Exotoxin Activity Associated with the Legionnaires Disease Bacterium

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Hemolysis occurred around growth of the Legionnaires disease bacterium on supplemented Mueller-Hinton agar containing sterile defibrinated blood from each of five mammalian species. Hemolysis was most pronounced with guinea pig or rabbit blood, was less intense with horse or sheep blood, and was slight with blood from a human donor. Sterile filtrates of allantoic fluid from embryonated hen's eggs that had been infected with this organism displayed hemolytic activity in a radial hemolysis assay with guinea pig cells in agar. Growth of the Legionnaires disease bacterium on F-G agar with 5% hen's egg yolk was surrounded by a zone of clearing and more circumscribed zones of iridescence and increased opacity on and in the medium. Attempts to detect activity analogous to that of *Escherichia coli* heat-labile or heat-stable enterotoxin in allantoic fluid from infected eggs or in cultures of the Legionnaires disease bacterium were not successful.

The pathogenesis of pulmonary and extrapulmonary disturbances in Legionnaires disease has not been defined. This report describes characteristics of cultures of the Legionnaires disease bacterium in vitro and in ovo that may be relevant to a hypothesis that this organism elaborates one or more exotoxins.

Bacterial strains. Strains designated Albuquerque 1, Burlington 1, Flint 1, Philadelphia 1, Philadelphia 2, and Pontiac 1 had been isolated as previously described (2). Knoxville 1 had been isolated from lung tissue from a fatal sporadic case of Legionnaires disease in Tennessee by Joseph E. McDade, Leprosy and Rickettsia Branch, Virology Division, Bureau of Laboratories, Center for Disease Control, by guinea pig passage and inoculation of embryonated hen's eggs. All strains had been maintained by serial subculture on F-G agar (6) with incubation at 35°C under 2.5% CO₂.

Allantoic fluid. Fluid was obtained by sterile aspiration from the allantois of embryonated hen's eggs, some of which had previously been infected with Philadelphia 2 by inoculation of the yolk sac with egg-grown organisms in yolk sac suspensions (11). Allantoic fluid was harvested from infected eggs on the day that embryonic death was detected (4 to 6 days after infection, day 11 to 13 of development). Fluid from control eggs was obtained on day 13 or 14 of development.

Hemolytic activity. Hemolytic activity of cultures of the Legionnaires disease bacterium

was assessed by growing the organisms at 35°C under 2.5% CO₂ on modified F-G agar containing various concentrations of ferric pyrophosphate soluble to which 5% defibrinated blood from one of five species had been added. A 12-ml amount of agar was dispensed into petri dishes (15 by 100 mm). Hemolysis by allantoic fluid was assessed by a radial hemolysis technique, employing 100- μ l samples in wells 9.5 mm in diameter cut into the F-G-blood agar, with incubation carried out under several combinations of temperature and atmosphere.

Digestion of egg yolk. Cultures of the Legionnaires disease bacterium were grown on F-G agar containing 5% hen's egg yolk (16). A 20-ml portion of agar was dispensed into petri dishes (15 by 100 mm), and the cultures were incubated at 35°C under 2.5% CO₂.

Enterotoxin activity. Strain Philadelphia 2 maintained by serial subculture on F-G agar was used to inoculate broths and solid media. The liquid media were Mueller-Hinton broth with 0.04% L-cysteine hydrochloride and 0.01% ferric pyrophosphate soluble and Trypticase soy broth with 0.6% yeast extract, 0.04% L-cysteine hydrochloride, 0.01% ferric pyrophosphate soluble, and 1.5% starch. The solid media were F-G agar and Trypticase soy agar with 0.6% yeast extract, 0.04% L-cysteine hydrochloride, 0.025% ferric pyrophosphate soluble, and 1.5% starch. Biphasic media incorporating 12.5 ml of each of the liquid media overlying 50 ml of the corresponding agar in 250-ml Erlenmeyer flasks were also prepared.

The solid media were inoculated by preparing spread plates of each medium, using 0.1 ml of undiluted Legionnaires disease bacteria; these plates were incubated at 37°C under 2.5% CO₂ until the appearance of confluent growth. The cultures on agar were then frozen and thawed, the liquefied material was centrifuged, and the supernatant fluids were filtered through 0.22- μ m filters. The broths and biphasic media were inoculated with an initial concentration of 10⁴ colony-forming units per ml, as determined by plating on F-G agar, and incubated at 37°C under 2.5% CO₂, either stationary or in a sealed container on a rotary shaker until turbid growth was achieved. The supernatant fluids of the broths or of the liquid phase of the biphasic cultures were then filtered through 0.22- μ m filters.

Filtrates of allantoic fluid, of eluates of agar-grown cultures, and of broth- or liquid-phase supernatant fluids were tested for activity resembling that of *Escherichia coli* heat-stable enterotoxin by the suckling mouse assay (5, 12). The same materials were examined for activity resembling that of *E. coli* heat-labile enterotoxin or cholera enterotoxin by the Y1 adrenal tumor (13) and Chinese hamster ovary (CHO) (9, 12) tissue culture assays and, in some cases, by a radioimmunoassay (8).

All six strains tested (Albuquerque 1, Burlington 1, Flint 1, Philadelphia 1, Philadelphia 2, and Pontiac 1) grew on F-G agar from which ferric pyrophosphate soluble had been omitted and to which 5% sterile defibrinated guinea pig, horse, human, rabbit, or sheep blood had been added. Hemolysis was visible within 2 days of inoculation of the plates but achieved maximum intensity only after 3 days of incubation and tended to develop only after the appearance of macroscopic growth of the organism (Fig. 1). Hemolysis was most pronounced on agar made with rabbit, or, particularly, guinea pig blood, was less intense on agar with horse or sheep blood, and was least apparent on agar with human blood.

Subsequent studies with six strains (Albuquerque 1, Burlington 1, Flint 1, Knoxville 1, Philadelphia 2, and Pontiac 1) indicated that growth and hemolysis on modified F-G agar with 5% guinea pig blood was as good or better in the presence of 0.0125 to 0.025% ferric pyrophosphate soluble (Fig. 2) as when the blood alone was relied upon as the source of iron. Inspection with a dissecting microscope of areas of growth on F-G agar with 5% guinea pig blood yielding individual colonies of the Legionnaires disease bacterium revealed no relative sparing of erythrocytes immediately surrounding each colony.

Cultures on F-G agar of allantoic fluid from

FIG. 1. Growth of Legionnaires disease bacterium strain Flint 1 on Mueller-Hinton agar with 0.04% L-cysteine hydrochloride and 5% sterile defibrinated guinea pig blood, showing clear hemolysis around area of confluent growth.

FIG. 2. Growth of Legionnaires disease bacterium strain Knoxville 1 on F-G agar (ferric pyrophosphate soluble at 0.025%) with 5% sterile defibrinated guinea pig blood, showing hemolysis extending around individual colonies.

infected eggs usually yielded growth of the Legionnaires disease bacterium. Sterile filtrates of allantoic fluid from infected, but not control, eggs regularly produced zones of hemolysis around wells in modified F-G agar with 5% guinea pig blood. The zones of hemolysis appeared only gradually after several days of incubation at room temperature. Refrigeration of

plates retarded the development and spread of the zones of hemolysis, whereas incubation at 35°C under 2.5% CO₂ led to early spontaneous lysis of erythrocytes in the agar. Zones of hemolysis often appeared as concentric multiple lamellae of different intensities. Serial dilution of filtrate from an infected egg in allantoic fluid from control eggs produced a progressive decline in hemolytic activity in the radial hemolysis assay (Fig. 3). Hemolytic activity in filtrates of allantoic fluid from infected eggs resisted heating at 60°C for 30 min and was still present after heating at 100°C for 15 min (Table 1).

All six strains tested (Albuquerque 1, Burlington 1, Flint 1, Knoxville 1, Philadelphia 2, and Pontiac 1) grew on F-G agar with 5% egg yolk, although growth was somewhat more luxuriant in parallel cultures on F-G agar without egg yolk. Development of macroscopic growth was followed by the appearance of a slowly spreading zone of clearing around the areas of growth of the bacteria, seen best with transmitted light. After several days of incubation, a second and narrower zone of opaque precipitate also became evident around areas of growth on the plates. When examined under reflected light, the portions of the agar surrounding areas of confluent growth had an oily or pearly sheen.

None of the samples tested showed any evidence of adenylate cyclase stimulation in Y1 or CHO cells, nor was any other morphological evidence of cytotoxicity observed. No antigen reacting in the radioimmunoassay for *E. coli* heat-labile enterotoxin was detected. None of the samples tested showed any activity in the infant-mouse assay for *E. coli* heat-stable enterotoxin. Control tests employing appropriate enterotoxinogenic *E. coli* strains indicated satisfac-

tory sensitivity of each of the assays employed.

Agar-grown cultures of various strains of the Legionnaires disease bacterium possess hemolytic activity, which is most pronounced with erythrocytes from guinea pigs, in which the lecithin content of the erythrocyte membrane is relatively high (15). Cell-free filtrates of allantoic fluid from eggs that have been infected with the Legionnaires disease bacterium possess heat-stable hemolytic activity. The clearing of the medium, more circumscribed cloudy precipitate, and iridescent sheen surrounding growth of the Legionnaires disease bacterium on F-G agar with egg yolk suggest that the organism may elaborate one or more enzymes that digest components of the yolk. These characteristics are relevant to the laboratory identification of this unusual organism.

Cultures of various species of *Clostridium* grown on McClung-Toabe or other egg yolk medium are characterized by clearing of the medium, formation of an opaque precipitate, development of a pearly iridescence, or a combination of these reactions, which are attributed to clostridial protease (18), lecithinase, and lipase (14), respectively. Gelatinase activity manifested by the Legionnaires disease bacterium (17) suggests that the organism may have the capacity for proteolysis. Whether the Legionnaires disease bacterium produces enzymes that attack triglyceride or phospholipid substrates is not yet established.

The hemolytic activity of the Legionnaires disease bacterium is compatible with the elaboration of a membrane-active exotoxin (3, 7). The best-described heat-stable membrane-active bacterial toxin is a lecithinase, the phospholipase C of *C. perfringens* (1, 10). It remains to be seen

FIG. 3. Radial hemolysis around 9.5-mm wells in F-G agar with 5% sterile defibrinated guinea pig blood by sterile filtrates of allantoic fluid from embryonated hen's eggs infected with Legionnaires disease bacterium strain Philadelphia 2. (A) Uninfected control; (B) fluid AF-8; (C) fluid AF-54; (D-H) fluid AF-60 undiluted and at serial twofold dilutions in allantoic fluid filtrate from uninfected control.

TABLE 1. Hemolytic activity of sterile filtrates of allantoic fluid from embryonated eggs after intravitelline inoculation of *Legionnaires disease bacterium*

Allantoic fluid	Diam of zone of hemolysis ^a		
	Untreated filtrate	Filtrate heated 60°C, 30 min	Filtrate heated 100°C, 15 min ^b
C-15 (uninfected egg)	Negative ^c	Negative	Negative
A-74 (infected egg)	18.0	19.0	13.0
A-75 (infected egg)	11.5	13.0	Trace positive ^d

^a Diameter in millimeters after incubation at room temperature for 1 week.

^b Evaporated samples reconstituted with sterile phosphate-buffered saline to 1.3 times initial volume before inoculation into wells.

^c No hemolysis visible at periphery of 9.5-mm wells.

^d Slight hemolysis at periphery of 9.5-mm wells.

whether the hemolytic and yolk-digesting activities of the *Legionnaires disease bacterium* are clinically relevant and whether they bear on the necrosis of inflammatory cells, disruption of alveolar epithelium, and hyaline membrane formation that characterize the histopathology of this disease (4, 19).

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