Killing of *Legionella pneumophila* by Human Serum and Iron-binding Agents

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**Abstract.** The inhibitory effect of serum on the growth and survival of *Legionella pneumophila* Bloomington 2 was investigated. When incubated in the presence of 20%-50% normal human serum for 10 h, viability was decreased by >99%. Heat-inactivated or <40% normal serum supplemented with 50 µM iron was not inhibitory. The addition of guinea pig complement to heat-inactivated serum resulted in killing of approximately 98% of the cells. Growth in buffered yeast extract broth was inhibited by the addition of ferric iron-binding compounds. Minimum bactericidal concentrations at 37°C were 10 µM apotransferrin, 35 µM 1,10-phenanthroline, and 50 µM deferoxamine. Addition of iron chelators to normal serum did not accelerate killing. Egg yolk-passaged "virulent" strains and agar-grown "avirulent" strains exhibited similar serum sensitivity. Results of this study indicate that complement and serum transferrin are antagonistic to the growth of *Legionella* in serum.

Bactericidal and bacteriolytic properties of serum on Gram-negative bacteria have been studied often during the past three decades [13, 21]. Although no single protocol has been established as the standard assay for serum activity, several factors have been identified that can modulate the bactericidal effect [21]. Included among these are antibody, complement, lysozyme [13], beta-lysin [7], and peptide PC-II [4]. Some of the bacteriostatic and bactericidal activity of serum has been attributed to the sequestering of iron by transferrin [2]; the effect of this protein can be abolished by increasing its level of iron saturation [3, 9].

With many kinds of Gram-negative bacteria, activation of the complement, apparently, is the primary and most rapid means of serum-mediated killing. Although serum is required for opsonization and phagocytosis of *Legionella* [20, 22, 23], it is not clear whether complement can reduce viability of cells of this genus in serum. A decrease was observed by one group [1], but not by two others [12; Caparon et al., Abstracts Ann. Mtg. ASM, 1984]. A fourth laboratory reported that cells of some but not all strains of *Legionella* are killed by serum [17]. In the present work, we have attempted to determine whether iron-binding agents might modulate the bactericidal action of serum complement with *Legionella*.

**Materials and Methods**

**Bacterial strains.** An avirulent strain of *Legionella pneumophila* Bloomington 2 (ATCC 33155) was maintained and subcultured >10 times on buffered charcoal yeast extract agar [8]. For the actual experiments, early stationary-phase cells were obtained from buffered yeast extract broth (BYEB) cultures shaken (200 rpm) at 37°C for 12 h. Virulent Bloomington 2 strains were obtained (courtesy of the Centers for Disease Control, Atlanta, Georgia) and maintained in embryonated hens' eggs [12], harvested, and tested on buffered charcoal yeast extract agar for viability and on trypticase soy agar for possible contaminants. *Legionella* is unable to grow on trypticase soy agar. Virulent cultures were then stored at −70°C. Immediately before use, cells were thawed at room temperature and partially purified by centrifugation [12].

**Media and buffers.** Buffered charcoal yeast extract agar and BYEB were formulated without supplemental iron, pyruvate, or α-ketoglutarate. MOPS buffer (1%) and 0.1 mM KHCO₃ were included in all media. Gelatin-veronal-buffered saline and phosphate-buffered saline supplemented with 0.15 mM CaCl₂ and 1.0 mM MgCl₂ (GVB ++, PSB ++) were prepared as described previously [11].

**Serum.** Venous blood was obtained from 18 healthy adults, aged 21–30. Volunteers had no history of Legionnaires' disease, pneumonias, or other chronic or debilitating diseases. Background antibody titers against *L. pneumophila* serogroups 1–6 were <1:64. Blood was drawn and allowed to clot. Serum was pooled and filtered through a 0.45-µm pressure filter (Millipore Corp., Bedford, Massachusetts) and stored at −20°C until used. Complement was inactivated by heating the serum at 56°C for 30 min immediately before use.
Iron-binding agents and complement. Apotransferrin (Sigma Chemical Corp., St. Louis, Missouri), 1,10-ortho-phenanthroline (Aldrich Chemical Co., Milwaukee, Wisconsin), and deferoxamine methane sulfonate (Desferal, Ciba Pharmaceutical Co., Summit, New Jersey) were obtained >99% iron-free. Stock solutions were prepared in deionized distilled water and filter sterilized. Guinea pig complement (Baltimore Research Laboratories, Baltimore, Maryland) was reconstituted 1:1 with heat-inactivated human serum.

Iron chelation assay. The original 12-h cell cultures were diluted in BYEB to a viable count of $5 \times 10^5$ colony-forming units/ml. The iron-binding agents were added to the test system 1 h before addition of the inoculum to obtain final volumes of 5 ml. Experiments, performed in triplicate, proceeded for up to 24 h at 37°C on a gyratory shaker (200 rpm). The pH value of the cultures remained between 7.0 and 7.5. Aliquots of 0.1 ml were removed and plated on buffered charcoal yeast extract agar. After purification of egg yolk-passaged *L. pneumophila* by differential centrifugation, cells were diluted in GVB++ to $5 \times 10^6$ colony forming units/ml. Subsequent procedures for the serum assay were as described above.

Serum bactericidal activity. Iron-binding agents and complement were mixed with serum and allowed to preincubate for 1 h prior to addition of the bacterial inoculum. The 12-h broth cultures were diluted in GVB++ and PBS++ or Hanks balanced salt solution to an absorbance value of 0.1 at 660 nm. The suspensions were diluted further in the various buffers to colony forming units/ml of $5 \times 10^5$, $5 \times 10^4$, $5 \times 10^3$, and $5 \times 10^2$. The dilutions, in 2.5-ml volumes, were incubated with 0%, 10%, 20%, 30%, 40%, or 50% normal serum or serum treated with heat, ferric sulfate, or iron chelators. BYEB was added to make a final volume of 5 ml. Components of the samples were adjusted so as to permit each test to contain identical quantities of BYEB compounds regardless of serum concentrations or treatment. The experiments, performed in triplicate, proceeded for up to 24 h at 37°C on a gyratory shaker (200 rpm). The pH remained between 7.0 and 7.5. Aliquots of 0.1 ml were removed and plated on buffered charcoal yeast extract agar. After purification of egg yolk-passaged *L. pneumophila* by differential centrifugation, cells were diluted in GVB++ to $5 \times 10^6$ colony forming units/ml. Subsequent procedures for the serum assay were as described above.

Measurements of iron and total iron-binding capacity. Iron concentrations in chemicals and media, as well as total serum iron and total iron-binding capacity were determined in an ACA-3 atomic absorption spectrophotometer (DuPont Corp., Wilmington, Delaware). The total iron-binding capacity of the pooled human serum was 65.8 μM; the serum iron was 17.3 μM. Nearly 100% of iron-binding capacity in serum is provided by transferrin [3]. Because each molecule of transferrin binds two atoms of iron, the pooled serum was calculated to contain 32.9 μM transferrin, of which 26.3% was iron saturated. The iron content of BYEB was 18–24 μM.

Results

A series of iron-binding compounds were examined for their bactericidal effects on *Legionella pneumophila*. Of the three iron-binding groups tested (i.e., proteinaceous, synthetic, and siderophoric), apotransferrin, 1,10-phenanthroline, and deferoxamine, respectively, were the most bactericidal. The minimum bactericidal concentrations of the three compounds were 10 μM, 35 μM, and 50 μM in BYEB. Viability decreased >90% within 19 h after addition of either 1,10-phenanthroline or deferoxamine, and within 13 h after addition of apotransferrin (Fig. 1). Preincubation of the chelators with either ferric pyrophosphate or ferric sulfate prior to the addition of the bacteria completely suppressed the activity of the iron-binding compounds. The quantities of iron needed were: for apotransferrin, 10 μM; for 1,10-phenanthroline, 40 μM; and for deferoxamine, 50 μM.

The viability of *L. pneumophila* decreased rapidly when incubated in the presence of 20%–50% normal human serum at 37°C. The decrease was >90% within 3 h and >99% within 10 h (Fig. 2A). The loss in viability occurred at a faster rate than that caused by the iron-binding agents. Addition of bactericidal quantities of the chelators caused no change in rate or total amount of killing (data not shown). In samples containing PBS++, killing was substantially decreased (Fig. 2B). Results in sam-