Pathogenicity of *Listeria monocytogenes* isolates in immunocompromised mice in relation to listeriolysin production

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Summary. The virulence of 74 *Listeria monocytogenes* isolates from clinical cases and food products and of 11 isolates of other *Listeria* species was tested in mice immunocompromised with carrageenan. Isolates of species other than *L. monocytogenes* were not lethal to such mice. All 29 clinical isolates of *L. monocytogenes* (serotypes 1/2a, 1/2b, 4b) and 33 of 42 isolates of various serotypes isolated mainly from dairy products killed all test mice (100% lethality) at an inoculum of 10^4 cfu/mouse. All lethal strains of *L. monocytogenes* were haemolytic and possessed the 58-Kda band specific for listeriolysin O as demonstrated by SDS-PAGE immunoblotting. The nine avirulent strains of *L. monocytogenes* had detectable haemolytic activity, but in six of them this activity was significantly weaker than in virulent strains and the 58-Kda band was not detected. The other three avirulent strains were highly haemolytic and possessed the 58-Kda band, which suggests that other factor(s) could be involved in the virulence of *L. monocytogenes*.

Introduction

The genus *Listeria* includes five recognised species,¹ of which only *L. monocytogenes* is regularly involved in human diseases. Pregnant women, neonates and more generally immunocompromised patients are particularly susceptible to listeriosis, although the disease sometimes occurs in apparently healthy individuals. Listeriosis is generally sporadic but large outbreaks have occurred in which the role of food products, mainly dairy products, has been clearly demonstrated.²

The virulence of *L. monocytogenes* for man has been correlated with pathogenicity in mice,^{3,4} particularly in mice immunocompromised by treatment with carrageenan.⁵ In this model, a large difference between the LD50 of virulent and avirulent species is observed. This characteristic, not observed in normal mice, affords a clear and rapid means of distinguishing between strains with a single dose inoculum and with the sole criterion being death of mice.

The major virulence factor of *L. monocytogenes* is an extracellular thiol-activated haemolysin, listeriolysin O (LLO).⁶ This has been identified as a protein of 58 Kda by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The role of LLO in virulence has been demonstrated in tests with non-haemolytic mutants obtained by transposon muta-

genesis.^{7–11} However, this has been done with laboratory strains and needs to be confirmed with a large and representative sample of strains from clinical cases and food products. The purpose of this study was to examine the association between the production of LLO and virulence in field isolates. Some *L. monocytogenes* isolates are avirulent;^{12–15} therefore, we first screened a large number of clinical and food isolates for virulence in mice immunocompromised with carrageenan.⁵ Having confirmed the existence of avirulent strains we then investigated the association of virulence with haemolysin production and with the possession of the 58-Kda protein band specific for LLO.

Materials and methods

Bacteria

Strains of *Listeria* used in this study are shown in the table; 70 strains were from the collection of the Centre Hospitalier Universitaire de Tours, France. Of the total of 74 *L. monocytogenes* isolates, 46% were from dairy products (raw milk and cheese) and 39% from human or animal clinical cases. The latter were either from sporadic cases (eight strains) or from outbreaks in man—Angers, France (four strains);¹⁶ Nova Scotia, Canada (three strains);¹⁷ Boston, USA (five strains);¹⁸ Los Angeles, USA (seven strains);¹⁹ Lausanne, Switzerland (two strains) (J. Bille, personal communication). Three other *L. monocytogenes* strains, kindly provided by P. Berche (Faculté de

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Table. Characteristics of *Listeria* strains used

Species	Source	Pathogenicity* in immunocompromised mice	Haemolysis† on sheep-blood agar	Serotype (number of isolates)‡	Total number of strains
<i>L. innocua</i>	Cheese, cow	—	—	4ab, 6a, ND (2)	4
<i>L. seeligeri</i>	Milk, silage	—	+	1/2a, 1/2b, ND	3
<i>L. ivanovii</i>	Milk, sheep	—	+++	5 (3)	3
<i>L. welshimeri</i>	Vegetation	—	—	6a	1
<i>L. monocytogenes</i>	Clinical cases	+	++	1/2a (2), 1/2b (3), 4b (24)	29
	Dairy products	+	++	1/2a (12), 1/2b (2), 1/2c, 3b, 4b, (10)	26
	?	+	++	3b, 3c, 4a, 4c, 4d, 4e, 7	7
	?	—	++	3a	1
	Dairy products	—	++	1/2a (2)	2
	Dairy products	—	+	1/2a (6)	6

*+, 100% mortality; —, no deaths in 5 days.

†—, No haemolysis; +, weak haemolysis; ++, clear haemolysis; +++, strong haemolysis.

‡ For number greater than one.

ND = not determined.

? = Unknown.

Médecine Necker-Enfants Malades, Paris, France), were used as controls—*L. monocytogenes* EGD (NCTC 7973), its hly[−] mutant obtained by transposon mutagenesis with Tn 1545, and the hly⁺ revertant of the latter strain.⁷

Species identification²⁰

Identification of *Listeria* was confirmed by testing the following properties: production of acid from D-xyllose (1%), L-rhamnose (1%) and α -methyl-D-mannoside (0.5%), haemolysis on Tryptose Soy Agar (TSA; Biomérieux) containing washed sheep red blood cells (5% v/v), and the Camp test reaction with *Staphylococcus aureus* (CIP 5710) and with *Rhodococcus equi* (CIP 5869).

Pathogenicity in immunocompromised mice

Bacterial strains were tested blindly in 5-week-old female Swiss OF1 mice (IFFA credo, France) weighing 20–25 g and kept under controlled temperature (21°C), humidity (60%) and light intensity. Carrageenan (Sigma, type II) dissolved in distilled water was injected intraperitoneally (200 mg/kg), 24 h before challenge. Bacterial isolates were grown for 24 h at 37°C on Brain Heart Infusion Agar (BHIA; Difco) and harvested in saline. Each suspension was standardised turbidimetrically, adjusted to 5×10^4 cfu/ml by dilution in saline and 0.2 ml was administered by intraperitoneal injection (i.e., 10^4 cfu/mouse). Sets of five mice received each isolate. In each experiment, control groups of mice were given known pathogenic and non-pathogenic strains, or saline alone. Mice were then observed for 6 days and death was recorded. The inoculum was checked by enumeration of bacterial colonies on TSA.

Titration of haemolytic activity

Haemolytic activity was assayed in the culture supernates of bacteria grown in Proteose-Peptide Broth treated with charcoal (CTB) as described previously.²¹ From an overnight culture, 125 μ l was inoculated into 5 ml of the same medium and incubated at 37°C for 10 h. Supernates were collected after centrifugation (10 000 *g* for 10 min at 4°C) and assayed for haemolytic activity by the modified method of Alouf *et al.*²² in flat-bottomed polystyrene microtitration plates (Greiner). A 100- μ l volume of a twofold dilution of culture supernate in phosphate-buffered saline (PBS, pH 6.0) was added to 50 μ l of sheep-red-blood-cell (SRBC) suspension (2.25%) in PBS supplemented with 40 mM DL-cysteine (Aldrich) and bovine albumin (Miles) 0.2%. Plates were then incubated at 37°C for 45 min, centrifuged (1000 *g* for 2 min), and the haemoglobin in the supernates was measured spectrophotometrically in a Titertek Multiskan apparatus (Flow Laboratories) at 541 nm. One haemolytic unit (HU) was defined as the amount of toxin causing the release of 50% of the haemoglobin of erythrocytes and was estimated from a standard graph.²²

Detection of LLO by electrophoresis and immunoblotting

Bacteria were grown overnight in CTB at 37°C and centrifuged (5000 *g* for 20 min at 4°C). Supernates were treated with trichloroacetic acid (TCA) 10% and precipitates were collected by centrifugation (11 000 *g* for 20 min at 4°C) then dissolved in 0.06 M Tris HCl buffer (pH 6.8). Protein concentration was determined by the method of Bradford.²³ Samples were then diluted in an equal volume of sample buffer (SDS 2%, glycerol 10%, mercaptoethanol 5%, bromophenol blue 0.002%, 0.02 M Tris HCl) and boiled for 3 min. SDS-

PAGE was performed in 7.5% resolving gels by the method of Laemmli²⁴ with approximately 100 µg of protein/well. After electrophoresis, separated proteins were transferred on to nitrocellulose membranes (Sartorius)²⁵ by means of a semi-dry fast electroblotter (Biometra). The nitrocellulose was then saturated overnight with gelatine 0.5% solution and incubated for 1.5 h in anti-listeriolysin O serum (kindly provided by P. Berche, Faculté de Médecine Necker-Enfants Malades, Paris, France)⁶ diluted 1 in 400 in PBS containing Tween 20 0.1% and gelatine 0.5% (PBS-T-G). After three washings in PBS containing Tween 20 0.1% (PBS-T) the nitrocellulose paper was incubated for 1 h with goat anti-rabbit immunoglobulin G linked to horseradish peroxidase (Nordic) diluted 1 in 1000 in PBS-T-G. After two washes in PBS-T and two in PBS, immunoreactive bands were revealed by treatment with 4-chloro-1-naphtol (HRP colour development reagent; Bio-Rad) 0.5 mg/ml and H₂O₂ 0.03% v/v.

Results

Confirmation of identity of *Listeria* species

The identity of the isolates as *Listeria* was confirmed by the criteria described in *Materials and methods*. We noted that 11 (13%) *L. monocytogenes* strains reduced α -methyl-D-mannoside in 4–7 days compared with 1–2 days for the other isolates.

Pathogenicity of isolates for immunocompromised mice

Mice were not affected by injection of carrageenan alone. After a slight decrease of weight (approximately

1.5 g) during the first 24 h, no sign of discomfort or toxicity was observed during the whole period of the experiment. Mice regained their initial weight 48 h after injection and then had a normal growth rate.

With an inoculum of 10⁴ cfu and a period of observation of 5 days, the 85 *Listeria* isolates could be classified as either pathogenic (four or five deaths out of five mice inoculated) or non-pathogenic (no death out of five mice). On re-testing two or three times, the six isolates that caused four deaths out of five were 100% lethal. No significant difference was observed in the mean time to death with pathogenic strains. Death occurred between 2 and 5 days after inoculation and, usually (75%), between 72 and 96 h.

The pathogenicity of *Listeria* isolates according to species, serotype and source is shown in the table. None of 11 isolates of species other than *L. monocytogenes* were pathogenic. Of the *L. monocytogenes* strains, all 29 clinical isolates (serotypes 1/2a, 1/2b, 4b) and 33 (79%) out of 42 isolates from other sources (mainly dairy products) were pathogenic. The nine non-pathogenic strains belonged to serotypes 1/2a (eight strains) and 3a (one strain), whereas no non-pathogenic strains were detected among the 10 strains of serotype 4b. The lack of pathogenicity of the nine *L. monocytogenes* strains was confirmed on re-testing.

The transposon-induced mutant, which lacks LLO (EGD hly⁻), was not pathogenic, in contrast to both the parent and revertant (EGD hly⁺) strains.

Association between pathogenicity and haemolysin production

All the strains were examined for haemolytic activity in a single experiment with the same batch of SRBC (fig. 1). The highest activities were produced

Fig. 1. Distribution of haemolytic activity in CTB culture supernates of *Listeria* strains: ▲, *L. ivanovii* (3 strains); △, *L. seeligeri* (3 strains); ●, *L. monocytogenes* lethal for mice (64 strains); ○, *L. monocytogenes* not lethal for mice (9 strains); □, *L. monocytogenes* EGD hly⁻ obtained by transposon mutagenesis. No haemolytic activity was detected in supernates of *L. innocua* (4 strains) and *L. welshimeri* (1 strain).

Fig. 2. Immunodetection of listeriolysin in culture supernates after electrophoresis on SDS-polyacrylamide gels. The blot was developed with rabbit anti-listeriolysin O serum (1 in 400). (A) Lane 1, mol.-wt markers; 2, *L. innocua*; 3, *L. seeligeri*; 4, *L. welshmeri*; 5 *L. ivanovii*; 6, mutant *L. monocytogenes* EGD hly⁻; 7, revertant *L. monocytogenes* EGD hly⁺; *L. monocytogenes* isolates lethal for mice from (8, 9) dairy products and from (10, 13, 14) clinical cases; 11, 12 weakly haemolytic *L. monocytogenes* isolates not lethal for mice. (B) *L. monocytogenes* isolates lethal for mice from (1, 2) clinical cases and from (3, 4, 14) dairy products; weakly haemolytic (5, 7, 11, 13) and highly haemolytic (6, 10, 12) *L. monocytogenes* isolates not lethal for mice; 8, wild strain *L. monocytogenes* EGD and (9) its mutant *L. monocytogenes* EGD hly⁻ used as controls.

by *L. ivanovii* strains (1600–2500 HU/ml). As expected, the Tn 1545-induced (*hly*⁻) mutant of *L. monocytogenes* EGD had no detectable haemolytic activity. The mean haemolytic activity of all other *L. monocytogenes* strains was 128 HU/ml. The nine non-pathogenic isolates belonged to two distinct groups—weakly haemolytic (six isolates with activity of 5–8 HU/ml) and highly haemolytic (three isolates with activities of 200, 500 and 600 HU/ml). The mean activity of the six weakly haemolytic strains (6.2 HU/ml) was significantly different from the mean activity of all other *L. monocytogenes* strains (167 HU/ml) ($t=12.7$, $p<0.0005$). Haemolytic activity correlated with intensity of haemolysis on blood agar (table).

Supernates of overnight cultures were tested for the presence of the 58-Kda LLO by immunoblotting with specific antiserum. This experiment was performed on 26 representative strains of *L. monocytogenes* and one strain of each other species. The *L. monocytogenes* strains included eight clinical isolates (all pathogenic) of various serotypes and 15 isolates from dairy products including the nine non-pathogenic strains. All pathogenic *L. monocytogenes* isolates possessed the LLO band (fig. 2). This band was also detected in *L. ivanovii* and in the three non-pathogenic, highly haemolytic *L. monocytogenes* strains. In contrast, LLO was not detected in isolates of *L. innocua*, *L. welshimeri* and *L. seeligeri*, or in the six weakly haemolytic and non-pathogenic *L. monocytogenes* isolates. The 58-Kda protein band was also absent from the *hly*⁻ transposon-induced mutant of *L. monocytogenes* EGD, being replaced, as previously described,⁷ by several bands of 45–55 Kda (fig. 2). The LLO band was present in the *hly*⁺ revertant of the *hly*⁻ strain.

Discussion

To investigate the variation in pathogenicity of field isolates of *Listeria* spp., a simple and reliable assay of virulence applicable on a large scale is required. The model of mice immunocompromised with carrageenan seemed to serve this purpose, because according to Stelma *et al.*,⁵ a single inoculum of 10^4 cfu was able to distinguish pathogenic from non-pathogenic strains. The relevance of this model to the natural infection is further supported by the well-established predilection of *L. monocytogenes* for immunocompromised hosts. Our results confirm and extend the previous observations of Stelma *et al.*⁵ on an enlarged sample of isolates. Indeed, all 29 *L. monocytogenes* isolates from clinical cases killed all test mice whereas the 11 isolates of other *Listeria* spp. were totally non-pathogenic. Such results are also in agreement with those obtained with normal mice²⁶ except in tests with *L. ivanovii* strains, which are generally found to be pathogenic in the latter model.³ The absence of pathogenicity observed in the present study with the *hly*⁻ transposon-induced mutant also confirms previous studies in which normal mice were used.⁷

In contrast to clinical isolates, a significant propor-

tion (21%) of *L. monocytogenes* isolates from food products were non-pathogenic in immunocompromised mice. It is important to note the different distribution of non-pathogenic strains in serotypes 1/2a and 4b, the two serotypes most frequently incriminated in human listeriosis: 40% (8 of 20) of 1/2a isolates were non-pathogenic, compared with none of 10 4b isolates. The higher proportion of virulent strains in serotype 4b is reflected in the fact that, although less frequent than isolates of serotype 1/2a in food products, they are more frequently isolated from clinical cases.^{27,28}

The immunocompromised mouse model was also used recently by Conner *et al.*²⁹ to test the pathogenicity of foodborne, environmental and clinical isolates of *L. monocytogenes*. As in the present study, they detected non-lethal isolates (7%), but in contrast, they also reported a large number of isolates with “intermediary pathogenicity”. However, the LD50 of isolates classified as “intermediary” was similar to that of fully pathogenic strains and was several orders of magnitude lower than that of “non-pathogenic” isolates, suggesting that the “intermediary” isolates could be classified justifiably as pathogenic. This apparent discrepancy between our results and those of Conner *et al.*²⁹ probably reflects the difference in the duration of the observation period; we found that a 5-day period gave unambiguous results, whereas results after 3 days, as used by Conner *et al.*, were ambiguous. Small differences in experimental procedures, such as the strain of mouse used, could also contribute to the observed differences between the two studies.

The main purpose of this study was to test the association between the production of LLO and the pathogenicity of *L. monocytogenes* isolates. All isolates classified as pathogenic in the immunocompromised mouse model produced a detectable amount of haemolysin and possessed the 58-Kda band specific for LLO, confirming previous observations.²¹ However, previous studies had not included non-pathogenic field isolates. These isolates could be classified into two categories. The first category included six isolates that produced a very small amount of haemolysin and no detectable LLO in SDS-PAGE immunoblotting. The absence of pathogenicity in isolates in this category is probably due to insufficient production of LLO, which has previously been demonstrated to be a crucial factor in the intracellular survival and multiplication of *L. monocytogenes*.^{30–32} We suggest that production of LLO above a minimal threshold level is necessary to allow intracellular growth of *L. monocytogenes*. All isolates of this category had a further remarkable characteristic—reduction of α -methyl-D-mannoside only after a significant delay, a property which was observed in only 8% of pathogenic isolates.

The other category of non-pathogenic isolates of *L. monocytogenes*, includes three strains that produced a large quantity of haemolysin and the 58-Kda band specific for LLO. The role of LLO in pathogenicity

has been clearly established. We can put forward two hypotheses to explain the lack of virulence in these strains. The first is that, although almost identical to the LLO of pathogenic strains, the haemolysin of this category of strains differs in amino-acid sequences which are crucial for intracellular growth but which are unrelated to the mechanism of haemolysis. Such a hypothesis has been suggested to account for the difference of virulence observed between haemolytic species of *Listeria*, i.e. *L. monocytogenes*, *L. ivanovii* and *L. seeligeri*.⁴ An alternative, and more likely, explanation is that another critical factor of pathogenicity is lacking in this category of non-pathogenic strains. Such a factor could be required, for example, for entry of organisms into cells, a crucial step in pathogenicity which is characteristic of *L. monocytogenes* when compared with other *Listeria* spp.³⁰ It has indeed been demonstrated that hly⁻ mutants of *L.*

monocytogenes are still capable of penetration but cannot survive within cells.^{9,30,31} In this context, we are currently investigating possible differences in surface antigens between pathogenic and non-pathogenic strains.

This study shows the existence of a significant proportion of potentially non-pathogenic strains of *L. monocytogenes* in food products, and highlights the importance of recognising such strains in the laboratory. Furthermore, it indicates that, in addition to LLO, other critical factor(s) responsible for pathogenicity may be present in virulent strains of *L. monocytogenes*.

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