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The mouse has been used almost exclusively as the experimental animal for host resistance studies in the United States. Host resistance models in mice have been validated and these types of studies are an integral part of the National Toxicology Program's (NTP) immunotoxicology testing program. Given that the Fischer 344 rat is the animal of choice for the NTP's toxicology studies, it was desirable to develop host resistance assays in this rat strain, eliminating the need to extrapolate doses between mouse and rat models. These studies were aimed at the development and the validation of a host resistance model to Listeria monocytogenes*, a gram-positive, facultative intracellular bacterium, in the Fischer 344 rat. The results demonstrated that* L. monocytogenes *infection in the Fischer 344 rat produces a very similar disease with respect to onset and duration as in the mouse. Animals inoculated with up to* 3×10^5 *viable colony forming units (CFU) of* L. monocytogenes *were capable of resisting overt disease when they had an intact immune system. Inoculation of animals with* 5×10^5 *cfu resulted in 50% mortality (5/10). However, pretreatment with cyclophosphamide (an immunosuppressive agent) followed by administration of* 5×10^5 L. monocytogenes *resulted in 100% mortality. Conversely, pretreatment with* Corynebacterium parvum *(an immune-enhancing agent) followed by administration of* 5×10^5 L. monocytogenes *resulted in 100% survival of animals. Overt disease without recovery was manifested in vehicle-treated animals inoculated with* 8×10^5 *and* 1×10^6 *microorganism, while earlier deaths and 100% survival resulted after pretreatment with cyclophosphamide or* C. parvum*, respectively. These data support the use of the inbred Fischer 344 rat for host resistance assays.*

One goal of immunotoxicological studies is to predict the risk associated with human exposure to xenobiotics. Although data on alterations of single immune parameters are helpful for anticipating human health

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effects, the immune system is dynamic, and there is a great deal of overlap in function allowing for compensation of injury to individual components. Host resistance models are one of the approaches used in immunotoxicology to assess the overall integrity of the immune system. These models evaluate the host's ability, following exposure to an agent of interest, to destroy or to inhibit the growth of microorganisms or tumors. Therefore, host resistance studies play an important role in understanding the clinical relevance of immune injury detected by in vitro assays. In the mouse, various host resistance models are currently used to detect injury to particular components of the immune system. For example, in the *Streptococcus pneumoniae* model, the major protection against the bacteria has been shown to involve humoral immunity and polymorphonuclear leukocytes [1]. In addition, natural killer (NK) cells and cytotoxic T (Tc) cells are predominantly responsible for protection in the B16F10 tumor model [2]. These studies deal with the development and validation of a host resistance model to *Listeria monocytogenes* in the Fischer 344 female rat.

L. monocytogenes is a gram-positive, facultative intracellular bacterium. Human disease associated with *L. monocytogenes* most often occurs in immune-compromised humans, including pregnant women and newborns. The foods most often associated with listeriosis outbreaks include milk and milk products like sour milk and cheeses [3]. The predominant sites of *L. monocytogenes* growth are in hepatocytes, Kupffer cells, and splenic macrophages, and it is believed the bacteria infect other cells via intercellular plasmic bridges [4].

The extensive study and characterization of *L. monocytogenes* infection and resistance has brought it center stage for use in immunotoxicological studies. The initial response (first 24 h) to *L. monocytogenes* infection resides in the action of neutrophils to prevent uncontrolled bacterial growth [4, 5]. The exact actions of neutrophils in the acute response have not been fully elucidated but several hypotheses exist. The simplest theory is that neutrophils have listericidal actions, either intrinsically or via the release of oxidative agents. Another explanation renders neutrophils lysing infected cells, enabling macrophages and natural killer cells to destroy the exposed organism. Finally, neutrophils produce substances that are vasoconstrictive, which could limit the spread of the bacteria, thereby increasing the effective log kill of activated macrophages and NK cells [6]. Although the precise role of neutrophils has yet to be determined, they are present in large quantities immediately following *L. monocytogenes* infection. In addition to neutrophils, macrophages and NK cells generate a rapid, nonspecific response to infection.

Host resistance to *L. monocytogenes* is also mediated via a T-cell–dependent pathway. Once a macrophage has phagocytized the organism,

it releases cytokines (predominantly IL-1, TNF-alpha, and IL-12), which activate other immune cells (e.g., NK cells) to release IFN-gamma, activating other macrophages to kill bacteria they contact. Stimulated T cells can also activate macrophages to destroy the bacteria through phagocytosis and intracellular killing. Once activation of macrophages has occurred, *L. monocytogenes* clearance is greatly enhanced. There is no evidence of humoral immunity having a role in host defense against primary *L. monocytogenes* infection.

Several host resistance models in rodents have been developed, and these types of studies are an integral part of both the National Toxicology Program (NTP) [7] and the Netherlands' National Institute of Public Health (NIPH) toxicology testing program [8]. Host resistance assays conducted by the NTP almost exclusively utilize the B6C3F1 (C57B1/6 × C3HHeN) hybrid mouse, while the NIPH's host resistance assays employ outbred Wistar rats. Given that the Fischer 344 rat is the animal of choice for the NTP's toxicology studies, it was desirable to establish a host resistance model in this strain. This would eliminate the need to extrapolate doses between mouse and rat models. Furthermore, the Fischer 344 rat is an inbred animal and as transplanted tumor host resistance models are developed in the rat, an inbred strain of experimental animal will be required.

MATERIALS AND METHODS

Bacterial Culture

L. monocytogenes strain 19303 (originally obtained from Herbert Weishimer of the Department of Microbiology and Immunology, Virginia Commonwealth University) was used in both the range-finding and the validation studies. This bacterial strain was also used in the tier II immunotoxicology validation studies supported and reported by the National Toxicology Program [7]. The stock culture was verified by the Department of Clinical Microbiology at Virginia Commonwealth University prior to the start of the study. Stock cultures were maintained at −80°C, and the viability of the bacterial stock was verified by performing colony counts on trypticase soy agar plates prior to and following inoculation of test animals.

Animals

Pathogen (coronavirus, mycoplasma, and Sendai virus)-free female Fischer 344 rats (6–8 weeks old) were purchased from Charles River Laboratories (Wilmington, MA). Animals were maintained under conditions specified by NIH guidelines. Upon arrival, the rats were

TABLE 1. Experimental Groups for the Range-Finding Study

Group	Inoculum
1	Vehicle (0.9% NaCl)
2	3×10^3 *L. monocytogenes*
3	1×10^4 *L. monocytogenes*
4	3×10^4 *L. monocytogenes*
5	1×10^5 *L. monocytogenes*
6	3×10^5 *L. monocytogenes*
7	1×10^6 *L. monocytogenes*

quarantined for 7 days prior to being put on study. Animals were randomized and housed individually in wire hanging stainless steel cages with an automatic watering system until they were infected with *L. monocytogenes*. Once inoculated, the rats were housed 4 animals per cage in shoebox cages on sawdust bedding and maintained on Agway Rat and Mouse Ration (NIH 07) and tap water *ad libitum*. All animals were housed in animal facilities at Virginia Commonwealth University where the temperature was maintained between 18 and 26°C, the relative humidity between 40 and 70%, and the light/dark cycle was on 12-h intervals.

Following randomization in the development study (referred to as the range-finding study from here on), the animals were assigned to treatment groups (8 animals/group) as shown in Table 1. For the validation study, animals were randomized and assigned to treatment groups (10 animals/group) as shown in Table 2. The concentrations of *L. monocytogenes* (CFU/animal) used in the validation study were selected

TABLE 2. Experimental Groups for the Validation Study

Group	Inoculum	Estimated % mortality[a]	Treatment
1	Vehicle (0.9% NaCl)	0	0.9% NaCl
2	1×10^5 CFU *Listeria*	10	0.9% NaCl
3	5×10^5 CFU *Listeria*	25	0.9% NaCl
4	8×10^5 CFU *Listeria*	75	0.9% NaCl
5	2×10^6 CFU *Listeria*	90	0.9% NaCl
6	0.9% NaCl	0	CPS
7	1×10^5 CFU *Listeria*	10	CPS
8	5×10^5 CFU *Listeria*	25	CPS
9	8×10^5 CFU *Listeria*	75	CPS
10	2×10^6 CFU *Listeria*	90	CPS
11	Vehicle (0.9% NaCl)	0	*C. parvum*
12	1×10^5 CFU *Listeria*	10	*C. parvum*
13	5×10^5 CFU *Listeria*	25	*C. parvum*
14	8×10^5 CFU *Listeria*	75	*C. parvum*
15	2×10^6 CFU *Listeria*	90	*C. parvum*

[a]Estimations were based on the results of the range-finding study.

TABLE 3. Relationship Between Calculated Inocula Concentration Predosing and Bacterial Count from Inocula Postdosing for the Range-Finding and Validation Studies

Range-finding study		Validation study	
Original inocula[a] (CFU/mL)	Postinocula (CFU/mL)	Original inocula[b] (CFU/mL)	Postinocula (CFU/mL)
1.0×10^4	1.05×10^4	1.0×10^5	8.10×10^4
1.0×10^5	7.65×10^5	8.0×10^5	8.17×10^5
1.0×10^6	1.08×10^6	2.0×10^6	1.90×10^6

Note. Original inocula represent a calculated number based on serial dilution from a stock of 2×10^9 CFU/mL[a] and 1.8×10^9 CFU/mL[b].

based on results found in the range-finding study and targeted at concentrations to produce a low (10–25%) and a high (75–90%) mortality rate.

Experimental Design

Inocula Preparation and Viability Verification

On the day prior to inoculation, the stock culture was enumerated. The stock solution was determined to be 2.0×10^9 CFU/mL at the time of inocula preparation for the range-finding study and 1.8×10^9 at the time of inocula preparation for the validation study. On the day of inoculation for each study, a vial of the same stock of *L. monocytogenes* was thawed. Based on the colony count of the frozen stock on the previous day, the inocula were prepared by making necessary dilutions with 0.9% NaCl in sterile injection vials in sufficient quantities to inoculate each rat with 1 mL of the inoculum. Dilutions were kept on ice. Inocula control animals received a volume of 0.9% NaCl equal to the volume (1 mL) of the inocula and were dosed at the same time as the treatment groups.

The inocula were counted after the animal injections were completed to determine whether a loss of viable bacteria occurred in the interim between removing the bacteria from the freezer and the inoculation of the animals. Table 3 shows the viability of the inocula from the range-finding and validation studies.

Test Articles

Cyclophosphamide (CPS, Sigma Chemical, St. Louis, MO), a known immunosuppressive agent, and *Corynebacterium parvum* (*C. parvum*, Ribi Immunochemistries, Hamilton, MT), a known immunoenhancing agent, were the two test articles used for the validation of this model. CPS was freshly prepared in 0.9% NaCl at a concentration of 20 mg/mL and animals received a single intravenous injection of 100 mg/kg 24 h before inoculation with *L. monocytogenes*. *C. parvum* was prepared in

0.9% NaCl at a concentration of 5 mg/mL and animals received a single intravenous injection of 25 mg/kg 7 days prior to inoculation with *L. monocytogenes*. Vehicle control animals received 0.05 mL 0.9% NaCl/10 g body wt intravenously 24 h before inoculation with *L. monocytogenes*.

Treatment and Inoculation of Animals

To facilitate intravenous injections, animals were vasodilated by placing them in shoebox cages without bedding on top of heating pads. Animals were closely monitored to ensure they did not overheat. After a brief heating period, the test articles, vehicle, or inocula were administered intravenously to the designated animal via one of the two lateral tail veins. Once injected, the animals were returned to their cages for the duration of the study.

Safety

All bacterial dilutions were performed in a vertical laminar flow containment hood, class B. Personnel used appropriate precautions, including wearing lab coats, gloves, masks, and safety glasses. For inoculations, air bubbles were purged from syringes into stoppered vials or alcohol-soaked gauze to prevent bacterial aerosols. All contaminated materials, including animal waste, were autoclaved prior to disposal. Dead animals were removed from cages and stored in a freezer until removed for incineration. All animal cages were sanitized by autoclaving.

Experimental Analyses

Observations

In the range-finding study, animals were observed for moribundity or mortality at the time of inoculation and twice daily thereafter for 14 days. For the validation study, moribundity and mortality checks were conducted twice daily at the beginning of the study. These checks were reduced to once a day when animals began to recover and no animals became moribund or died over a 2-day period. The number of animals alive, the number of animals dead, and the cumulative number of animals dead were recorded at each check period. Animals that were moribund were euthanized and scored as having died at the subsequent observation point.

Body Weight Analyses

In the range-finding study, animals were weighed on the day prior to inoculation and weekly until the end of the study. In the validation

study, animals were weighed beginning the week before *L. monocytogenes* inoculation (the day prior to treatment with *C. parvum*) and then weekly until the end of the study. Data were analyzed as body weight and body weight change over the course of the experimental period.

Statistical Analyses

For body weight analysis, Bartlett's test was used to evaluate the homogeneity of the data [9]. For homogeneous data, Dunnett's test was used to compare the test article treatment groups with the vehicle groups [10]. For nonhomogeneous data, the Wilcoxon rank sum test was used for statistical comparison [11]. Statistical significance was reported as $^{*}p < 0.05$ and $^{**}p < 0.01$ unless otherwise indicated. Survival time data were analyzed by the Fit Model [12] using JMP statistics from SAS Institute, Inc.

RESULTS

Observations

At the onset of the range-finding study, all animals appeared clinically normal, and the animals inoculated with 0.9% NaCl remained normal throughout the experimental period. Animal inoculated with 1×10^6 CFU of *L. monocytogenes* became lethargic and showed signs of dehydration beginning the morning of day 2 following bacterial inoculation, with the first death in that dose group occurring the same afternoon.

At the onset of the validation study, all animals appeared clinically normal. Animals inoculated with 0.9% NaCl remained clinically normal throughout the experimental period. On day −2, animals in groups 13, 14, and 15 (Table 2) all appeared slightly unthrifty and had dark yellow urine stains on their perineal areas. The automatic watering system lines feeding these animals' cages was found to be clogged, resulting in water deprivation and the production of very concentrated urine. The animals began drinking as soon as the lines were open and by the following day, their coats were returning to normal appearance.

Body Weights and Survival Data

Animal weights for the range-finding study are shown in Figure 1. Rats inoculated with 0.9% NaCl showed a mean increase in body weight of 6.98 g over the experimental period. Rats inoculated with 3×10^3, 1×10^4, and 3×10^4 CFU/animal all gained weight similar to the 0.9% NaCl control group such that their mean body weights were not significantly different from those of the control group during the entire experimental

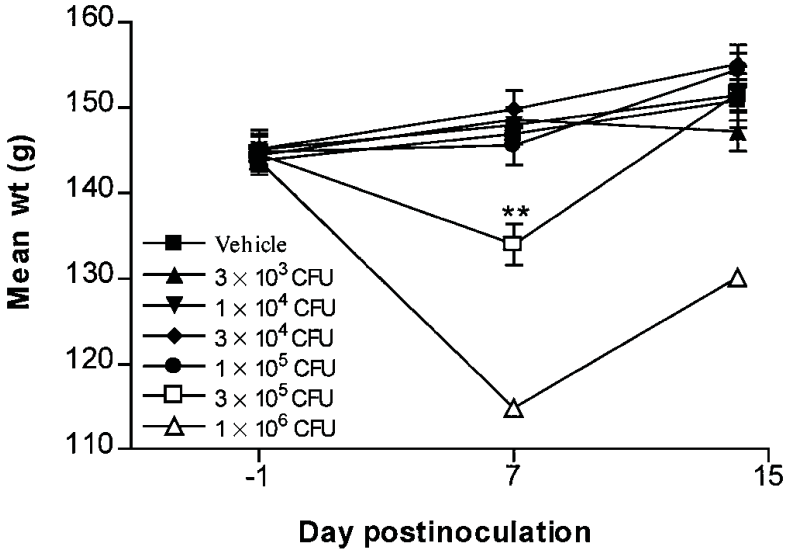

FIGURE 1. Mean body weights of Fischer 344 rats inoculated with *L. monocytogenes* in the range-finding study. Rats were inoculated with the indicated concentrations of *L. monocytogenes* on day 0. The points and bars on the curve represent the mean weight ± SE of 8 animals per group except for the 1×10^6 CFU group on days 7 and 14, where the points represent a single animal. Statistical significance is denoted as $^{**}(p < 0.01)$.

period. Although there was no significant difference in mean body weights, animals receiving 1×10^5 CFU showed significant decrease in body weight change during the first week postinoculation as compared to vehicle controls. Rats inoculated with 3×10^5 CFU/animal showed a significant decrease in weight during the first week of infection as compared to the vehicle control group but gained weights during the second week such that by the last day of the study, their body weight did not differ significantly from those of the vehicle control group. Only one animal in the experimental group inoculated with 1×10^6 CFU/animal survived. By day 7, this animal's weight had decreased from 137.4 to 114.8 g. During the second week of infection, the animal began to recover and her body weight increased to 130.1 g by the end of the study.

Animal weights for the inoculum control groups in the validation study are shown in Figure 2. Vehicle rats, treated and inoculated with 0.9% NaCl, showed an increase in mean body weight of 22.27 g over the experimental period. Animals inoculated with 1×10^5 CFU demonstrated no statistically significant differences in their mean body weights at the end of the first week postinoculation, but their mean weight gain was significantly decreased as compared to the inoculum control group (9.2 g as compared to 15.6 g). The animals recovered during the second week postinoculation and by the end of the study neither their mean weight nor mean weight gain differed significantly from the control group. Animals inoculated with 5×10^5 CFU experienced significant weight loss during the first week postinoculation, with 4 animals

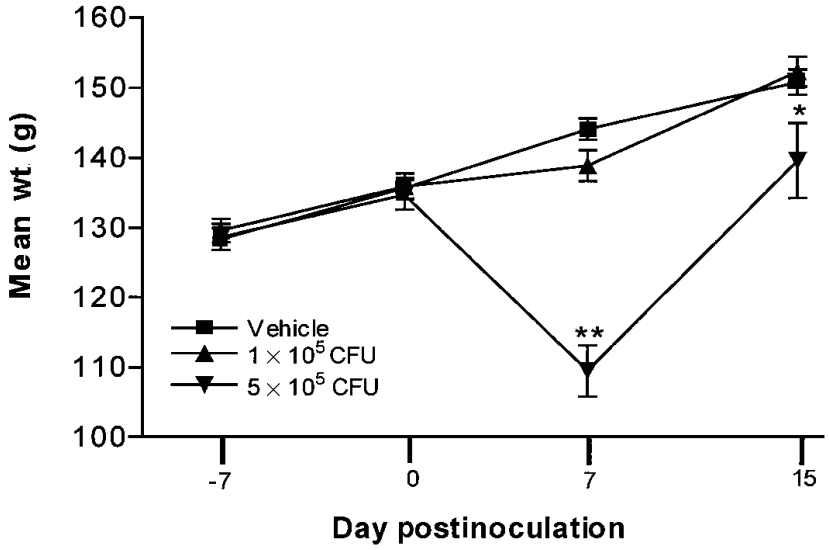

FIGURE 2. Mean body weights of fischer 344 rats with *L. monocytogenes* inoculated in the validation study. Rats were inoculated with the indicated concentrations of *L. monocytogenes* on day 1. The points on the curves represent the mean weight ± SE of 10 animals per group. Data for animals inoculated with 8×10^5 and 2×10^6 are not shown because these animals died within the first week of the study. Statistical significance is denoted as $^*(p < 0.05)$ and $^{**}(p < 0.01)$.

dying during this period. Although the surviving animals gained weight during the second week postinoculation, their mean body weights as compared to the control group were still significantly decreased at the termination of the study. All animals inoculated with 8×10^5 or 2×10^6 CFU and treated with 0.9% NaCl died within the first week postinoculation (data not shown). At the 3 highest concentrations of inoculum, animals treated with the CPS died before day 7 postinoculation. One animal inoculated with 1×10^5 CFU and treated with cyclophosphamide survived until day 7. Following administration of *C. parvum* but prior to inoculation with *L. monocytogenes*, the mean body weights of animals significantly decreased when compared to their respective controls. During the 2 weeks following inoculation, animals treated with *C. parvum*, including those receiving all concentrations of *L. monocytogenes*, experienced an increase in mean body weight (data not shown).

Figure 3 displays the survival results for the range-finding study. Groups of animals inoculated with concentrations of *L. monocytogenes* up to and including 3×10^5 showed 100% survival. In the group of rats receiving 1×10^6 *L. monocytogenes*, the first deaths occurred on day 2.5 postinoculation. Deaths occurred over the next 3 days, with the last death occurring on day 6.5. One animal in this group survived until the end of the experimental period (day 14). This animal showed evidence of infection as manifested by its unthrifty appearance and loss of body weight as described previously. Using the Product Limit Survival Estimates, the group receiving 1×10^6 *L. monocytogenes* was statistically different from the control group (probability > $\chi^2 = 0.000$).

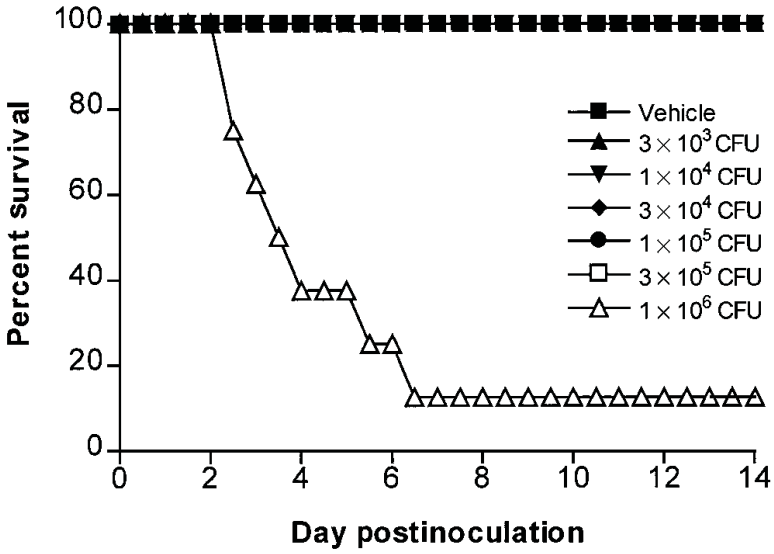

FIGURE 3. Survival of female Fischer 344 rats inoculated with *L. monocytogenes* in the range-finding study. Rats were inoculated with the indicated number of *L. monocytogenes* on day 0. The points on the curve represent percent survival for each dose group at the indicated time. Groups consisted of 8 animals per group on day 0.

Survival data for animals inoculated with concentrations of *L. monocytogenes* ranging from 1×10^5 to 2×10^6 CFU and treated with NaCl in the validation study are shown in Figure 4. All of the animals inoculated with 1×10^5 CFU survived until the termination of the study. In the group of animals inoculated with 5×10^5 CFU, the first animal died on day 4.5 and by the end of the study there was a 50% survival rate. Animals in the group inoculated with 8×10^5 CFU began to die on day 3 of the study and 100% mortality was seen by day 6. In the group of animals inoculated with 2×10^6 CFU, there was 100% mortality by day 2.5.

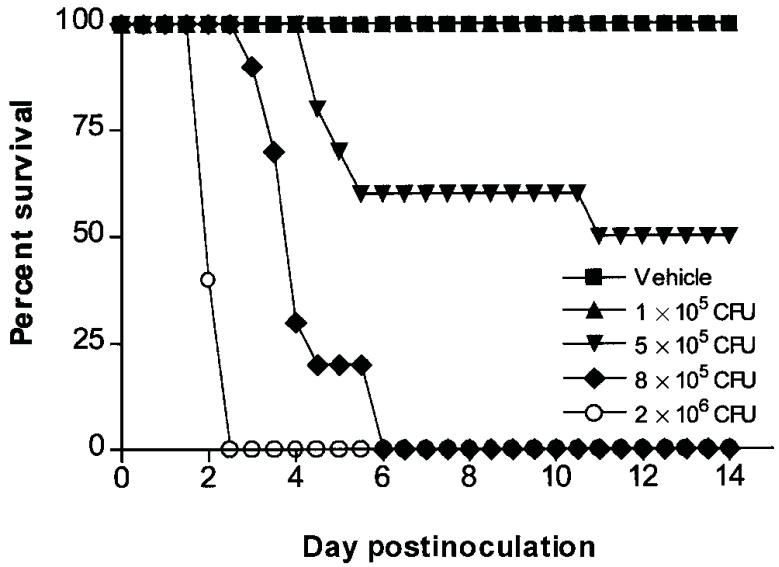

FIGURE 4. Survival of NaCl vehicle-treated female Fischer 344 rats inoculated with *L. monocytogenes* in the validation study. Rats were inoculated with the indicated number of *L. monocytogenes* on day 0. The points on the curve represent percent survival for each dose group at the indicated time. Groups consisted of 10 animals per group on day 0.

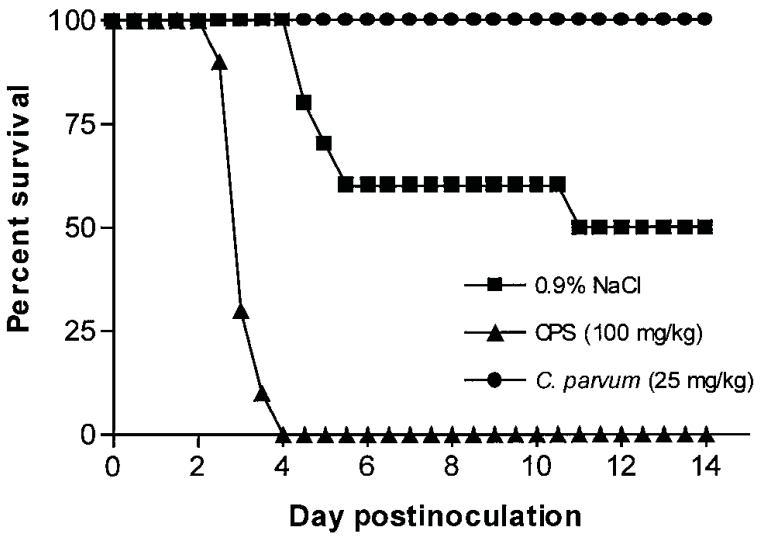

FIGURE 5. Effects of cyclophosphamide (CPS) and *Corynebacterium parvum* on percent survival of female fischer 344 rats following inoculation with 5×10^5 CFU of *L. monocytogenes*. Rats were administered either *C. parvum* on day −6, 0.9% NaCl (Vehicle) or CPS on day 0. All animals were inoculated via the intravenuous route with 5×10^5 CFU of *L. monocytogenes* on day 1. The points on the curve represent percent survival for each dose group at the indicated time. Groups consisted of 10 animals per group on day 0.

At all inoculum levels, animals treated with CPS showed mortality at an earlier time point than their NaCl-treated controls and reached 100% mortality by the termination of the study. No deaths were seen in CPS-treated control animals (inoculated with 0.9% NaCl). Conversely, there was no mortality in groups of animals treated with *C. parvum*, regardless of the inoculum level (data not shown).

Figure 5 shows the percent survival for groups of animals inoculated with 5×10^5 CFU of *L. monocytogenes*. Animals treated with the NaCl vehicle began to die on day 4.5, with the last death observed on day 11.0, resulting in 50% mortality. Animals treated with CPS exhibited significantly decreased resistance as evidenced by earlier deaths and 100% mortality within the group. *C. parvum* was observed to significantly increase host resistance as evidenced by 100% survival within the *C. parvum*-treated groups.

DISCUSSION

Over the past two decades, a tiered approach to immunotoxicology has undergone development in the United States and in Europe. In the United States, the National Toxicology Program (NTP) has focused on mouse models [7], while Europe, championed primarily by the Netherlands' National Institute of Public Health (NIPH), has concentrated on rat models [13]. These two systems differ not only in the test animal of choice, but also in their division of assays into tiers. In the European community, tier I immunotoxicity testing in the rat is included in the general toxicity testing and is based solely on immunopathology

without functional tests. Tier I testing in the mouse, as accepted by the NTP, follows general toxicity testing and includes immunopathology as well as assays for cellular, humoral, and innate immunity. For tier II testing, both approaches include host resistance studies. Rat host resistance assays have been developed for bacterial, parasitic, viral, and autoimmune diseases [8], while mouse models have been developed for bacteria [1, 14], viruses [15], parasites [16], and syngeneic tumors [2].

As efforts toward international harmonization emerge, there is a drive to move toward the rat as the animal of choice for immunotoxicology testing. Historically, the NTP has recommended the use of inbred Fischer 344 rats while the NIPH in the Netherlands has used the outbred Wistar rat for toxicology testing. One advantage of using the Fischer 344 over the Wistar rat is the ability to conduct assays such as the cytotoxic T-lymphocyte assay (CTL) and syngeneic tumor models that require the homogeneity of an inbred species. Therefore, the purpose of these studies was to develop a bacterial model of host resistance in the Fischer 344 rat using *L. monocytogenes*.

The data presented here show that the Fischer 344 female rat is susceptible to *L. monocytogenes* and the course of the disease is similar to that seen in the B6C3F1 mouse and the Wistar rat. In the Wistar model, 75% mortality (3/4) was seen in 5-week-old male rats inoculated with 2×10^6 *L. monocytogenes* with a mean interval between infection and death of 4.3 days [17]. Inoculation with 3.4×10^3 CFU has been shown to cause death in 5/12 B6C3F1 mice, with mortality occurring on days 5 and 6 [18]. Likewise, 50% (5/10) mortality was seen in Fischer 344 rats inoculated with 5×10^5 CFU *L. monocytogenes*, with animals dying between days 2 and 6. In the Fischer 344 rat model, as well as in the Wistar rat and the B6C3F1 mouse models, higher inoculum levels caused earlier deaths at shorter intervals.

Furthermore, immunoenhancing and immunosuppressive agents altered the outcome of *Listeria* infection in the Fischer 344 rat. The alkylating agent CPS preferentially kills small lymphocytes and decreases lymphocyte response to mitogens while showing less activity against mononuclear and polymorphonuclear leukocytes [19]. In this regard, CPS would be expected to interfere with T-cell activation of macrophages and, therefore, reduce resistance to *L. monocytogenes* infection. In concordance, as compared to 50% mortality in animals with an intact immune system, 100% mortality (10/10) was seen in animals receiving 5×10^5 CFU of *L. monocytogenes* after being immunosuppressed with CPS. Furthermore, deaths occurred earlier in CPS immunosuppressed animals (beginning on day 2.5) than in nonimmunosuppressed animals (beginning on day 4.5) given the same inoculum. Conversely, *C. parvum* increases the number and activation state of liver macrophages [20] and therefore would be expected to increase resistance to

Listeria. In agreement, pretreatment with *C. parvum* followed by administration of 5×10^5 CFU *L. monocytogenes* resulted in 100% survival of Fischer 344 rats (10/10). In summary, at all concentrations of *L. monocytogenes* tested, treatment with the immunoenhancing agent *C. parvum* was protective and treatment with the immunosuppressive agent CPS resulted in 100% mortality.

These data show that the Fischer 344 rat, like the Wistar rat and the B6C3F1 mouse, is a suitable host for *L. monocytogenes* bacterial host resistance.

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