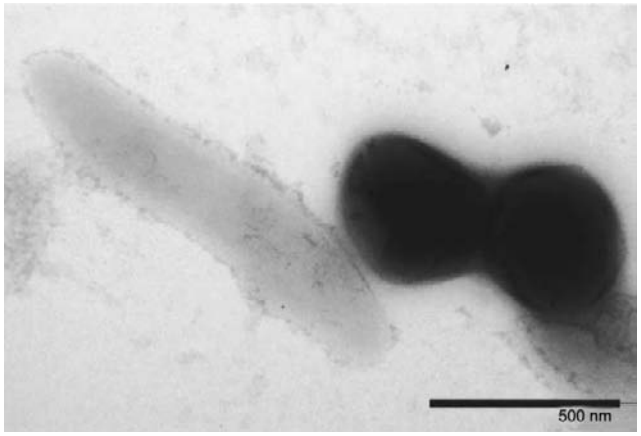

Figure 3. Negative-staining electronic microscopy showing Mimivirus *(right)* and *Tropheryma whipplei* bacillus (*left;* bar, 500 nm).

were able to induce infection, although no evidence of a lytic cycle was observed (unpublished data). Finally, despite much effort that involved a wide variety of cells and cell lines, we were unable to induce Mimivirus infection in cells other than amoebae and macrophages (unpublished data). These data suggest that a high inoculum of Mimivirus can produce pneumonia. However, in mice, the target cell of the virus has not been identified.

AN INDEX CASE OF MIMIVIRUS PNEUMONIA IN A LABORATORY TECHNICIAN

Both before and after its characterization, Mimivirus was handled in our laboratories with the same precautions used for other environmental (but potentially pathogenic) bacteria. The technicians who staff our routine diagnostic laboratory are annually screened (serologically) for exposure to the microorganisms with which they work. From these results, we do not have any evidence that any technician ever had a laboratory-acquired infection. However, in December 2004, one of our technicians developed subacute pneumonia, with dry cough, fever, and chest pain. This technician was in charge of performing Mimivirus serologic testing and Western blotting and, thus, handled relatively large amounts of Mimivirus antigens. Radiography of his chest revealed bilateral basilar infiltrates [19] (figure 4). The illness did not respond to amoxicillin-clavulanate treatment prescribed on day 15 after onset of symptoms, but after an additional 2 weeks, the illness resolved, and our technician, thankfully, made a full recovery. Serum samples were screened against pneumonia agents, and the results were negative for all agents except for Mimivirus, for which we observed seroconversion. Moreover, testing by 2-dimensional Western blotting revealed a reaction against 23 identified proteins of Mimivirus, including 22 proteins with unknown functions and 4 encoded by genes with no homologues (figure 5). Thus, we had few doubts that our technician had experienced a laboratory-acquired Mimivirus infection that manifested as pneumonia.

PREVALENCE OF ANTIBODIES TO MIMIVIRUS IN PATIENTS WITH PNEUMONIA.

Three studies have investigated the prevalence of antibodies to Mimivirus in specific human populations. The first study tested serum samples obtained from 376 Canadian patients with community-acquired pneumonia and 511 healthy subjects. A total of 9.66% of patients with pneumonia had antibodies to Mimivirus, compared with 2.3% of control subjects [22]. Patients with pneumonia who had antibodies to Mimivirus were more likely to be hospitalized from a nursing home and to be rehospitalized after discharge [20]. The second study included 26 patients from Marseille, France, who acquired pneumonia while in an intensive care unit, as well as 50 control serum samples (from blood donors). Antibodies to Mimivirus were detected in samples obtained from 5 patients but in none of the control samples. Mimivirus DNA was amplified from a bronchoalveolar lavage specimen obtained from a patient who had relapsing pneumonia and who did not have available convalescent-phase serum samples. All other bronchoalveolar lavage specimens tested along with negative controls failed to yield a specific PCR product [20]. The final serosurvey involved 157

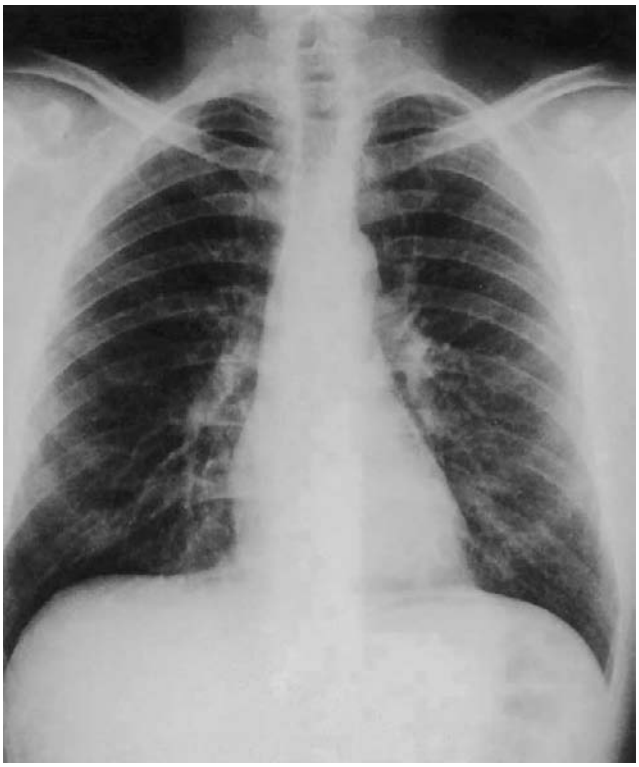

Figure 4. Chest radiograph of a laboratory technician who was infected with Mimivirus, showing bilateral basilar infiltrates.

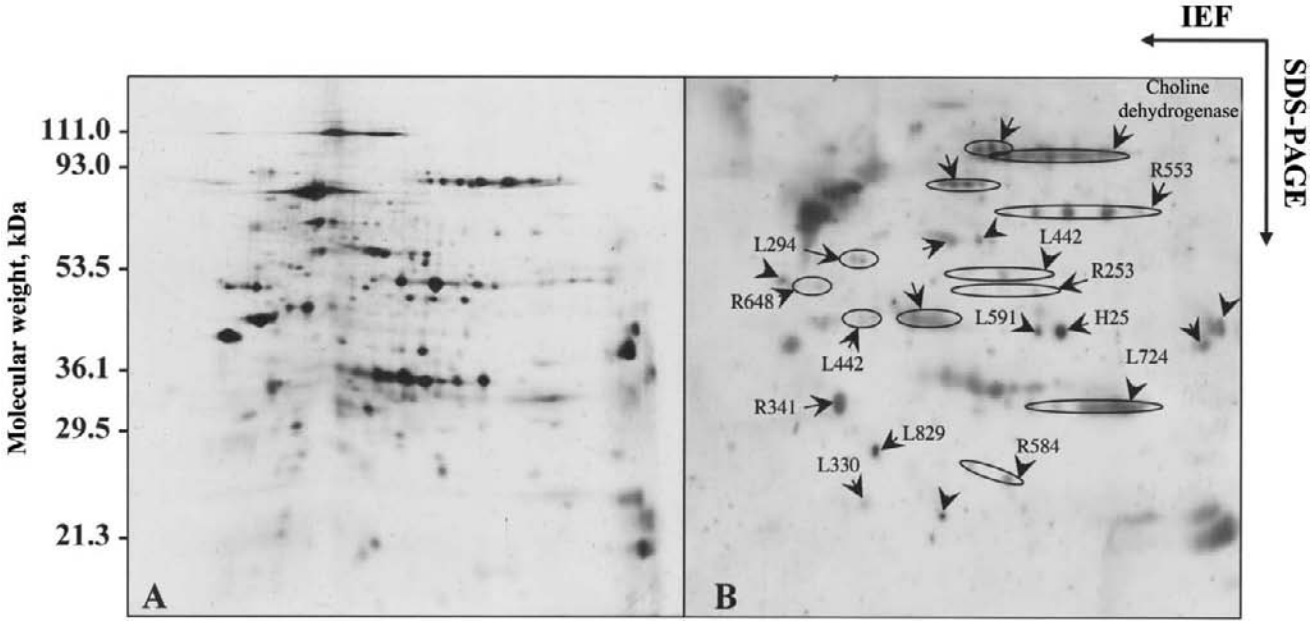

Figure 5. Western blot recognition in human serum of Mimivirus proteins resolved by 2-dimensional electrophoresis. Purified and solubilized Mimivirus extract was separated using first-dimension immobilized pH gradient strips (pI range, 3–10) followed by a 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis in the second dimension. The 2-dimensional gel was either visualized by silver staining *(A)* or transferred to nitrocellulose and probed with serum from the infected patient *(B)*. Spots or a train of spots specifically recognized are indicated. The serum sample shows a strong reaction against several spots, including 23 spots that were not detected when the membrane was probed with serum that had been obtained from the same patient a few months earlier. With the exception of the choline dehydrogenase–like proteins encoded by the gene R135, all identified candidates correspond to proteins of unknown functions. Among the 13 characterized proteins, 4 are encoded by ORFans genes of Mimivirus. These proteins correspond to L330, L724, and both the N- and C-terminal extremities of L442 protein, which appears as cleaved. IEF, immunoelectroforesis.

intensive care unit patients with pneumonia (among whom there were 210 episodes of pneumonia). Serum specimens obtained from these patients were tested against a panel of antigens from "conventional" pneumonia agents and amoeba-associated microorganisms, including Mimivirus [1]. We found evidence of infection with "conventional" pathogens in 28 cases and with amoeba-associated pathogens in 18 cases. Among this latter group, more patients had seroconversion to Mimivirus than to any other pathogen (5 cases), and seroconversion was more common among patients with ventilator-associated pneumonia than among those with community-acquired pneumonia (31.6% and 10.5% of patients, respectively). Taken together, these 3 studies reveal a significant rate of seroconversion in patients with either community-acquired pneumonia (especially among patients who had been hospitalized from a nursing home) or nosocomial pneumonia.

MIMIVIRUS AS AN EMERGING PATHOGEN

From the progress reported above, we now have some evidence that Mimivirus may be a human pathogen that causes pneumonia. To summarize, first, the virus multiplies in amoeba, a well-known "Trojan horse" for pneumonia agents [3]. Second, we have been able to experimentally induce pneumonia in mice inoculated with the virus. Third, one of our laboratory technicians acquired the virus and developed pneumonia. Fourth, in several serologic studies, we have encountered patients with pneumonia who apparently experienced seroconversion to Mimivirus. And finally, we amplified Mimivirus DNA from a patient with unexplained pneumonia. Thus, Mimivirus fulfills Koch's criteria (table 2). However, several issues remain poorly understood or controversial. First, Mimivirus does not efficiently replicate in co-culture with any of the mammalian cells tested to date (table 3). Second, serologic cross-reactions among pathogens are commonly observed, and these may account for the apparent seroreactivity of Mimivirus antigens. We have a preliminary observation of a cross-reaction between serum specimens obtained from patients with tularemia (unpublished data), although this finding cannot entirely explain the high seroprevalence of Mimivirus antibodies. Finally, we have yet to obtain isolates of the virus from patients with pneumonia. Clearly, there is a need for additional studies (e.g., more-comprehensive PCR-based surveys of natural amoebal communities

Table 2. Application of Koch's postulates to Mimivirus as a pathogen.

Postulate	Finding
Presence in cases of pneumonia	Yes: antibodies were recovered in 3 studies, and DNA was recovered in 1 case [20]
Absence in control cases	Yes [20]
Experimental model	Yes: it causes pneumonia in mice; Mimivirus was recovered from the lung [46]

or epidemiological investigation of patients who demonstrated apparently specific anti-Mimivirus antibody responses) to resolve these issues.

HOW MIMIVIRUS IS CHANGING OUR PERCEPTION OF WHAT VIRUSES ARE

Our perception of viruses has changed dramatically since their existence was first demonstrated as "filterable" infectious agents and since they were first to be visualized with the advent of electron microscopy [32]. For example, early work revealed that viruses were obligate intracellular parasites that possessed a replicative cycle in the host cell, into which they disappeared after decapsidation, only to subsequently reassemble into their virion form; this led to 2 very different hypotheses to explain their nature. Some scientists, such as Nobel laureate McFarlane Burnet (also a rickettsiologist), believed that viruses were viable organisms, whereas others, such as Nobel laureate W. Stanley, believed that they were merely complex molecules [32]. Today, after several decades of research, our understanding of viral diversity and the biological properties of viruses is profound enough to give us the ability to construct entirely "synthetic" viruses [42, 43]. Throughout this progress, we have had to reconsider how best to define "virus;" with the discovery and characterization of a virus of the size and complexity of Mimivirus, we are perhaps required to reconsider once more. Nonetheless, some biologically defined boundaries between viruses and "living" cells remain; that the Mimivirus does not possess genes encoding rRNA or ribosome-associated proteins suggests that an integral capacity for complete protein translation is lacking in even the most complex viral genomes [37]. Furthermore, despite its complexity, Mimivirus (like all other viruses) possesses a capsid, whereas "living" cells do not.

Table 3. Contradiction to Lwoff definition of viruses by Mimivirus

Definition	Mimivirus
One-dimension inferior to 0.022 micron	Bigger
Contains DNA or RNA	Contains both
Presence of energy metabolism enzymes	Unknown
Parasite	Yes
No binary multiplication	Yes
Postgenomic definition: no ribosomal proteins or ribosomal gene	Yes

Indeed, the ubiquity of capsids among viruses may indicate their common ancestry [47]. This hypothesis is supported by the occurrence of a double-barred trimmer coat protein in icosahedral double-strand DNA viruses infecting each of the 3 domains of life [18].

In conclusion, the search for new bacterial pathogens that cause pneumonia led to the discovery of what is currently the largest virus ever encountered, and characterization of this organism has yielded results that have pushed our knowledge of viral diversity to new limits. However, such steps have also blurred our current understanding of what viruses are.

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Potential conflicts of interest. All authors: no conflicts.

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