Difference in Virulence of Environmental Isolates of Legionella pneumophila

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Endemic nosocomial Legionnaires disease has occurred at our medical center for several years. Two subtypes of Legionella pneumophila serogroup 1 (UH-1 and RH-1) have been isolated in approximately equal numbers from potable water. The widespread isolation of these nonpathogenic isolates permits a comparison between virulence factors of the two subtypes. L. pneumophila serogroup 1 strains were selected for study based on differences in disease incidence caused by these subtypes. Organisms were identified by colony morphology, monoclonal antibody agglutination (16), and plasmid content (13). Isolated colonies of each subtype were streaked on BCYE agar, harvested at 72 h, and frozen at -70°C in 50% glycerol-50% Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.) for future use.

For each series of animal inoculations, a fresh suspension of organisms was thawed and grown on BCYE agar for 72 h. The organisms were suspended in sterile water, and the optical density was adjusted to 45% A530, which corresponded to approximately 1.2 x 10^6 CFU/ml. Serial dilutions were made in sterile water to obtain the desired concentrations for inoculations, which ranged from 1.2 x 10^4 to 1.2 x 10^6 CFU/ml. Samples of inoculum suspension were plated on BCYE agar in triplicate to verify the accuracy of the estimated inoculum concentration. Direct bacterial counts of the inoculum were performed by the method of Shepard and McRae (18), except that slides with circular wells 6 mm in diameter were used (Cell Line Associates, New Field, N.J.) and that the organisms were heat fixed only and stained by the Gimenez method (10).

Killed L. pneumophila cells used for control inoculations were prepared in two ways. Suspensions of UH-1 or RH-1 (1.2 x 10^6 CFU/ml) were killed by heating them to 121°C for 20 min. Similar suspensions were killed by exposure to 253-nm UV light at a distance of 8 cm (equaling approximately 1.6 mW/cm^2) from the source (General Electric bulb G 30T8) for 30 min. Samples of each suspension of killed L. pneumophila were cultured on BCYE agar to document sterilization.

Infection. Male Hartley strain guinea pigs (Lab Supply, Indianapolis, Ind.) weighing 250 to 300 g were housed three to a cage and given standard commercial feed and autoclaved tap water. The cages were placed in a biological isolation rack which exhausted effluent air through a filter (pore size, 0.3 μm; Lab Products Co., Rochelle Park, N.J.). Animals received single 1-ml intraperitoneal doses of either UH-1 or RH-1, ranging from 1.2 x 10^5 to 1.2 x 10^6 CFU.

Control animals received single 1-ml intraperitoneal injections of either heat-killed or UV-killed suspensions of approximately 1.2 x 10^6 CFU of UH-1 or RH-1. Other controls received single intraperitoneal injections of 1 ml of sterile water. Weights and temperatures were recorded daily for 1 week and then twice a week for the next 2 weeks. Hypothermic, unresponsive animals were anesthetized with sodium pentobarbital (The Butler Co., Columbus, Ohio) and then exsanguinated by cardiac puncture.

Histopathology and cultures. All dead animals were autopsied. Lung and spleen tissue sections from some animals were fixed in 10% neutral Formalin and processed for
TABLE 1. Dose-response data for subtypes UH-1 and RH-1

<table>
<thead>
<tr>
<th>L. pneumophila subtype</th>
<th>CFU/inoculum (mean ± SD)*</th>
<th>Mortality rate (no. dead/no. tested (%))</th>
<th>Days to death (mean ± SD)**</th>
</tr>
</thead>
<tbody>
<tr>
<td>RH-1</td>
<td>(2.2 ± 0.8) x 10^6</td>
<td>8/9 (88)</td>
<td>2.0 ± 1.1</td>
</tr>
<tr>
<td>RH-1</td>
<td>(1.9 ± 0.6) x 10^6</td>
<td>15/17 (88)</td>
<td>3.5 ± 0.5</td>
</tr>
<tr>
<td>RH-1</td>
<td>(2.0 ± 0.8) x 10^6</td>
<td>0/11 (0)</td>
<td>5.3 ± 1.5</td>
</tr>
</tbody>
</table>

* Number of replicate determinations (n) for CFUs is equal to the number of animals at each dose level. If less than six animals, n = 6. In determining mean days to death, n = the number of animals which died at each respective dose. NA, Not applicable.

** Heat killed; CFU determination was made before heating.

Uninfected Sterile H_2O 0/12 (0)  NA

of times until death was performed by the regression method of Cox (4).

Four animals were excluded from analysis because of sacrifice for rectal prolapse; three from the RH-1 group and one from the UH-1 group. If these animals are included in the analysis of data, the results are not significantly altered.

RESULTS

Mortality and survival. Mortality rates are shown in Table 1. The average mean infecting dose of L. pneumophila by CFU count was 98% of the average mean direct count for UH-1 and 94% of that for RH-1. Because of the good correlation between the CFU and the direct counts, only the CFU counts are shown in the table. All cultures of heat-killed and UV-killed L. pneumophila were sterile. The LD_{50} of UH-1 was 7.4 x 10^6 CFU (95% confidence limits, 3.6 x 10^6 to 15.1 x 10^6 CFU) and was significantly lower than the RH-1 LD_{50} of 9.1 x 10^6 CFU (95% confidence limits, 5.6 x 10^6 to 15.1 x 10^6 CFU), as determined by the method of Spearman and Karber (7) (P = 0.0001, Z test of the difference between two population parameters).

The mean time until death increased as the inoculum of either subtype decreased (Table 1). Animals given the highest dose (approximately 10^8 CFU) of either subtype died quickly, whereas guinea pigs given RH-1 at doses from 10^5 to 10^7 CFU tended to live longer than those given an equivalent dose of UH-1. Life table analysis of times to death in individual animals, as determined by the regression method of Cox (4), demonstrated that at doses of 10^6 and 10^7 CFU the time until death in animals given RH-1 was significantly longer than in animals given an equivalent dose of UH-1 (P = 0.0008).

Illness. Animals given either UH-1 or RH-1 became febrile between 1 and 5 days after injection. Animals that died became hypothermic as a preterminal event. Controls given killed organisms developed fever for 1 to 2 days after injection and then became afibrile. Uninfected controls remained afibrile throughout the period of observation.

Guinea pigs infected with either UH-1 or RH-1 experienced a mean maximum weight loss of 15% by 4 to 5 days after infection. Guinea pigs that died lost weight from the time of infection until death, whereas survivors began to gain weight at a rate equal to that of uninfected animals by 5 days after injection. Controls given killed organisms had a small (5%) initial weight loss 1 to 2 days after injection and then gained weight at a rate equal to that of uninfected controls. Uninfected controls gained 2% of their initial body weight daily. There were no subtype-associated differences in either fever pattern or weight loss.

Histology and cultures. The lungs from infected animals showed variable degrees of involvement, ranging from focal interstitial inflammation to extensive alveolar consolidation with edema, proteinaceous debris, and inflammatory cells filling the alveoli. Histology of the spleens ranged from apparently normal to scattered areas of necrosis with inflammatory infiltrate. These findings did not vary between animals given UH-1 and animals given RH-1. L. pneumophila was seen by direct fluorescent antibody stain in 100% of the lung and spleen tissue samples examined in both UH-1 and RH-1 groups.

In autopsied animals infected with UH-1, L. pneumophila was cultured from 33 of 37 (89%) peritoneal exudates, 28 of 37 (75%) spleen tissue samples, and 31 of 37 (84%) lung tissue samples. In autopsied animals infected with RH-1, L. pneumophila was cultured from 19 of 22 (86%) peritoneal exudates, 20 of 22 (91%) spleen tissue samples, and 21 of 22...
(95%) lung tissue samples. There were no dose-related or subtype-related differences in the number of organisms seen by direct fluorescent antibody stain or recovered in culture.

Serologies. The overall seroconversion rate for the survivors from the UH-1 group was 31 of 35 (89%), and for those from the RH-1 group it was 35 of 39 (90%). Seroconversion data are not shown for individual dose levels. The 50% infective dose of UH-1 was 5.8 x 10^3 CFU (95% confidence interval, 0.8 x 10^3 to 3.8 x 10^3 CFU), and the 50% infective dose of RH-1 was 1.4 x 10^4 CFU (95% confidence interval, 0.5 x 10^4 to 4.1 x 10^4 CFU). These results are different, but the difference is not statistically significant (P = 0.21, Z test of the difference between two population parameters).

Seroconversion occurred in all three controls injected with heat-killed UH-1 and in two of three controls injected with UV-killed UH-1. The three controls given heat-killed RH-1 showed seroconversion, as did the three controls given UV-killed RH-1. None of the 12 guinea pigs given sterile water showed seroconversion.

Because the mortality curves were so steep for the two subtypes, a second series of inoculations was carried out with intermediate doses between 10^6 and 10^8 CFU (Table 2). The mortality rates from these injections confirm both the accuracy of our original mortality curves (Fig. 1) and the LD_{50} for each subtype calculated from our original data.

**DISCUSSION**

Winn and Chandler (20) recently reviewed the role of virulence factors in *Legionella* infections, including variations in the environment, host susceptibility, cellular and molecular mechanisms of damage, laboratory techniques, and alterations of intrinsic virulence between strains. Several investigators have been able to show alterations in the virulence of *L. pneumophila* after passage on artificial media or through living hosts. Our study is the first to examine and demonstrate differences in virulence between two unaltered environmental isolates of the same serogroup. McDade and Shepard (14) compared the virulence of *L. pneumophila* cultured in guinea pigs, in chicken eggs, and on modified Mueller-Hinton medium. They observed a 10^4-fold increase in the number of *L. pneumophila* (Philadelphia 2) required to produce an LD_{50} in guinea pigs after passage of the strain on modified Mueller-Hinton agar at weekly intervals for 6 months compared with the dose required for an LD_{50} with cells obtained from *L. pneumophila*-infected guinea pig spleens. They also observed a >10^3-fold increase over the dose required for an LD_{50} with cells obtained from *L. pneumophila*-infected chicken egg yolk sacs. Wong et al. (21) demonstrated a decrease in the LD_{50} of *L. pneumophila* (Knoxville 1) for guinea pigs from >10^10 CFU (when passed a multiple but unspecified number of times on modified Mueller-Hinton agar) to approximately 10^5 CFU after as few as three passages in cultured human embryonic lung fibroblasts. Ormsbee et al. (15) independently confirmed these findings, noting a 10^4-fold increase in the LD_{50} of *L. pneumophila* for guinea pigs after 12 passages on modified Mueller-Hinton agar and a 10^3-fold return towards the original LD_{50} after three further passages in chicken eggs. Huebner et al. (11) compared the virulence of the Philadelphia and Pontiac isolates of *L. pneumophila* in guinea pigs and found no difference between the two, although the passage history of the strains used was not given. Fitzgeorge et al. (8) showed that two environmental isolates of *L. pneumophila* (from serogroups 1 and 3) were of similar virulence after three passages on BCYE agar and that both strains were significantly more virulent than a serogroup 1 strain which had been passed multiple times on unspecified artificial media. None of the studies described above have demonstrated differences in the virulence of environmental isolates unaltered by multiple passages either on artificial media or through living hosts.

Brown et al. (2) isolated two strains of *L. pneumophila* from a hospital environment. Their epidemiologic data led them to suggest that the plasmid-bearing strains could be less virulent than plasmidless isolates; however, no in vivo virulence data were presented. We repeatedly isolated two strains (UH-1 and RH-1) from our hospital environment. The plasmidless isolate (UH-1) was isolated from most (24 of 25) cases of nosocomial Legionnaires disease in our medical center (17). The present study shows that when guinea pigs are given equivalent doses of UH-1 and RH-1, UH-1 has a lower 50% infective dose and LD_{50} and causes death more quickly than RH-1. We conclude that unaltered environmental UH-1 is more virulent than RH-1 in the animal model used.

**TABLE 2. Intermediate dose-response data for subtypes UH-1 and RH-1**

<table>
<thead>
<tr>
<th><em>L. pneumophila</em> subtype</th>
<th>CFU/inoculum (mean ± SD)</th>
<th>Mortality rate (no. dead/no. tested) (%)</th>
<th>Days to death (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>UH-1</td>
<td>(6.0 ± 0.8) x 10^7</td>
<td>5/5 (100)</td>
<td>3.2 ± 0.4</td>
</tr>
<tr>
<td>RH-1</td>
<td>(4.4 ± 1.2) x 10^7</td>
<td>0/6 (0)</td>
<td>NA</td>
</tr>
<tr>
<td>UH-1</td>
<td>(3.0 ± 0.4) x 10^7</td>
<td>4/6 (67)</td>
<td>4.5 ± 1.7</td>
</tr>
<tr>
<td>RH-1</td>
<td>(2.2 ± 0.6) x 10^7</td>
<td>0/6 (0)</td>
<td>NA</td>
</tr>
<tr>
<td>UH-1</td>
<td>(6.0 ± 0.8) x 10^6</td>
<td>2/6 (33)</td>
<td>4.5 ± 0.7</td>
</tr>
<tr>
<td>RH-1</td>
<td>(4.4 ± 1.2) x 10^6</td>
<td>0/6 (0)</td>
<td>NA</td>
</tr>
<tr>
<td>UH-1</td>
<td>(3.0 ± 0.4) x 10^6</td>
<td>0/6 (0)</td>
<td>NA</td>
</tr>
<tr>
<td>RH-1</td>
<td>(2.2 ± 0.6) x 10^6</td>
<td>0/6 (0)</td>
<td>NA</td>
</tr>
</tbody>
</table>

* For CFU, n = 6; for mean days to death, n = the number of animals dying at each respective dose. NA, Not applicable.

**FIG. 1. Mortality curves for UH-1 and RH-1.** Data points are combined from Tables 1 and 2. UH-1 had a lower LD_{50} than RH-1.
This difference in virulence may help to explain the prevalence of clinical isolation of UH-1 in our hospital and the prevalence of another plasmidless clinical isolate of *Legionella pneumophila* in another institution (2), even though other strains were also present in the potable water. Furthermore, it may help to explain why the nearly ubiquitous environmental existence of *L. pneumophila* does not correlate with the more limited occurrence of clinical Legionnaires disease.

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LITERATURE CITED