Pathogenic Analysis of Aeromonas Hydrophila Septicemia*

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ABSTRACT

Aeromonas hydrophila has emerged as a potential pathogen in the immunocompromised host. Various aeromonal infections, including septicemia, have also been reported in apparently healthy individuals. For years, researchers have disagreed over the epidemiologic roles of aeromonads in gastroenteritis. Isolation rates of aeromonads by stool culture among patients with gastroenteritis are not consistently high. Carriers of this bacterium also exist. The septicemic course is, however, often fulminant and fatal, and may lack an obvious focus. Pathogenic mechanisms are complex and largely unresolved.

The objective of this study is to report the necropsy findings from a uremic patient who presented with typical aeromonal septicemia of obscure origin asking if such investigation could give insight into some of the questions mentioned previously. Western blot immunostaining for aerolysin (β-hemolysin of aeromonads) was used to evaluate whether or not such a virulence factor is involved in the process of septic dissemination. The autopsy showed that the skin and liver contained microabscesses. The upper gastrointestinal mucosae and spleen contain patchy putrefactive lesions with adjacent focal hemorrhage. Perimortem blood cultures grew Aeromonas hydrophila. A conventional Western blot analysis of the culture supernatant failed to show aerolysin. A control Aeromonas sobia American Type Culture Collection (ATCC) strain produces readily detectable aerolysin. It is concluded that this isolate may be aerolysin-deficient or one secreting low levels of aerolysin; these would require more sensitive methods of detection. The primary focus of infection might be the upper gastrointestinal tract. Other virulence factors including the bacterial proteases and/or phospholipases might be responsible for the pathogenesis of septic dissemination.

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Introduction

Aeromonas hydrophila (A. hydrophila) is a polarly flagellated, small, gram-negative (G−) rod-shaped bacteria that produces oxidase. It is a facultative anaerobe and ferments glucose. The organism exists in an aquatic environment and is also part of the normal intestinal flora of leeches. A. hydrophila can cause infections in various cold-blooded and warm-blooded animals. In humans, aeromonads are associated with septicemia, pneumonia, gastroenteritis, endocarditis, and hepatobiliary and soft tissue infections. In patients with renal failure, aeromonads may cause peritonitis, septicemia, and the contamination of dialysate. An obvious focus of infection cannot be found in many septicemic patients. Most investigators believe that the infections are of nosocomial origin or that they arise through intact or defective intestinal mucosa. In practice, Aeromonas spp. are grouped among the organisms whose association with gastroenteritis is probable.

Pathological features of human aeromonal gastroenteritis and septicemia are, however, seldom reported in detail in the literature. A case is described in which a uremic patient died within 30 hours of presentation of a fulminant septicemic infection caused by A. hydrophila. These pathological findings are presented in order to characterize gastrointestinal (GI) involvement in cases of septicemia caused by Aeromonas Spp.

Materials and Methods

Species Identification

Blood samples taken upon admission and at postmortem grew A. hydrophila.* The species was identified by the Vitek Instrument 120† followed by a positive Voges-Proskauer (VP) reaction using a microbiological identification kit.‡

Cytotoxic Assay

Cell-free supernatants of Aeromonas broth cultures were prepared according to the method of Cumberbatch et al. Briefly, the organisms were plated on 5 percent sheep blood agar. Several colonies were suspended in Brain-Heart Infusion (BHI) broth§ to 1 McFarland unit as measured by turbidimetry (Vitek colorimeter, product number 52-1210). Eight milliliter suspensions were incubated in 50-ml Erlenmeyer flasks at 37°C and 100 rpm agitation for 8 to 24 hours. The cultures were spun at 12,000 g for 45 minutes at 4°C. The supernatants were sterilized by passage through 0.45 μm nitrocellulose filters. The filtrates were stored at −20°C or used directly in the cytotoxic and blotting experiments. Cytolytic capacities of the sterile culture supernatants were analyzed through incubation with normal fresh necropsy tissues in small pieces for 30 hours at 4°C or at room temperature. Plain BHI broths were used as negative controls. At different times during incubation, treated tissues were processed for routine H & E histologic evaluation.

Electron Microscopy

Aliquots of 24-hour broth cultures were washed in saline and spun at 2,500 rpm for 10 minutes. The pellets were resuspended in saline and processed for electron microscopic study in 2 percent phosphotungstic acid (PTA) (negative staining) on carbon-coated formvar. Formalin-fixed hepatic and splenic tissues were further fixed in 3 percent glutaldehyde and processed in 1 percent (W/V) osmium tetroxide and a graded series of alcohols. Processed specimens were embedded in epoxy.

* Bactec NR-860, Becton Dickinson Diagnostic Instrument Systems, Sparks, MD 21152.
† bioMerieux Vitek Inc., Hazelwood, Mo 63042.
‡ Organon Teknika Corp., Durham, NC 27712.
§ BBL, Becton Dickinson Microbiology Systems, Cockeysville, MD 21031.
‖ Acrodisc, Gelman Sciences, Ann Arbor, Mi 48106.
resin. Thin sections of representative blocks were stained with uranyl acetate and lead citrate and examined with a Philips EM 201 transmission electron microscope (TEM).§

IMMUNOBLOTTING

Supernatants of the 8-, 16- and 24-hour cultures were analyzed for aerolysin (β-hemolysin/cytotoxin of A. hydrophila) by immunoblotting after sodium dodecyl sulfate electrophoresis in 12 percent acrylamide slabs.** Aeromonas sobia (A. sobia)†† with clearer β-hemolysis served as a positive control. Primary mouse monoclonal antibody to aerolysin was used.‡‡ Commercially available, avidin-labeled monoclonal anti-mouse or anti-rabbit secondary antibodies and biotin-labeled horseradish peroxidase (HRP), or HRP-conjugated sheep anti-mouse secondary antibody were used. The blocking agent was 1 percent nonfat goat milk in phosphate-buffered saline. Prestained low-range molecular weight protein standards were used.** The cellular extracts of the bacterial pellets obtained from different time points of broth cultures were also subjected to blotting. The pellets were first suspended in saline to 1 McFarland unit. Eighty microliters of each suspension were boiled in 20 μL of Laemmli loading buffer (5×) for 3 minutes before electrophoresis.

Results

CASE HISTORY

The patient was a 66-year-old white man with chronic renal failure that was thought to be secondary to hypertension and nephrosclerosis. He had received regular hemodialysis since 1986. He had a GI hemorrhage in 1987 secondary to the Mallory-Weiss syndrome. He was an ethanol user. One month before this presentation, he had been admitted to hospital for digitalis overdose. Skin tests at that time showed immune anergy. His most recent regular dialysis had occurred two days before this visit. One day prior to this admission in late July, 1993, he presented with left knee pain that migrated toward the ankle. Laboratory data at a local hospital showed metabolic acidosis and hyperkalemia of 6.3 meq/L. He was intubated on his transfer to the West Virginia University hospitals (WVUH). Peripheral blood cell counts performed at WVUH showed anemia (12.2 g/dL Hgb), leukopenia (3300/μL) with left shift (25% Band), and thrombocytopenia (58000/μL). Prothrombin and partial thromboplastin times were mildly prolonged. A routine x-ray of the left leg was normal. The initial impression of the treating physician was sepsis. The patient was hemodialyzed through femoral catheterization because of a somewhat thrombosed radial shunt; his blood pressure, however, continued to drop. He died shortly after dialysis. A complete necropsy was performed 8 hours later.

GROSS FINDINGS

At autopsy, the skin showed multiple purple discolorations representing the old bruised lesions of long-term uremia. A few small vesicles with erythematous bases were identified in the region of the thighs. The entire gastric mucosa was dark red. The rugae were swollen and coated with patchy fibrinous exudate. Multiple enlarged subserosal lymph nodes were present. The entire duodenum and small intestines also showed diffuse swelling and congestion. The cecal mucosa showed patchy congestion. The colon was grossly unremarkable. The liver showed mottled brown cut surfaces. The kidneys were small and contained focal congestions and numerous small cysts on the entire cut surfaces. The rest of the organs all showed varying degrees of congestion.

MICROSCOPIC FINDINGS

Microscopically, the liver tissue contains focal septic microabscesses with hemorrhage and shrunken hepatic cords in the adjacent area. The small petechial skin lesions also consist of epidermal microabscesses with dermal congestion and hemorrhage. The upper GI tract shows extensive involvement of patchy anucleated shrunken mucosae with adjacent hemorrhage and mild focal lymphocytic infiltrate (figure 1). The dilated submucosal vessels and lymphatics contain fibrinous thrombi with gram-negative (G−) bacilli. The subserosal nodes contain subcapsular hemorrhage and fibrinous material with similar organisms. The spleen contains similar putrefactive foci without noticeable microabscesses in the evaluated sections. The other organs, including the lung, show only focal dilated vessels containing fibrinous material and G− bacilli.

SPECIAL STUDIES

The sterile culture supernatants of the A. hydrophila isolate were able to induce putrefactive disintegration of the incubated tissues in vitro at room temperature but not

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† Philips Electron Optics, The Netherlands.
** Bio-Rad Laboratories, Hercules, Ca 94547.
†† ATCC 9071 reference strain, American Type Culture Collection, Rockville, MD 20852.
‡‡ Provided by Dr. J. T. Buckley from the Department of Microbiology, Victoria University, Victoria, British Columbia, Canada.
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Figure 1. Microscopic pathology of the patchy putrefactive gastric lesion associated with bacterial colony and adjacent hemorrhage (Hematoxylin & eosin; 40x magnification).

at 4°C. Disintegration was marked by the loss of nuclei, cellular swelling, and disruption similar to the lesions described in autopsy.

The colonies of *A. hydrophila* clinical isolate showed small and somewhat discrete zones of hemolysis on 5 percent sheep blood agar at 40 hours of growth. *A. sobia*, the ATCC control strain, showed larger confluent zones of β-hemolysis (figure 2). The broth culture pellets of clinical isolate were pink, and those of the control strain were white. Western blot analysis of the sterile 24-hour culture supernatants failed to show a 52-kDa aerolysin molecule on the clinical *A. hydrophila* isolate (figure 3). An aerolysin band was identified on the control *A. sobia* isolate that was grown and tested in identical conditions. The secretion was present at 8 hours and continued to increase through 24 hours of culture. The extracts of bacteria pellets from both strains do not reveal aerolysin molecules in the amount applied for electrophoresis.

Ultrastructurally, the liver and spleen contained numerous bacillary organisms similar to those obtained from blood cultures. Cellular debris and apparently hemolyzed ghost red blood cells were also seen. Fimbriae and typical monorichous coiled polar flagella at a wavelength of approximately 1.7 μm were identified in cultured organisms by PTA-negative staining. These are only rarely partially identified in tissue sections with regular transmission electron microscopy.

Discussion

The main difficulty in assessing the significance of various lesions in our case was the striking patchy putrefactive disintegration in many organs. Postmortem decomposition did not contribute significantly in this case because of the short postmortem period before autopsy.

Figure 2. Hemolytic patterns of *A. hydrophila* isolate (Top) and *A. sobia* control (Bottom) on 5 percent sheep blood agar at the fortieth hour of culture at 37°C.
The unique distributions of the putrefactive lesions differ from the usual pattern of autolysis as well. Regular postmortem autolysis occurs early in the pancreas, intestinal linings, and adrenal glands. Putrefactive lesions in this case were associated with organisms, and occurred abundantly in the liver, spleen, and upper GI tract. A small part of the changes may result from postmortem growth of aeromonads in tissues. The premortem colonization, tissue cytolysis, and vascular dissemination, however, were important initial processes in vivo leading to distinctive putrefactive lesions with hemorrhage, fibrinous emboli with organisms, lymphadenitis, and microabscesses.

Most *A. hydrophila* isolates are capable of producing aerolysin, which can cause hemolysis and tissue cytolysis. They also secrete protease, phospholipase, and other substances that may cause similar pathological changes as well. Aerolysin causes severe congestion and hemorrhage of intestinal mucosae in the rabbit model. It is lethal to mice at very low concentrations. In our case, hemolysis and cytolysis were observed in the vessels and tissues at both the microscopic and ultrastructural levels. However, immunoblotting failed to show aerolysin. The faint hemolysis on blood agar of the aerolysin-deficient clinical isolate compared with the clear β-hemolysis of the aerolysin-producing ATCC strain suggests that the former hemolysis might be due to α-hemolysis, non-specific phospholipase activities, or both. These possibilities had been addressed by Ljungh and Wadstrom.

It is also possible that our isolate secretes a variant or a small amount of β-hemolysin that could not be recognized by the monoclonal antibody clone used or at the amount of supernatant tested. More sensitive methods, such as the chemiluminescence detection system or nucleic acid hybridization/amplification, are required to rule out the presence of aerolysin activity.

For years, determining the epidemiologic roles of aeromonads in gastroenteritis has been controversial. This has been partly due to the existence of various species and strains of aeromonads that vary in their pathogenicity. The results of the western blot analysis suggest that our isolate is another pathogenic variant of *A. hydrophila*. Consequently, the striking putrefactive tissue changes might be the important and unique pathogenic processes of this variant. Further investigation is necessary to elucidate the exact mechanisms involved in these processes.

The GI tract can also become the embolic target of any septicemia. Pathological changes of organs in septicemia were described in a baboon model injected intravenously with *Escherichia coli*. The alterations in the GI tract ranged from mucosal congestion to multiple bleeding spots. In that report, bacterial disseminations existed mainly in the dilated vessels of the intestines. They also elicited early pneumonic lesions including fibrin emboli, interstitial edema, and polymorphonuclear leukocytic reactions.

In this case, the aeromonal organisms existed in vessels, in the altered lamina propria portions of mucosae, and on the desquamated cryptal and surface epithelia. These findings suggested that the upper GI tract was the ini-
tial focus of infection. The paucity of pulmonic and colonic lesions also argued against bacterial dissemination from another origin as the only cause of gastric lesions. If it were otherwise, all the organs would have similar numbers of lesions produced during the bacteremic process. The bulky upper GI and hepatic lesions in this case suggested that the primary route of infection was oral.

It is thought that bacterial migration to the mesenteric lymph nodes and further to systemic circulation might be important mechanisms of dissemination of the GI organisms in septic conditions. The overall pathological changes in this case also resembled those in a tied ileal loop model in which aeromonads were inoculated into reversibly tied rabbit small intestine. Therefore, it is our conclusion that oral ingestion of A. hydrophila from environmental sources might be the cause of gastric lesions and subsequent septicemia. Several virulence characteristics of our isolate were evaluated and were found to be an aero-lysin-deficient variant or one that secretes low levels of aerolysin; it thus requires further characterization with more sensitive methods.

It is believed that the combination of the host factors (uremic, immunocompromised) and the microorganism factors (virulence) resulted in this fatal outcome. Analysis of this case may help to define the pathogenic mechanisms and epidemiologic distributions of the virulent variants. In addition, strategy aimed at neutralization of virulence factors might be developed and add another therapeutic modality beyond present antibiotics.

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