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Disseminated *Ureaplasma* infection as a cause of fatal hyperammonemia in humans

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Abstract

Hyperammonemia syndrome is a fatal complication affecting immunosuppressed patients. Frequently refractory to treatment, it is characterized by progressive elevations in serum ammonia of unknown etiology, ultimately leading to cerebral edema and death. In mammals, ammonia produced during amino acid metabolism is primarily cleared through the hepatic production of urea, which is eliminated in the kidney. *Ureaplasma* species, commensals of the urogenital tract, are Mollicutes dependent on urea hydrolysis to ammonia and carbon dioxide for energy production. We hypothesized that systemic infection with *Ureaplasma* species might pose a unique challenge to human ammonia metabolism by liberating free ammonia resulting in the

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SUPPLEMENTARY MATERIALS

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hyperammonemia syndrome. We used polymerase chain reaction, specialized culture, and molecular resistance profiling to identify systemic *Ureaplasma* infection in lung transplant recipients with hyperammonemia syndrome, but did not detect it in any lung transplant recipients with normal ammonia concentrations. Administration of *Ureaplasma*-directed antimicrobials to patients with hyperammonemia syndrome resulted in biochemical and clinical resolution of the disorder. Relapse in one patient was accompanied by recurrent *Ureaplasma* bacteremia with antimicrobial resistance. Our results provide evidence supporting a causal relationship between *Ureaplasma* infection and hyperammonemia, suggesting a need to test for this organism and provide empiric antimicrobial treatment while awaiting microbiological confirmation.

INTRODUCTION

Hyperammonemia syndrome (HS) is an uncommon but usually fatal disorder that affects immunosuppressed patients, including about 4% of lung transplant recipients (1, 2). Patients with HS show progressive elevation in serum ammonia concentrations, accompanied by worsening neurological abnormalities, cerebral edema, and death. The low frequency of this disorder has precluded systematic study, but therapies that suppress endogenous ammonia production and augment ammonia secretion have been reported to be ineffective (2). *Ureaplasma urealyticum* and *Ureaplasma parvum* are fastidious organisms that are commonly found as part of the normal microbiome of the human urogenital tract but are infrequently recognized because they do not grow in routine bacteriological cultures. *Ureaplasma* species differ from many other bacteria in the microbiome in that they produce urease. The hydrolysis of urea and generation of ammonia as a byproduct of this reaction in the urine are inconsequential to the host; however, systemic infection with *Ureaplasma* organisms may pose a severe threat to mammalian ammonia clearance (3–5). Here, we used longitudinal *Ureaplasma* PCR and culture to provide evidence supporting a causal role of systemic infection with *Ureaplasma* species in the pathogenesis of the HS and suggest approaches to diagnose and treat this disorder.

RESULTS

Index case

A 44-year-old man with pulmonary sarcoidosis underwent bilateral lung transplantation at Northwestern Memorial Hospital (Chicago, IL) with induction immunosuppression consisting of methylprednisolone (500 mg) and basiliximab (20 mg). Primary graft dysfunction necessitated 2 days of extracorporeal membrane oxygenation. Maintenance immunosuppression included tacrolimus (target trough level, 8 to 12 ng/ml), mycophenolate mofetil (1000 mg twice daily), and prednisone (0.5 mg/kg). He received antimicrobial prophylaxis with trimethoprim-sulfamethoxazole, valganciclovir, and voriconazole. A 7-day course of vancomycin and cefepime was administered to treat *Staphylococcus aureus*, *Stomatococcus* species, and viridans group streptococci isolated from bronchoalveolar lavage (BAL) fluid. Seven days after transplant, he developed acute mental status changes. Routine blood, urine, and BAL fluid cultures were negative, and head computed tomography was normal. Serum ammonia, which had been normal on postoperative day 4, was 61 μ M on day 5 (laboratory reference range, 0 to 53 μ M) and 107 μ M on day 7 (Fig. 1). Testing to

exclude known abnormalities in urea cycle metabolism was negative (table S1). Venovenous hemodialysis for ammonia removal was initiated; tacrolimus and protein in the tube feedings were temporarily discontinued; and lactulose, rifaximin, sodium phenylacetate/benzoate, and L-carnitine were administered according to an established protocol (table S2) (2). Despite these therapies, the serum ammonia rose to 291 μ M on day 10. On the basis of a reported association between *Mycoplasma hominis* infection and hyperammonemia (6), the patient received azithromycin (500 mg daily for 5 days), beginning 10 days after transplant. Serum ammonia concentration began to decrease the day after azithromycin initiation and was normal on day 15 (Fig. 1A), and this normalization was accompanied by recovery of his mental status. Recurrent hyperammonemia developed on postoperative day 31 (Fig. 1B) and worsened despite reinstitution of the therapies above, including parenteral azithromycin. On postoperative day 40, he died from a neurologic event, and autopsy revealed cerebral edema and pulmonary consolidation.

Molecular diagnostics

We undertook a careful microbiologic examination of specimens collected during the patient's hospitalization (Table 1, microbiologic results for non-*Ureaplasma* organisms are presented in table S3). Examination of plasma by polymerase chain reaction (PCR) first detected *U. urealyticum* 9 days after transplantation, 1 day before the initiation of azithromycin therapy. This specimen was negative for *M. hominis* by PCR and culture. Examination of plasma for *U. urealyticum* by PCR was positive 1 day before starting azithromycin and negative immediately after treatment (Fig. 1A and Table 1). Samples of bladder, liver, spleen, and lung tissue obtained less than 6 hours postmortem tested positive for *U. urealyticum* and negative for *U. parvum*, *M. hominis*, and *Mycoplasma pneumoniae* by PCR. *U. urealyticum* was cultured from the lung at autopsy and was resistant to azithromycin and erythromycin [minimum inhibitory concentration (MIC) values, 256 μ g/ml] but susceptible to levofloxacin (MIC, 1 μ g/ml), tetracycline (MIC, 0.5 μ g/ml), and doxycycline (MIC, 0.25 μ g/ml). Sequencing of this *U. urealyticum* isolate identified a A2058G mutation in the 23S ribosomal RNA (rRNA) gene operon 2 (*Escherichia coli* numbering system) associated with macrolide resistance (7, 8). No other mutations were detected in this gene or the genes for proteins L4 or L22 of the ribosomal unit. Native lung tissue from the recipient was negative for these organisms.

Additional patients with hyperammonemia and control cohort

We then performed microbiologic analysis of other patients who died of HS at three different centers, as well as 20 lung transplant recipient controls with normal ammonia concentrations (6). The first patient was a 59-year-old man who underwent single-lung transplantation at Washington University (St. Louis, MO) for emphysema, developed HS refractory to the therapies described above 10 days after transplant, and died from cerebral edema with herniation. Freshly frozen, paraffin-embedded lung tissue collected at autopsy tested positive for *U. urealyticum* but negative for *U. parvum* and *M. hominis* by PCR. The second patient was a 64-year-old man who had undergone bilateral sequential lung transplantation at Mayo Clinic (Rochester, MN) for pulmonary fibrosis and died 16 days after transplantation from cerebral edema. Liver function was normal, and liver biopsy revealed normal architecture; glutamine synthetase levels were normal, as described in a

report previously published on this patient (6). This patient was shown to have systemic *M. hominis* infection (6). We performed PCR and found that residual BAL fluid and blood collected shortly before his death were positive for both *M. hominis* and *U. parvum*. The third patient was a 59-year-old man who underwent bilateral lung transplant for idiopathic pulmonary fibrosis at Loyola Medical Center (Chicago, IL). On postoperative day 12, he developed mental status changes, and his ammonia concentration was 549 μ M. Despite treatment similar to that outlined in table S2, the ammonia concentration increased to greater than 1700 μ M, and the patient expired. Freshly frozen, paraffin-embedded lung tissue collected at autopsy tested positive for *U. urealyticum* but negative for *U. parvum* and *M. hominis* by PCR. We obtained the donor BAL collected before lung harvest, as well as the recipient's BAL collected immediately after implantation, and found that both tested positive for *U. urealyticum* but negative for *U. parvum* and *M. hominis*. The recipient's native lung tissue was negative for all three organisms.

We then tested BAL fluid and serum collected from 20 lung transplant recipients who had documented normal ammonia concentrations 1 to 7 days after transplant; all were PCR-negative for *U. urealyticum*, *U. parvum*, and *M. hominis*. Cultures could be obtained from five of these patients and were also negative for the same three organisms (Table 1).

Prospective validation

On the basis of these results, we tested for *Ureaplasma* organisms in blood and BAL fluid from a 52-year-old female who underwent bilateral lung transplantation at Washington University (St. Louis, MO) for interstitial lung disease complicated by refractory hyperammonemia 9 days later. Both specimens were positive for *U. parvum* by PCR, and BAL fluid culture was positive for *U. parvum* but negative for *U. urealyticum* or *M. hominis*. Two days after initiating therapy with levofloxacin and azithromycin, the ammonia concentration normalized, and her mental status returned to normal (Fig. 2). After 7 days of antimicrobial treatment, both BAL fluid and blood were negative for *U. parvum*, and there has been no relapse of hyperammonemia in 6 months of follow-up.

Another recent patient with HS was successfully treated, providing additional prospective validation of the relationship between *Ureaplasma* and HS. This was a 55-year-old female who underwent bilateral sequential lung transplant for idiopathic pulmonary fibrosis at Massachusetts General Hospital (Boston, MA). Because the lymphocyte crossmatch was positive at the time of transplant, she was treated with peri- and postoperative plasmapheresis, followed by intravenous immunoglobulin and rituximab. The remainder of her immunosuppression regimen consisted of pre-/postoperative mycophenolate and intraoperative methylprednisolone 1 g intravenously and then 125 mg every 12 hours for a total of eight doses, followed by tapering doses; basiliximab 20 mg intravenously on postoperative days 2 and 6; and tacrolimus on postoperative day 4, titrated to achieve a trough concentration of 10 to 14 ng/ml. On day 10, she demonstrated altered mental status. Computed tomography of her brain demonstrated no evidence of acute pathology, but an electroencephalogram showed bifrontal rhythmic periodic discharges concerning for nonconvulsive epileptic seizure activity. Magnetic resonance imaging revealed newly detected multiple microhemorrhages. At that time, her ammonia concentration was found to

be elevated at 135 μM . Lactulose (20 g three times a day), rifaximin (550 mg two times a day), sodium benzoate (5 g two times a day), arginine (4 g three times a day), and levocarnitine (7 g/day) were initiated. Her tube feeds were adjusted to lower protein content. Hemodialysis was also initiated to facilitate removal of ammonia. Additionally, tacrolimus was discontinued on postoperative day 20 when hyperammonemia failed to resolve and replaced with cyclosporine, similar to the index patient. Although ammonia concentration transiently decreased, this response was dependent on hemodialysis. Even a temporary discontinuation of hemodialysis led to an increase in ammonia to greater than 200 μM . This pattern persisted even at day 42, with no significant improvement in mental status or consistent decrease in ammonia. At this time, her BAL and blood were tested, and both were found to be positive for *U. parvum* by PCR and culture. The patient received levofloxacin, and the ammonia concentration decreased to 30 to 35 μM , despite discontinuation of hemodialysis and other adjunctive methods for treating hyperammonemia. At 4 months after transplant, there has been no relapse of HS.

DISCUSSION

HS is an often fatal complication of systemic immunosuppression, including that associated with lung and other solid organ transplantation; however, its low frequency has hampered systematic study of its etiology and precludes randomized trials of potential therapies (1, 6, 9–12). Here, we present evidence suggesting that systemic infection with *Ureaplasma* species plays a causal role in the development of hyperammonemia. We found evidence of systemic infection with either *U. urealyticum* or *U. parvum* in all patients with HS after lung transplantation and none of 20 control lung transplant patients without the disorder. In three affected patients, treatment with antimicrobials targeting *Ureaplasma* species was associated with resolution of both biochemical and neurological abnormalities. Recurrent hyperammonemia in one of these patients was accompanied by disseminated infection with a strain of *Ureaplasma* that had likely acquired antibiotic resistance.

Our suggestion that disseminated *Ureaplasma* infection is responsible for HS is consistent with the described clinical features of the disease and is biologically plausible. Ammonia is generated as a byproduct of amino acid metabolism and is normally converted to urea in the liver, with the urea then being excreted in the urine. In addition, the brain and skeletal muscle can metabolize limited amounts of ammonia through the generation of glutamine. Glutamine is then metabolized in the kidney to regenerate ammonia, which is excreted in the urine. *Ureaplasma* organisms have developed a metabolic strategy that uses the free energy released during the hydrolysis of urea into CO_2 and ammonia to create a membrane potential based on the differential concentrations of the ammonium cation outside and inside the bacterial membrane. This membrane potential is then used to phosphorylate adenosine diphosphate to adenosine triphosphate via a chemiosmotic mechanism. In patients colonized with *Ureaplasma* organisms in the urogenital tract, the hydrolysis of urea and the production of ammonia by the organisms are inconsequential; however, disseminated infection threatens ammonia excretion. First, the organism releases free ammonia generated by the hydrolysis of urea into the circulation. Second, the released ammonia is converted back to urea in the liver providing more substrate to the organism. The hydrolysis of urea during *Ureaplasma* bacteremia might also reduce the efficiency of ammonia clearance during

dialysis, which removes ammonia both as urea and as free ammonia, providing an explanation for why patients with hyperammonemia respond poorly to this therapy. Dietary protein restriction and inhibitors of protein catabolism, also suggested as therapies for patients with hyperammonemia, would be predicted to have little effect on the altered ammonia metabolism during disseminated *Ureaplasma* infection, because a cycle of organismal urea hydrolysis and hepatic urea generation is unlikely to be significantly affected by the small changes in ammonia generation induced by these strategies. Indeed, all of these modalities were ineffective in the absence of antimicrobial therapy in all the hyperammonemia patients we examined.

Ureaplasma species are part of the normal microbiome of the urogenital tract. Systemic spread of the organism in response to immunosuppression is therefore a plausible mechanism for dissemination in immunocompromised hosts. However, we cannot exclude the possibility of other sources of *Ureaplasma*, including transmission from donor organs. Data from the patient at Loyola Medical Center suggest that transmission of this organism from the donor is a possible mechanism, because donor BAL before the lung procurement was positive for *Ureaplasma* species. HS has been described in other immunocompromised states, including heart-lung (9), kidney (13), liver (14), intestinal (15), islet cell (16), and bone marrow (17) transplantation; however, the syndrome has been most frequently reported in lung transplant recipients. This might result from increased recognition of the disorder in this population or reflect increased risks associated with lung transplantation, including the possibility of donor transmission. For example, exposure of the lung directly to the systemic venous circulation and impaired lymphatic drainage in the donor lung after transplantation have been suggested to increase the risk of pneumonia in the first 4 to 6 weeks after transplantation (18–20). In support of this hypothesis, we were able to detect or culture *Ureaplasma* from all of the lung tissue and BAL samples collected from patients with hyperammonemia available for our analysis. Most patients who are affected with HS have undergone bilateral lung transplantation, as observed here. Whether this is a significant risk factor as compared to single-lung transplantation and whether it is related to increased pathogenic load in the bilateral donor lung block or the lack of pulmonary lymphatics in both lungs remain to be determined.

A limitation of our study is that it does not include patients with other immunosuppressed states. Therefore, further studies should be performed to investigate the pathogenic role of *Ureaplasma* in bone marrow and other solid organ transplants, as well as critically ill patients or those undergoing chemotherapy. Additional limitations include small sample size for prospective validation of recipient infection as well as donor transmission. Further prospective studies will be required to determine the incidence of donor and recipient colonization as well as the source of *Ureaplasma* dissemination in the host. Additionally, the mechanisms by which *Ureaplasma* species alter human ammonia metabolism should be investigated, perhaps using laboratory and animal models. Nevertheless, given the absence of proven treatments for HS, our findings support testing for *Ureaplasma* species in patients with HS, as well as empirical use of antimicrobial therapy against *Ureaplasma* species while awaiting test results. A nucleic acid amplification test (NAAT) such as PCR provides the fastest testing strategy; culture may be especially useful to assess antimicrobial susceptibility

if a NAAT is positive. Our results suggest that in lung transplant recipients with HS, both blood or plasma and BAL fluid should be tested. *Ureaplasma* organisms are generally susceptible to macrolides, fluoroquinolones, and tetracyclines, although resistance to all these classes has been reported and, as observed here, may emerge during therapy. Until predictors of resistance emergence and contemporary resistance patterns of *Ureaplasma* species are better understood, it seems prudent to treat patients with HS with combination therapy. In addition, screening of patients and donors for *Ureaplasma* species before or immediately after transplantation might help inform future consideration of global or targeted prophylaxis.

MATERIALS AND METHODS

Culture

U. urealyticum, *U. parvum*, and *M. hominis* culture was performed at Mayo Clinic and Quest Diagnostic Nichols Institute. Specimens were cultivated on A8 agar plates (Hardy Diagnostics) in an anaerobic atmosphere at 35°C for 6 weeks. Culture plates were examined by inverted light microscopy at least weekly. Suspect colonies were confirmed by real-time PCR (21). Recovered isolates were subcultured and incubated for 48 hours. Subculture material was placed into M5 transport medium (Remel) and frozen at –20°C for transport to the University of Alabama at Birmingham (UAB) for susceptibility testing.

Antibiotic susceptibility testing

Minimal inhibitory concentration values were determined by broth microdilution in 10B broth (22), in accordance with Clinical and Laboratory Standards Institute guidelines (<http://clsi.org/blog/2011/11/11/clsi-publishes-a-guideline-on-methods-for-antimicrobial-susceptibility-testing-for-human-mycoplasmas-m43/>) (22).

PCR and molecular resistance profiling

PCR for *U. parvum*, *U. urealyticum*, and *M. hominis* was performed at Mayo Clinic as described before (21). Genomic *U. urealyticum* DNA was purified using the QIAamp DNA Blood Mini Kit (Qiagen). Conserved regions of ribosomal protein L4 and L22 genes and two whole 23S rRNA operons were amplified using published primers (23, 24). PCR was performed with Platinum PCR SuperMix (Invitrogen) on a Veriti 96-well thermal cycler (Applied Biosystems). PCR products were cleaned by USB ExoSAP-IT (Affymetrix) and sequenced using Sanger technology at UAB. Sequences were compared to corresponding gene sequences of 14 macrolide-susceptible American Type Culture Collection (ATCC) type strains of *U. parvum* (serovar 1, ATCC 27813; serovar 3, ATCC 27815; serovar 6, ATCC 27818; and serovar 14, ATCC 33697) and *U. urealyticum* (serovar 2, ATCC 27814; serovar 4, ATCC 27816; serovar 5, ATCC 27817; serovar 7, ATCC 27819; serovar 8, ATCC 27618; serovar 9, ATCC 33175; serovar 10, ATCC 33699; serovar 11, ATCC 33695; serovar 12, ATCC 33696; and serovar 13, ATCC 33698) using CLC DNA Workbench 7.5 (Qiagen).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Clinical course of index patient

(A) Initial occurrence of hyperammonemia in index patient. (B) Recurrence of hyperammonemia in the index patient and response to therapy.

Fig. 2.
Prospective validation of the association of *Ureaplasma* with hyperammonemia after lung transplantation.

Table 1
Results of testing for *Ureaplasma* species and clinical profile of the patients

COPD, chronic obstructive pulmonary disease; PF, pulmonary fibrosis; CF, cystic fibrosis; IPF, idiopathic pulmonary fibrosis.

Subject	Source tissue	Test	Result	Clinical profile
Index patient	Plasma (day 5)	PCR	Negative	44 years; male; sarcoidosis; bilateral lung transplant; survival, 40 days
	Plasma (day 9, before azithromycin)	PCR	<i>U. urealyticum</i>	
	Plasma (day 15, on azithromycin)	PCR	Negative	
	Plasma (day 33, on azithromycin, hyperammonemia recurrence)	PCR	<i>U. urealyticum</i>	
	Bladder (fresh, postmortem)	PCR	<i>U. urealyticum</i>	
	Lung (fresh, postmortem)	PCR	<i>U. urealyticum</i>	
	Lung (fresh, postmortem)	Culture	<i>U. urealyticum</i> *	
	Liver (fresh, postmortem)	PCR	<i>U. urealyticum</i>	
	Spleen (fresh, postmortem)	PCR	<i>U. urealyticum</i>	
Expired patient 1 [†]	Lung (formalin-fixed paraffin-embedded)	PCR	<i>U. urealyticum</i>	59 years; male; COPD; bilateral lung transplant; survival, 10 days
Expired patient 2 [‡]	BAL	PCR	<i>U. parvum</i> , <i>M. hominis</i>	64 years; male; IPF; bilateral lung transplant; survival, 16 days
	Whole blood (EDTA)	PCR	<i>U. parvum</i> , <i>M. hominis</i>	
Expired patient 3 [‡]	BAL	PCR	<i>U. urealyticum</i>	59 years; male; IPF; bilateral lung transplant; survival, 21 days
	Lung (formalin-fixed paraffin-embedded)	PCR	<i>U. urealyticum</i>	
	Liver (formalin-fixed paraffin-embedded)	PCR	<i>U. urealyticum</i>	
Prospective case 1	BAL (day 12 before <i>Ureaplasma</i> treatment)	PCR and culture	<i>U. parvum</i>	52 years; female; IPF; bilateral lung transplant; alive at 6 months
	Blood (day 12 before <i>Ureaplasma</i> treatment)	PCR	<i>U. parvum</i>	
	BAL (day 19, after treatment)	PCR and culture	Negative	
	Blood (day 19, after treatment)	PCR	Negative	
Prospective case 2	BAL (day 42 before <i>Ureaplasma</i> treatment)	PCR and culture	<i>U. parvum</i>	55 years; female; IPF; bilateral lung transplant; alive at 4 months
	Blood (day 42 before <i>Ureaplasma</i> treatment)	PCR	<i>U. parvum</i>	
Negative controls (n = 15) [‡]	BAL (archived specimens)	PCR	Negative	Patients with documented normal ammonia concentrations. Mean age, 54.5 ± 5.3 years. Had COPD (50%), PF (35%), or CF (15%).
	Serum (archived specimens)	PCR	Negative	
Negative controls (n = 5) [§]	BAL	PCR and culture	Negative	

Subject	Source tissue	Test	Result	Clinical profile
	Whole blood	PCR and culture	Negative	

* Isolate found to be macrolide-resistant and to carry A2058 23S rRNA gene mutation.

† Posthumous testing.

‡ Collected between days 1 and 7 after transplantation.

§ Collected between days 7 and 14 after transplantation.