Oral infectivity and bacterial interactions with mononuclear phagocytes

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Summary. The purpose of this study was to clarify the association between the oral infectivity of a bacterial strain and its susceptibility to ingestion by mononuclear phagocytes or ability to survive within them. Ten bacterial strains tested—all of known oral infectivity—comprised *Salmonella typhimurium*, *Listeria monocytogenes* (three strains), *Escherichia coli* (two strains), *Proteus mirabilis*, *Enterococcus faecalis*, *Bacteroides fragilis*, and a *Bacteroides* sp. The phagocytic uptake of each strain was measured as the bacteria to phagocyte ratio after mononuclear phagocytes in mouse peritoneal exudate were permitted to ingest bacteria *in vivo* for 3 min. The three *Listeria* strains were the most susceptible to phagocytic uptake and the *Salmonella* strain was relatively resistant. The intracellular survival of each strain was studied during a subsequent 2 h *in-vitro* incubation of the mononuclear phagocytes that had been permitted to ingest bacteria *in vivo*. The strains with the best intracellular survival were *Ent. faecalis* and two of the three *Listeria* strains. The ability of *S. typhimurium* to survive intracellularly was intermediate but better than that of the two *E. coli* strains. Oral infectivity was not consistently correlated with susceptibility to ingestion by mononuclear phagocytes or ability to survive within them.

Introduction

Different species or strains of enteric bacteria vary in invasiveness.^{1–2} Pathogenic organisms such as *Salmonella* spp. and *Listeria monocytogenes* given by the natural (oral) route are able to migrate (“translocate”) from the intestine to other sites, such as the liver and spleen, and cause systemic disease.^{2–6} Translocation of potentially pathogenic members of the normal intestinal microflora, such as *Escherichia coli*, *Klebsiella pneumoniae*, *Proteus mirabilis*, *Pseudomonas aeruginosa* and *Enterococcus faecalis*, can be induced by bringing about intestinal overgrowth. This has been studied in gnotobiotic mice^{6–10} and in antibiotic-treated rodents colonised with an antibiotic resistant strain.^{11–17} Normal animals are resistant to overgrowth and to translocation of such bacteria given orally, even in large doses.^{13–14, 16, 18} Relatively non-pathogenic, strictly anaerobic members of the normal intestinal flora such as *Bacteroides* spp. can rarely be induced to translocate, even in gnotobiotic mice with high intestinal concentrations of the organism^{7–8} or in antibiotic-treated rodents with intestinal overgrowth of an antibiotic resistant strain.¹⁷

Earlier studies showed that mononuclear phagocytes could transport inert particles (1 µm latex beads)

and possibly viable bacteria from the intestinal lumen to the draining mesenteric lymph node (MLN).¹⁹ A similar mechanism of particle transport had been described for alveolar macrophages migrating from the terminal alveolus of the lung to the draining tracheobronchial lymph node.²⁰ Because *Salmonella* spp. and *L. monocytogenes* can survive and possibly replicate in phagocytic leucocytes,^{21–23} and because they readily translocate from the intestine, it seemed reasonable to speculate that facultative intracellular parasitism is associated with oral infectivity.

Thus, the aim of this study was to determine if the oral infectivity of a bacterial strain was related to the susceptibility of that strain to ingestion by mononuclear phagocytes or its ability to survive within them.

Materials and methods

Mice

Female Swiss Webster mice (18–22 g; Harlan-Sprague Dawley, Inc., Indianapolis, IN, USA) were used in all experiments.

Bacteria

Bacterial strains were chosen for their strong or weak ability to produce systemic infection in orally

Table I. Oral infectivity of 10 bacterial strains for mice, expressed as the recovery of viable organisms from mesenteric lymph node (MLN) and liver

Bacterial strain	Oral infectivity* for		Reference
	mice in whose caeca the infecting organism was present at $< 10^7$ /g	gnotobiotic or antibiotic-treated mice in whose caeca the infecting organism was present at 10^{10-11} /g†	
<i>S. typhimurium</i> 14028	+++	ND	table II
<i>L. monocytogenes</i> +H1a	++	ND	table II
<i>L. monocytogenes</i> -H1a	+	ND	table II
<i>L. monocytogenes</i> +H4b	+	ND	table II
<i>Ent. faecalis</i> M20	Nil‡	+++	8, 10, 13-14
<i>E. coli</i> C25	Nil‡	++	6-8, 11, 16, table III
<i>E. coli</i> M14	Nil‡	++	8, 10
<i>P. mirabilis</i> M13	Nil‡	++	8, 10
<i>B. fragilis</i> 9032	Nil‡	+	table III
<i>Bacteroides</i> sp. M25	Nil‡	+	17

ND, not done.

* + + +, Bacterial strain recovered from the MLN and liver of the majority of mice, with occasional deaths; ++, bacterial strain recovered from the MLN of the majority of mice, and from the liver of at least an occasional mouse, with no mortality; +, bacterial strain recovered from the MLN of at least an occasional mouse, with no mortality.

†Caecal overgrowth followed either oral inoculation of the bacterial strain into gnotobiotic mice, or oral inoculation of an antibiotic-resistant strain into antibiotic-treated mice.

‡High numbers (e.g., 10^9) given orally are rapidly eliminated from the gut. The strain is either not recovered from extra-intestinal tissues, or recovered from the MLN of an occasional mouse.^{10, 14, 16, 18}

Table II. Caecal colonisation and oral infectivity of *S. typhimurium* 14028 and *L. monocytogenes* (three strains) in normal mice killed 48 h after oral administration of 10^9 bacteria

Oral inoculum	Mean log ₁₀ (SEM) viable bacteria/g caecum (n = 6)			No (%) of mice, in groups of 21–24, yielding infecting organisms* from		Mean log ₁₀ (SEM) viable bacteria in		Mortality (%)
	Aerobic and facultative gram-negative bacilli	Aerobic and facultative gram-positive bacteria	Strict anaerobes	MLN	liver	MLN	liver	
Saline	5.4 (0.1)	7.8 (0.3)	9.3 (0.2)	0/24 (0)	0/24 (0)	—	—	0/24 (0)
<i>S. typhimurium</i> 14028	6.4 (0.3)†	8.2 (0.2)	9.4 (0.2)	19/21 (90)‡	12/21 (57)§	3.3 (0.2)	2.4 (0.3)	3/24 (13)
<i>L. monocytogenes</i> + H1a	4.5 (0.6)	8.2 (0.1)¶	9.4 (0.2)	12/23 (52)¶¶	13/23 (57)§	3.4 (0.2)	2.9 (0.3)	0/23 (0)
<i>L. monocytogenes</i> – H1a	5.1 (0.5)	8.2 (0.2)¶	9.3 (0.1)	0/24 (0)	0/24 (0)	—	—	0/24 (0)
<i>L. monocytogenes</i> + H4b	5.3 (0.3)	8.5 (0.1)¶	9.1 (0.1)	4/24 (17)	3/24 (13)	1.8 (0.2)**	2.2 (0.8)	0/24 (0)

*The orally administered strain was the only organism recovered from MLN or liver.

†*S. typhimurium* was the predominant species in all mice tested.‡Significantly greater than in mice given +H1a ($p < 0.05$) and all other inocula ($p < 0.01$).§Significantly greater than in mice given saline, -H1a, or +H4b ($p < 0.01$).¶*L. monocytogenes* present in the caecal flora (up to 10^6 /g) of individual mice as shown by growth on listeria selective agar.¶Significantly greater than in mice given +H4b ($p < 0.05$), -H1a ($p < 0.01$) or saline ($p < 0.01$).**Significantly less than the number recovered from mice inoculated with 14028 or +H1a ($p < 0.01$).

infected mice. The ability of each strain to migrate from the intestine has either been previously recorded, or is presented here (tables I, II and III). *S. typhimurium* ATCC 14028, *L. monocytogenes* ATCC 43249 serotype 1/2a, and *L. monocytogenes* ATCC 43248 serotype 1/2a were obtained from the American Type Culture Collection, Rockville, MD, USA. *S. typhimurium* 14028 has been reported to be virulent for mice by intraperitoneal inoculation.²⁴⁻²⁵ The two *L. monocytogenes* strains were derived from a single parent strain isolated from a guinea-pig lymph node.²⁶ *L. monocytogenes* 43248 (termed -H1a) is a spontaneous non-haemolytic variant of its wild-type

counterpart 43249 (termed +H1a); these two strains are identical in all characters tested so far except for haemolysin production, mouse virulence, invasiveness in cultured epithelial cells and phospholipase production.^{4, 26-27} *L. monocytogenes* CDC G283 serotype 4b (termed +H4b), isolated in a food-borne outbreak of listeriosis, was obtained from the Centers for Disease Control, Atlanta, GA, USA. Both *L. monocytogenes* +H1a and +H4b, but not -H1a, were haemolytic when grown on Tryptic Soy Agar (Difco) supplemented with sheep blood 5%. *B. fragilis* VPI 9032, a human clinical isolate, was obtained from the Department of Anaerobic Microbiology, Virginia

Table III. Caecal colonisation and oral infectivity of *E. coli* C25 and *B. fragilis* 9032 in antibiotic-treated mice killed 48 h after oral administration of 10^9 bacteria

Oral inoculum*	Mean log ₁₀ (SEM) viable bacteria/g caecum (n = 6–7)				Number of mice (%) in groups of 19 and 22 yielding infecting organism from MLN†	Mean log ₁₀ (SEM) viable bacteria in MLN†
	Aerobic and facultative gram-negative bacilli	Aerobic and facultative gram-positive bacteria	Strict anaerobes	Infecting organism‡		
Saline	6.2 (0.5)	7.8 (0.3)§	8.7 (0.2)	—	—	—
<i>E. coli</i> C25	10.2 (0.1)§	4.6 (0.5)	None detected	10.1 (0.1)	17/22 (77)	1.3 (0.1)
<i>B. fragilis</i> 9032	7.3 (1.2)	4.8 (0.9)	10.5 (0.1)§	10.3 (0.2)	5/19 (26)	1.1 (0.1)

*Before oral inoculation with *E. coli* C25 or *B. fragilis* 9032, mice were treated for 3 days with bacitracin and streptomycin or penicillin and neomycin, respectively.

†Indigenous caecal bacteria (streptomycin-sensitive *E. coli* or an *Enterobacter* sp.) were also recovered (10–80 bacteria/MLN) from four of 24 saline-treated mice, four of 22 *E. coli*-treated mice, and two of 19 *B. fragilis*-treated mice.

‡*E. coli* C25 was enumerated on MacConkey agar supplemented with streptomycin 100 µg/ml, and *B. fragilis* 9032 on bacteroides bile esculin agar. Caecal organisms from control mice gave negligible growth on these media.

§Significantly increased compared to the other two treatment groups ($p < 0.01$).

||Significantly greater than in mice given *B. fragilis* 9032 ($p < 0.01$).

Polytechnic Institute and State University, Blacksburg, VA, USA. *E. coli* C25, a streptomycin-resistant, human clinical isolate, was obtained from Dr R. Berg, Louisiana State University, Shreveport, LA, USA; this strain has been widely used in studies of bacterial translocation in rodents.^{6–8, 11, 16} The remaining strains were isolated from Swiss Webster mice during studies of intestinal bacterial translocation in our laboratory.^{16–17} All bacterial strains were maintained at -70°C in Brain Heart Infusion Broth (Difco) supplemented with glycerol 10%, except the *Bacteroides* strains which were maintained at -70°C in Cooked Meat Broth²⁸ (Difco).

Extracellular haemolysin production by *L. monocytogenes*

Production of extracellular haemolysin (listeriolysin) by *L. monocytogenes* was assayed by the method of Tabouret *et al.*²⁹ with minor modifications. Filter-sterilised supernates from 24-h Tryptic Soy Broth (Difco) cultures were diluted in phosphate-buffered saline supplemented with 6 mM cysteine hydrochloride, incubated for 30 min at 35°C to activate the haemolysin,³⁰ mixed 9 parts to 1 part with washed sheep red blood cells (RBCs) 10%, incubated at 35°C for 30 min and centrifuged to pellet the RBCs; the degree of haemolysis was measured as the absorbance at 540 nm. Haemolytic units were expressed as the highest dilution of bacterial supernate showing 50% haemolysis.

Oral infectivity of *B. fragilis* 9032, *E. coli* C25, *S. typhimurium* 14028 and *L. monocytogenes* + H1a, –H1a, +H4b

In this paper, oral infectivity is defined as the ability of an organism to translocate to extra-intestinal tissues (MLN and liver) after oral inoculation. Separate groups of mice were inoculated orally, by means of a “feeding needle”, with 0.1 ml of suspensions con-

taining log₁₀ 9 saline-washed cells of *B. fragilis*, *E. coli*, *S. typhimurium* or *L. monocytogenes* (strain +H1a, –H1a, or +H4b). All bacterial cells were obtained from overnight tryptic soy broth cultures grown aerobically, with the exception of *B. fragilis* which was grown anaerobically in supplemented²⁸ brain heart infusion broth. Control mice were inoculated with saline. To facilitate intestinal colonisation with streptomycin-resistant *E. coli* C25, mice were given drinking water containing bacitracin (Sigma) 2 mg/ml and streptomycin sulphate (Sigma) 2 mg/ml for 3 days before oral inoculation. To facilitate intestinal colonisation with penicillin-resistant *B. fragilis* 9032, mice were given drinking water containing penicillin G (Sigma) 200 units/ml and neomycin sulphate (Upjohn Co.) 4 mg/ml for 3 days before oral inoculation. Mice were killed 2 days after oral inoculation. In mice given antibiotics, the treatment was continued for the duration of the experiment. Viable bacteria in the caecum (tissue with contents), MLN and liver were counted and identified as previously described.^{13–16} For the selective isolation of the orally inoculated strains from the caecal flora, caeca were also plated as appropriate on listeria selective agar,³¹ MacConkey agar supplemented with streptomycin 100 µg/ml,^{15–16} or Bacteroides Bile Esculin Agar²⁸ (Difco). The limits of detection of the assays were 10 bacteria/MLN, 15 bacteria/liver, and 500 bacteria/caecum. The combined results of two separate experiments are presented (11–18 mice/treatment group in each experiment). MLN from all mice in all experiments were examined. Livers were examined from all mice in experiments with salmonella and listeria. The caecal microflora was characterised in three or four mice/treatment group in each of the two experiments.

In-vivo phagocytic uptake and in-vitro intracellular survival of bacteria in mononuclear phagocytes

Bacterial uptake and intracellular survival in mononuclear phagocytes were assayed by the method of

Leijh *et al.*³² with minor modifications as described previously.³³ Briefly, mice were given an intraperitoneal injection of 1 ml of proteose peptone 10% in tap water, and 48 h later an intraperitoneal injection (1 ml) of $\log_{10}$ 8 saline-washed bacteria of a single strain, prepared as described for oral inoculation (above). After 3 min (the time of maximal phagocytic uptake³²), mice were killed by cervical dislocation, and peritoneal exudate cells were harvested in ice-cold Hanks's balanced salts solution containing gelatin 0.1%, washed at least four times (4 min, 110 g) in the same medium, and resuspended in 4 ml to a concentration of 7×10^6 cells/ml. After the final wash, the numbers of bacteria in the supernates were 10–100-fold lower than the corresponding numbers of intracellular bacteria and, therefore, were unlikely to interfere with assay of the latter. As in previous reports,^{32–34} Wright-Giemsa stains demonstrated that c. 80% of the peritoneal exudate cells were macrophages, with $\geq 95\%$ viability as determined by both propidium iodide and trypan blue dye exclusion. This preparation of peritoneal exudate cells will be termed mononuclear phagocytes for the purposes of the present report. Washed mononuclear phagocytes were agitated by rotation of the suspension at 4 rpm at 37°C for a maximum of 120 min. At various time intervals, a 0.5-ml sample was removed, mixed with 0.5 ml of ice-cold Hanks's balanced salts solution and centrifuged for 4 min at 110 g; the cell pellet was then resuspended in 1 ml of bovine serum albumin (Sigma) 0.01% in ice-cold distilled water and subjected to lysis by three cycles of freezing and thawing in liquid nitrogen and warm (37°C) water. Complete lysis of phagocytes was verified microscopically. The numbers of viable intracellular bacteria were determined, after serial dilution in phosphate-buffered saline, by appropriate culture methods.³⁵

At the initial time point in this assay (0 min), phagocytic uptake was measured by calculating the bacteria to phagocyte ratio ('phagocytic index').³⁶ By the method of Leijh *et al.*,³² the rate constant (K_k) for in-vitro intracellular killing between 0 and 120 min was calculated as: $K_k = [\ln N_{(t=0)} - \ln N_{(t)}]/t$ where N was the number of bacteria and t was the time in minutes.

Four points should be borne in mind. Firstly, the two anaerobic *Bacteroides* strains could be used in this assay despite the aerobic incubation because there was no loss of viability after exposure of either strain to atmospheric oxygen for 24 h. Secondly, the fate of intracellular bacteria had to be interpreted in a dynamic milieu in which phagocytes could still continue to ingest extracellular bacteria.³ Assessment of viable intracellular bacteria is reliable only when they exceed the extracellular bacteria by 10–100 times.³² Assay of intracellular bacteria was not possible after 120 min because the numbers of extracellular bacteria in the leucocyte supernates increased substantially, probably due to phagocyte degradation and liberation of viable bacteria, coupled with continued multipli-

cation. In addition, the number of peritoneal exudate cells decreased by at least 50% from 120 to 240 min and the cell suspension began to stick to the sides of the test tube, presumably due to a release of leucocyte DNA. Thirdly, repeated centrifugation was used to decrease the numbers of extracellular bacteria in the leucocyte suspensions. Some investigators have used antibiotics to kill extracellular bacteria (e.g., gentamicin to kill extracellular listeria^{37–38}), but we found, as have others,^{39–40} that gentamicin affected the viability of intracellular bacteria. Finally, adherent macrophages were not used because they might have decreased listericidal activity.⁴¹

Scanning electronmicroscopy of peritoneal exudate cells

Preparations of washed mononuclear phagocytes, containing viable intracellular bacteria, were viewed by scanning electronmicroscopy to determine if bacteria were adherent to the leucocytes. Washed mononuclear phagocytes were fixed in 0.1 M cacodylate buffer containing glutaraldehyde 3%, dehydrated in ethanol, critical-point dried with CO₂, sputter-coated with gold palladium, and examined at 20 kV with a Hitachi S-450 scanning electronmicroscope.

Statistical analysis

Statistical analyses were performed with StatView SE+Graphics (Abacus Concepts, Berkeley, CA, USA). Bacterial numbers ($\log_{10}$ transformed), phagocytic indices, and intracellular killing constants (K_k) were analysed by a one-way analysis of variance followed by Fisher's test for least significant difference. Mice with no detectable bacteria in MLN or liver were not considered in computing the differences in the numbers of bacteria in these sites among various treatment groups. The frequency of bacterial recovery from MLN and liver was determined by χ^2 analysis with continuity correction.

Results

Quantitation of extracellular haemolysin production by listeria

The haemolytic properties (see above) of the three strains of *L. monocytogenes* were confirmed by four separate assays, all of which showed 512, 256 and 32 units of extracellular haemolysin for strains +H1a, +H4b and –H1a, respectively.

Oral infectivity of *S. typhimurium* 14028 and *L. monocytogenes* +H1a, –H1a and +H4b

Table II shows the caecal colonisation and oral infectivity of *S. typhimurium* 14028 and *L. monocytogenes* +H1a, –H1a and +H4b. Oral inoculation with $\log_{10}$ 9 viable cells had little effect on the caecal

Fig. 1. Phagocytic uptake of strains expressed as the number of viable intracellular bacteria/ml divided by the number of mouse peritoneal mononuclear phagocytes/ml (standardised to 7×10^6). Intracellular bacteria were obtained from phagocytes that had been permitted to ingest bacteria *in vivo* for 3 min. Each value represents the mean of 5-9 separate assays; bars are 95% confidence intervals. Significant differences ($p < 0.05-0.01$) occurred between any two strains that can be connected by a line that starts without an arrowhead and ends with an arrowhead. Lm, *L. monocytogenes*; Ef, *Ent. faecalis*; Ec, *E. coli*; Bf, *B. fragilis*; St, *S. typhimurium*.

microflora except for *S. typhimurium*, which became the predominant facultative gram-negative organism. Also, the total numbers of caecal facultative gram-negative bacilli were 10-fold greater in mice inoculated with *S. typhimurium* than in control mice, but this was not statistically significant. *S. typhimurium* was recovered in relatively high numbers from the MLN of nearly every mouse and from the liver of c. 50%, and occasional mortality was observed (table II).

Each of the three *L. monocytogenes* strains colonised the caecum in relatively low numbers; there was considerable animal to animal variation, from no detectable listeria to a maximum of $\log_{10} 5$ /g of caecum (table II). There were no noticeable differences in intestinal colonisation by the three strains. However, a spectrum of oral infectivity was observed: -H1a was not recovered from the MLN or liver of any mouse, +H4b was recovered from the MLN and liver of an occasional mouse, and +H1a was recovered in relatively high numbers from the MLN and liver of c. 50% of mice. There were no deaths in mice inoculated with the *L. monocytogenes* strains.

Oral infectivity of *B. fragilis* 9032 and *E. coli* C25

Table III shows the caecal colonisation and oral infectivity of *B. fragilis* 9032 and *E. coli* C25. Neither strain could be expected to colonise the caecum without the administration of antibiotic, or to translocate to the MLN in the absence of caecal overgrowth.^{10,14,16,18} Therefore, mice were treated with bacitracin and streptomycin to enhance caecal colonisation of *E. coli* C25 (table III) and induce caecal overgrowth.^{6,8,11,16} To compare the translocation of *B. fragilis* present in similarly high caecal concen-

trations, 36 mice were treated with neomycin and penicillin and the caecal concentration of *B. fragilis* was monitored in each animal. Although there was considerable animal to animal variation, 19 of 36 treated mice were colonised with *B. fragilis* at concentrations of $\log_{10} 9.8-10.9$ /g of caecum. In these mice, translocation of bacteria to the MLN was, as expected, less striking than in animals colonised by *E. coli* (table III). In this experiment, the liver was not cultured because high caecal concentrations of *E. coli* or *B. fragilis* do not usually induce systemic spread.^{6-7,10-11}

Uptake of eight strains of intestinal bacteria by peritoneal mononuclear phagocytes

Fig. 1 presents the phagocytic index of eight strains of intestinal bacteria with varying oral infectivity (table I). *L. monocytogenes* -H1a was ingested more efficiently than the other strains; 1 ml of a suspension of peritoneal mononuclear cells (7×10^6 cells/ml) contained an average of $\log_{10} 7.1$ listeria. *S. typhimurium* 14028 was one of the least efficiently ingested strains; 1 ml of mononuclear cell suspension contained an average of only $\log_{10} 5$ salmonella. The general observation was that phagocytic uptake (fig. 1) was not consistently correlated with oral infectivity (table I).

One ml of peritoneal mononuclear cell suspension contained an average of $\log_{10} 4.4$ and 4 of *P. mirabilis* and *Bacteroides* sp., respectively ($n = 6$). These data are not presented as phagocytic indices in fig. 1 because repeated centrifugation failed to produce a concentration of extracellular bacteria 10-fold lower than the corresponding concentration of intracellular bacteria. Therefore, we concluded that the phagocytic indices of

Fig. 2. Percentage survival of strains of bacteria incubated *in vitro* with mouse mononuclear phagocytes for 0, 30, 60 and 120 min (mean values of 4–7 separate assays): —○— *L. monocytogenes* + H1a, —▲— *Ent. faecalis* M20, —□— *L. monocytogenes* - H1a, —●— *S. typhimurium* 10428, —▲— *L. monocytogenes* + H4b, —■— *B. fragilis* 9032, --○-- *E. coli* M14, --△-- *E. coli* C25.

Fig. 3. Survival of strains incubated for 120 min *in vitro* with mouse mononuclear phagocytes. Survival expressed as the intracellular killing constant (K_k). Each value represents the mean of 5–9 separate assays; bars are 95% confidence intervals. Significant differences ($p < 0.05$ – 0.01) occurred between any two strains that can be connected by a line that starts without an arrowhead and ends with an arrowhead. Abbreviations as in fig. 1.

P. mirabilis and *Bacteroides* sp. could not be quantified with accuracy, but were lower than the phagocytic index of *S. typhimurium*. When the phagocytic uptake of this strain of *P. mirabilis* (M13) was studied previously with mice purchased from a different animal supplier, 1 ml of peritoneal mononuclear cell suspension contained an average of $\log_{10}$ 4.8 *P. mirabilis* cells.³³

To verify that the washed peritoneal exudate cells did not contain adherent extracellular bacteria at the outset of the assay for intracellular survival, they were viewed by scanning electronmicroscopy. At least 200 leucocytes were observed for each bacterial strain

tested. No adherent extracellular bacteria were seen except for *P. mirabilis*, 2–10 cells of which were seen adhering to one in 20 leucocytes.

Intracellular survival of eight strains of intestinal bacteria in peritoneal mononuclear phagocytes

The intracellular survival of eight bacterial strains is presented as the percentage survival after 0, 30, 60 and 120 min (fig. 2) and as the intracellular killing constant (K_k) during the period of 120 min (fig. 3). According to the formula for K_k , a negative value would indicate an increase in the numbers of intracellular bacteria, a

Table IV. Relationship between oral infectivity of 10 bacterial strains and their phagocytic uptake and intracellular survival in mononuclear phagocytes

Strain	Oral infectivity*	Phagocytic uptake†	Intracellular survival‡
<i>S. typhimurium</i> 14028	Very high	Low	Intermediate
<i>L. monocytogenes</i> + H1a	High	High	High
<i>L. monocytogenes</i> - H1a	Intermediate	High	High
<i>L. monocytogenes</i> + H4b	Intermediate	High	Intermediate
<i>Ent. faecalis</i> M20	Intermediate	High	High
<i>E. coli</i> C25	Intermediate	Low	Low
<i>E. coli</i> M14	Intermediate	Low	Low
<i>P. mirabilis</i> M13	Intermediate	Low	Low
<i>B. fragilis</i> 9032	Low	Low	Intermediate
<i>Bacteroides</i> sp. M25	Low	Low	Not tested

*Relative infectivity based on information in table I.

†Relative phagocytic uptake based on information in fig. 1 and reference 33.

‡Relative intracellular survival based on information in fig. 3 and reference 33.

result not observed with any of the strains tested. As shown in fig. 2, all strains decreased in number during the 120 min of this assay. Surprisingly, the three strains best able to survive intracellularly were *L. monocytogenes* + H1a and - H1a and *Ent. faecalis*, with > 60% of the intracellular bacteria persisting for 120 min (fig. 2). This result was reflected in correspondingly low K_k values (fig. 3). Another surprising result was that the K_k of *S. typhimurium* (a relatively virulent organism) was not significantly different from that of the relatively avirulent *B. fragilis*. The general observation was that intracellular survival (fig. 3) was not consistently correlated with oral infectivity (table I).

Because we could not eliminate sufficient numbers of extracellular bacteria, the intracellular survival of *P. mirabilis* and a *Bacteroides* sp. could not be accurately quantified. In a previous study,³³ slightly more *P. mirabilis* were ingested than in the present study, permitting repeated centrifugation to remove sufficient numbers of extracellular *P. mirabilis* (i.e., resulting in 10-fold fewer extracellular *P. mirabilis* than intracellular bacteria). In this latter study, *E. coli* M14 was also tested, its intracellular survival being similar to that of *P. mirabilis* M13.

Discussion

The results showed (tables II and III) that pathogenic species, such as *S. typhimurium* and *L. monocytogenes*, could translocate after oral inoculation into mice; that facultative species, such as *E. coli*, translocated after antibiotic-induced intestinal overgrowth; and that strictly anaerobic species, such as *B. fragilis*, translocated only in an occasional mouse even after antibiotic-induced overgrowth. These studies of bacterial translocation were performed so that strains with a documented spectrum of oral infectivity could be tested for their ability to interact with mononuclear phagocytes. These results are summarised in table IV.

In studying the interactions between salmonella and macrophages, Wells and Hsu⁴² concluded that the

virulence of salmonella strains was inversely related to the phagocytic index. *S. typhimurium*, a virulent strain in our study (table I), was relatively resistant to phagocytosis (fig. 1). However, *P. mirabilis* and a *Bacteroides* sp. were even more resistant to phagocytosis than *S. typhimurium*, yet these two strains were clearly less virulent than *S. typhimurium*. Curiously, *L. monocytogenes* strains + H1a, - H1a and + H4b, and *Ent. faecalis* had similar phagocytic indices (fig. 1), yet these strains differed in oral infectivity (table I). Therefore, the degree of bacterial phagocytosis by mouse mononuclear phagocytes was not correlated with oral infectivity in mice (table IV).

Because salmonellae and listeria translocate from the intestine with relative ease,²⁻⁵ we postulated that their ability to survive in mononuclear phagocytes²¹⁻²³ might be associated with oral infectivity. None of the strains appeared to multiply in mouse mononuclear phagocytes, including the orally invasive *S. typhimurium* and *L. monocytogenes* + H1a (figs. 2 and 3). This is not an unusual observation. Czuprynski *et al.*⁴³ used proteose peptone-elicited mouse macrophages in a similar antibiotic-free assay and observed that the numbers of viable intracellular *L. monocytogenes* decreased by *c.* $\log_{10}$ 0.5 in 2 h. Buchmeier and Heffron^{24,44} reported that *S. typhimurium* 14028 (the strain used in the present study) survived moderately well in splenic and bone marrow-derived macrophages, but less well in peritoneal macrophages (resident and proteose peptone-elicited). Hsu³ concluded that the pathogenesis of salmonellosis was unlikely to be directly related to the ability of the organisms to survive in host macrophages. Thus, our observations that salmonella and listeria did not survive efficiently (or multiply) in mononuclear phagocytes were consistent with those of others.

We expected that pathogenic *Salmonella* and *Listeria* strains would survive better intracellularly than species of the normal enteric microflora. This expectation was sometimes but not always fulfilled. Buchmeier and Heffron⁴⁴ recently compared *S. typhimurium* 14028 with two *E. coli* strains and noted that it survived intracellularly and inhibited macrophage

phagosomal-lysosomal fusion to a greater extent than did the *E. coli* strains. Consistent with this observation, intracellular *S. typhimurium* 14028 survived significantly better than the two *E. coli* strains (fig. 3). However, several other observations were inconsistent. For example, intracellular survival of the relatively avirulent *B. fragilis* was similar to that of *S. typhimurium*, *L. monocytogenes* + H4b and the two *E. coli* strains (fig. 3). Also, there were no significant differences in intracellular survival between *L. monocytogenes* strains + H1a and -H1a and *Ent. faecalis*. As summarised in table IV, when all the bacterial strains in this study were considered as a group, survival within mononuclear phagocytes (fig. 3) was not consistently correlated with oral infectivity (table I).

Because listeriolysin has been considered a primary virulence factor for *L. monocytogenes*,^{5, 26-27, 45-46} some comment seems appropriate. Kuhn *et al.*⁴⁵ observed that, after oral inoculation, bacterial translocation to extra-intestinal tissues occurred with a haemolytic *L. monocytogenes* strain, but not with a non-haemolytic mutant. Using two of the *L. monocytogenes* strains of the present study, others have reported that -H1a was relatively avirulent compared to +H1a by either the oral⁴ or the intraperitoneal route.^{4, 26} After orally inoculating *L. monocytogenes* +H1a, -H1a, and +H4b into mice, we agree with Kathariou *et al.*⁴⁷ and Tabouret *et al.*,²⁹ who concluded that listeriolysin production was not directly proportional to virulence for mice. Kuhn *et al.*⁴⁵ noted that haemolysin production had no effect on uptake of listeria by mouse peritoneal macrophages. In the present study, differ-

ences in phagocytic uptake of *L. monocytogenes* +H1a, +H4b and -H1a were not significant (fig. 1). Kuhn *et al.*⁴⁸ also reported that loss of haemolysin (transposon-induced mutation) reduced intracellular survival. In the present study, intracellular survival was not consistently correlated with haemolysin production; *L. monocytogenes* +H1a and -H1a produced 512 and 32 haemolytic units, respectively, yet these strains had similar intracellular survival. Furthermore, the intracellular survival of both +H1a and -H1a was significantly better than that of +H4b, an intermediate haemolysin producer. However, listeriolysin is not the only virulence factor in *L. monocytogenes*,^{27, 45, 47-48} and in a recent review, Portnoy *et al.*⁴⁹ summarised information on a number of genes with products that probably participate in pathogenicity.

In conclusion, the results indicated that the ability of a bacterial strain to interact with mononuclear phagocytes was not consistently associated with oral infectivity (table IV). As a caveat, it should be mentioned that proteose peptone-elicited peritoneal mononuclear cells were tested in these experiments and that either unelicited mononuclear phagocytes or mononuclear phagocytes from other tissues (e.g., spleen, liver, bone marrow) might have behaved differently. However, this study suggests that oral infectivity is not generally correlated with bacterial uptake or intracellular survival in mononuclear phagocytes.

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