Interactions of *Escherichia coli* and *Proteus mirabilis* with mouse mononuclear phagocytes

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Summary. Five strains of enterobacteria (three of *Escherichia coli* and two of *Proteus mirabilis*) were studied to assess and compare their phagocytic uptake and intracellular killing by mouse macrophages. Each strain was injected intraperitoneally into separate groups of mice and peritoneal exudate cells were harvested after 3 min for phagocytosis to occur *in vivo*. Acridine orange staining showed that there were approximately 10-fold fewer intracellular *P. mirabilis* than *E. coli* cells. The average numbers of viable intracellular bacteria per leucocyte were 0·03 and 0·02 for *P. mirabilis* strains M13 and H1, respectively, and 0·48, 0·45, and 0·28 for *E. coli* strains M14, A-D M5 and H40. Thus, both *P. mirabilis* strains were ingested less readily than any of the three *E. coli* strains ($p < 0\cdot01$). The rates of in-vitro intracellular killing were similar for all five strains of bacteria. The intracellular killing constants (K_k) for the three mouse isolates were 0·017, 0·016 and 0·020 min for *E. coli* M14 and A-D M5, and *P. mirabilis* M13, respectively; the K_k s for the two human isolates were 0·026 and 0·029/min for *E. coli* H40 and *P. mirabilis* H1, respectively. The K_k s for all five strains were not significantly different. Assuming that the numbers of viable intracellular bacteria at the beginning of the assay represented 100% viability, 6–17% of the intracellular bacteria remained viable after 2 h, reflecting $\log_{10}$ 3·9–5·6 bacteria $(6-8) \times 10^6$ peritoneal exudate cells. Intravenous injection of these five strains into separate groups of mice demonstrated that the *P. mirabilis* strains were more virulent than the *E. coli* strains. Injection of each *P. mirabilis* strain was associated with ruffled fur and death, whereas mice given any of the three *E. coli* strains remained visibly healthy and none died. Consistent with these observations, quantitation of viable bacteria in the liver and spleen showed that greater numbers of *P. mirabilis* M13 than of *E. coli* M14 or A-D M5 persisted in these organs; similarly greater numbers of *P. mirabilis* H1 than of *E. coli* H40 persisted in the liver and spleen. Because the rates of intracellular killing of these five strains were similar, the relative virulence of both strains of *P. mirabilis* appeared to be associated with decreased phagocytic uptake rather than differences in intracellular survival.

Introduction

Ingestion and killing of bacterial pathogens by tissue macrophages is a primary mechanism of host defence. It is well known that some relatively pathogenic bacterial species can resist intracellular killing and even replicate within tissue macrophages. Classical examples of these facultative intracellular pathogens include *Listeria monocytogenes* and *Salmonella* sp.^{1,2} Although these species are not considered to be members of the normal intestinal flora, it is well documented that they can

use the intestinal tract as a portal of entry to invade otherwise sterile tissues such as the liver and spleen.³ There is direct electronmicroscopic evidence that *L. monocytogenes*⁴ and *Salmonella* spp.⁵ can be exported from the intestinal tract within tissue phagocytes in the intestinal mucosa. We have postulated recently that, like *Salmonella* spp. and *L. monocytogenes*, intestinal bacteria such as *Escherichia coli* and *Proteus* sp. may translocate across the intestinal tract within mononuclear phagocytes. We have proposed that phagocytes transporting intestinal bacteria fail to accomplish intracellular killing and then liberate viable bacteria in an extraintestinal site.^{3,6} For macrophages to play an

active role in the transport of intestinal bacteria, these bacteria must be capable of surviving within the phagocytes. The ability of mononuclear phagocytes to ingest and kill facultative pathogens such as *L. monocytogenes* and *Salmonella* spp. has been relatively well studied. However, there are comparatively few reports describing the uptake and killing of normal intestinal bacteria by mononuclear phagocytes.

The aim of the present study was to determine the ability of murine macrophages to ingest and kill five strains of normal enterobacteria—three *E. coli* strains and two *P. mirabilis* strains.

Materials and methods

Mice, bacterial strains, and preparation of bacterial inocula

Female, 20–24-g Swiss-Webster mice were used in all experiments. Five strains of bacteria in the family Enterobacteriaceae were examined; *E. coli* strains M14 and A-D M5 and *P. mirabilis* strain M13 were isolated from mouse caecal contents and *E. coli* H40 and *P. mirabilis* H1 were human clinical isolates. Each strain was identified by the API 20E system (Analytab Products, Plainview, NY). (*E. coli* A-D is a lactose-negative variant⁷). Each strain was grown in Brain Heart Infusion Broth (Difco) supplemented with glycerol 10% and frozen at -70°C until needed for experiments. Overnight Tryptic Soy Broth (Difco) cultures of individual species were washed twice in sterile saline and resuspended to $c. 10^8$ bacteria/ml (determined by densitometry) in Hanks's Balanced Salts Solution (HBSS; Gibco) with gelatin 0.1%. The numbers of viable bacteria were confirmed by quantitative plating on to MacConkey agar containing lactose 10%.

Electronmicroscopy of bacterial cells

Transmission electronmicroscopy of negatively stained cells was used to visualise the surface structures on the cells in the bacterial inocula described above. Bacterial cells were washed once, resuspended in normal saline, placed on carbon-coated Formvar grids for 30–90 s, negatively stained with 12 mM uranyl acetate containing bacitracin (Sigma) 50 $\mu\text{g}/\text{ml}$ and 12 mM oxalic acid (Sigma) at pH 6.6, and examined at 80 kV with a Jeol 100 CX transmission electronmicroscope.

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

Intracellular killing of bacteria was assessed by the method of Leijh *et al.*¹ with minor modifications. Briefly, mice were given intraperitoneal (i.p.) injections of 1 ml of Proteose Peptone (Difco) 10% in tap water; 48 h later,

separate groups of 10–12 mice were given i.p. injections of 1 ml of one of the five bacterial suspensions prepared as described above. The numbers of viable bacteria/ml in these inocula were (6×10^7) – (1×10^8) . After 3 min (a time corresponding to maximal phagocytic uptake¹), mice were killed by cervical dislocation and 3 ml of HBSS with heparin 1U/ml were injected i.p. The abdominal cavity was gently massaged for 1 min and the peritoneal exudate cells were harvested into ice cold HBSS with gelatin 0.1%, washed four times (4 min, 110 *g*) in the same medium, and resuspended to a concentration of $(6-8) \times 10^6$ cells/ml in 6 ml. After the last wash, the numbers of bacteria in the supernatants were 10-fold lower than the corresponding numbers of intracellular bacteria, indicating that the residual extracellular bacteria should not affect the subsequent quantitation of intracellular bacteria. As reported by other investigators,^{1,8} Wright-Giemsa stain of cells prepared by cytospin centrifugation demonstrated that approximately 80% of the peritoneal exudate cells appeared to be macrophages. Viability of the peritoneal exudate cells was consistently $\geq 95\%$ as determined by trypan blue dye exclusion. Washed peritoneal exudate cells were rotated at 4 rpm at 37°C for a maximum of 120 min. At various intervals, 0.5-ml samples were removed and mixed with 0.5 ml of ice cold HBSS, centrifuged for 4 min at 110 *g*, and 1 ml of bovine serum albumin (BSA) 0.01% in ice cold distilled water was added to the cell pellet. The cells were subjected to three freeze-thaw lysis cycles with liquid nitrogen and warm water (37°C) and phagocyte lysis was verified microscopically. The numbers of viable intracellular bacteria were determined after serial dilution in phosphate buffered saline with gelatin 0.1% (PBSG), followed by plating on to MacConkey 10% lactose agar.

Phagocytic uptake was quantified by a method similar to that described by Ofek and Sharon.⁹ At the beginning of the assay for intracellular killing (0 min), the bacteria-to-phagocyte ratio was determined by dividing the number of viable intracellular bacteria/ml by the total number of peritoneal exudate cells/ml.

Data were analysed by a one-way analysis of variance and then tested for least significant difference. The numbers of viable bacteria in the in-vitro intracellular killing assay were transformed to $\log_{10}$ values before statistical analysis. The rate constant (K_k) for in-vitro intracellular killing was calculated according to Leijh *et al.*¹ as: $K_k = [\ln N_{(t=0)} - \ln N_{(t)}]/t$. The data for in-vitro intracellular killing and in-vivo phagocytic uptake represent pooled data from 5–9 similar experiments performed on separate days. Each experiment included use of either all three mouse isolates (*E. coli* M14 and A-D M5, *P. mirabilis* M13) or both human isolates (*E. coli* H40, *P. mirabilis* H1).

At the beginning and end of the in-vitro intracellular killing assay (0 and 120 min), acridine orange stains of intact leucocytes (prior to lysis) were viewed and photographed by fluorescence microscopy to assess visually the viability of intracellular bacteria. With this methodology, viable bacteria fluoresce green and non-viable bacteria appear yellow-green to yellow to red;¹⁰

only brilliant apple green bacteria were considered to be viable, and bacteria with even slight shades of yellow were considered to be non-viable. To present the results of the acridine orange stain optimally, a representative colour transparency was subjected to computer image analysis. With a Dage-MIT series 68 Newvicon® camera, the colour transparency was displayed on a television monitor and the video image was digitalised with an image processing system (System S75 software; International Imaging Systems) and stored as an eight-bit, 512 × 512 pixel image in the frame buffer memory of a Masscomp MC 5500 minicomputer. This image was transferred to an Iris 2400 Turbo Work Station monitor and photographed from the screen on to Kodak Professional Copy Film. With this technique, fluorescent green (viable) bacteria have a dull appearance, whereas yellow-green to red (nonviable) bacteria are noticeably brighter.

In-vivo clearance of bacteria by the kidneys, spleen and liver

Separate groups of 20 mice were given intravenous (i.v., tail vein) injections of one of the five test strains suspended in 0.25 ml of sterile saline. Each inoculum was prepared as described above and the total number of viable bacteria suspended in 0.25 ml sterile saline was $(1-5) \times 10^8$. In each of two replicate experiments, four mice from each group were killed 15 min and 1, 4, 24 and 48 h after bacterial injection. The spleen, kidneys and liver were aseptically excised, in that order, for quantitation of viable bacteria. Kidneys (treated as one tissue) and spleen were separately homogenised in 2 ml of PBSG and the liver was homogenised in 3 ml of the same medium. Tissue homogenates were serially diluted in PBSG, planted on to MacConkey lactose agar and incubated at 35°C for 24–48 h. Bacteria were counted as

the total number of viable bacteria per tissue and these numbers were transformed to $\log_{10}$ values for statistical analysis by one-way analysis of variance followed by testing for least significant difference. The limits of detection of the assay were $\log_{10}$ 2.0 for the spleen and kidneys and $\log_{10}$ 2.2 for the liver; for the purpose of statistical analysis, values below the limit of detection of the assay were assigned a value of $\log_{10}$ 1.9 for the spleen and kidneys and $\log_{10}$ 2.1 for the liver.

Results

Electronmicroscopy of bacterial cells

The table shows the types of surface appendages observed on the bacterial cells in the inocula used in these experiments. At least 200 cells of each strain were examined. All five strains had peritrichous flagella. Fimbriae were also easily detected on the two *P. mirabilis* strains. Fimbriae were never observed on *E. coli* A-D M5, but a sparse distribution of fimbriae was seen on occasional (c. 0.5%) cells of *E. coli* M14 and H40.

In-vivo phagocytic uptake of bacteria

Three min after i.p. injection of *E. coli* strains M14, A-D M5 or H40, c. 25–50% of the harvested peritoneal exudate cells contained ingested bacteria, as viewed by acridine orange staining. These bacteria were unevenly distributed within individual phagocytes; c. 10% of the phagocytes contained many bacteria while the remainder contained few or no bacteria. At this time, virtually all intracellular

Table. Surface appendages, phagocytic indices and intracellular killing constants (K_k) of *E. coli* strain M14, AD M5 and H40 and *P. mirabilis* strains M13 and H1

Strain	Source	Flagella*	Fimbriae	Phagocytic index†	K_k (min)‡ mean (SEM)
<i>E. coli</i> M14	Mouse	+	sparse	0.48 (0.16)	0.017 (0.006)
<i>P. mirabilis</i> M13	Mouse	+	+	0.03 (0.01)§	0.020 (0.003)
<i>E. coli</i> A-D M5	Mouse	+	—	0.45 (0.11)	0.016 (0.003)
<i>E. coli</i> H40	Man	+	sparse	0.28 (0.06)	0.026 (0.009)
<i>P. mirabilis</i> H1	Man	+	+	0.02 (0.01)§	0.029 (0.003)

*Peritrichous flagella were noted on c. 50% of the cells of each strain.

†Number of viable intracellular bacteria/total number of peritoneal exudate cells after in-vivo phagocytosis for 3 min.

‡The rates of intracellular killing for the three mouse isolates were similar, $p > 0.50$. The rates of intracellular killing for the two human isolates were also similar, $p > 0.50$. No significant differences were noted when results with all five strains were compared simultaneously but individually ($p > 0.1$), but when the results obtained with the mouse isolates and the human isolates were pooled, the difference between the mouse and human isolates was significant ($p < 0.05$).

§Significantly reduced compared with each *E. coli* strain, $p < 0.01$.

bacteria appeared to be viable (apple green fluorescence). Nomarski differential interference optics was used to verify the intracellular position of the bacteria. Consecutive examination of cells with Nomarski and epifluorescence revealed that the bacteria were in optimal focus when the leucocytes were optically sectioned through the cytoplasm; focusing at the apical or basal surface of the leucocytes, the image of the bacteria was out of focus. Approximately 10-fold fewer peritoneal exudate cells, harvested from mice given *P. mirabilis* M13 or H1, contained bacteria and the number of intracellular bacteria per leucocyte appeared to be reduced. As noted with the three *E. coli* strains, virtually all intracellular *P. mirabilis* appeared to be viable at this time, as determined by acridine orange staining. The average number of viable intracellular bacteria per leucocyte was determined for each of the five bacterial strains by plate counts. Consistent with the visual observations from the acridine orange stains, significantly lower bacteria-to-leucocyte ratios were attained with the two *P. mirabilis* strains than with each of the three *E. coli* strains (table).

In-vitro intracellular killing of *E. coli* M14 and A-D M5 and *P. mirabilis* M13

The results of the intracellular killing of *E. coli* M14 and A-D M5 and *P. mirabilis* M13 are presented in two ways in an attempt to clearly illustrate our interpretations of these data (fig. 1). At each time point throughout the 2-h assay (including 0 min), there were fewer viable intracellular bacteria of *P. mirabilis* M13 than of *E. coli* M14 or A-D M5 ($p < 0.01$, fig. 1a), reflecting the lower numbers of intracellular *P. mirabilis* M13 present at the beginning of the assay. The three curves presented in fig. 1a appear to have similar slopes and the K_k s indicated that these three strains were killed intracellularly at similar rates (table). Fig. 1b shows the intracellular killing of *E. coli* M14 and A-D M5 and *P. mirabilis* M13 expressed as the percentage of viable intracellular bacteria. Here, there appeared also to be no differences in the rates of intracellular killing of these strains; 14–17% of the intracellular *E. coli* M14 or A-D M5 or *P. mirabilis* M13 cells were viable at the end of the 2-h assay, representing substantial numbers of intracellular bacteria (fig. 1a).

The presence of viable intracellular bacteria at the conclusion of the assay was confirmed microscopically by observations of cells stained with acridine orange. Fig. 2 is a typical acridine orange stain of peritoneal exudate cells after incubation *in*

Fig. 1. In vitro intracellular killing of *E. coli* M14 (▲) and A-D M5 (■) and *P. mirabilis* M13 (●) at various times after in-vivo phagocytosis for 3 min by mouse peritoneal exudate cells. (a) Numbers of viable bacteria recovered from $(6-8) \times 10^6$ peritoneal exudate cells. (b) Percentage of viable intracellular bacteria (number of bacteria recovered at time 0 = 100%). Results are expressed as means, bar = SEM.

vitro for 2 h with *E. coli* M14. It shows a peritoneal exudate cell with many viable intracellular bacteria. In most microscopic fields visualised, the majority of intracellular bacteria appeared dead at this time; however, microscopic fields similar to that shown in fig. 2 were not difficult to find. Similar observations were made with acridine-orange-stained preparations of peritoneal cells exposed for 2 h to *P. mirabilis* M13 and *E. coli* A-D M5.

In-vitro intracellular killing of *E. coli* H40 and *P. mirabilis* H1

Results of in-vitro intracellular killing of *E. coli* H40 and *P. mirabilis* H1 are shown in fig. 3. The kinetics of intracellular killing of these two human isolates paralleled those obtained with the three mouse isolates. At each time point throughout the 2-h assay there were fewer viable intracellular *P.*

Fig. 2. Computer image analysis of acridine-orange-stained mouse peritoneal exudate cells after in-vivo phagocytosis of *E. coli* M14 for 3 min, followed by 2 h of in-vitro incubation. Bacteria appear unevenly distributed within mononuclear phagocytes with *c.* 40 intracellular bacteria in one phagocyte (far right) and few or no bacteria in the remaining phagocytes. The non-viable bacteria appear swollen and brighter than the viable bacteria; the phagocyte on the far right contains nine non-viable bacteria (arrows) and at least 30 viable bacteria. Bar, 5 μ m.

mirabilis H1 than *E. coli* H40 cells ($p < 0.01$, fig. 3a), reflecting the lower number of intracellular *P. mirabilis* H1 recovered at the beginning of the assay. The K_k s of *E. coli* H40 and *P. mirabilis* H1 were also similar (table). Fig. 3b shows the percentage of viable intracellular *E. coli* H40 and *P. mirabilis* H1 at various times during the 2-h assay. Although only 5% of *E. coli* H40 and 17% of *P. mirabilis* H1 cells remained viable at the conclusion of the assay, these percentages reflected substantial numbers of bacteria (fig. 3a). Acridine orange staining of peritoneal exudate cells again demonstrated that viable intracellular bacteria were detected easily in percentages consistent with those presented in fig. 3b.

It should be noted (table) that the K_k s for the three mouse isolates (*E. coli* M14 and A-D M5, *P. mirabilis* M13) were similar to each other ($p > 0.5$) and that the K_k s for the two human isolates (*E. coli*

H40, *P. mirabilis* H1) were also similar to each other ($p > 0.5$). The three mouse isolates appeared to be killed at slower rates than the two human isolates (table). These differences were not statistically significant when an analysis of variance was used to compare all five strains simultaneously but individually ($p > 0.1$); however, when the results obtained with the three mouse isolates and the two human isolates were pooled into two separate groups, the difference between the mouse and human isolates was significant ($p < 0.05$).

In vivo clearance of E. coli M14 and A-D M5 and P. mirabilis M13 by liver, spleen and kidneys

The persistence of *E. coli* M14 and A-D M5 and *P. mirabilis* M13 after i.v. injection was monitored as the numbers of viable bacteria in the liver, spleen and kidneys (fig. 4) during a 48-h period. All livers, spleens and kidneys appeared grossly normal at each time throughout this assay and there were no

Fig. 3. In-vitro intracellular killing of *E. coli* H40 (▲) and *P. mirabilis* H1 (●) at various times after in-vivo phagocytosis for 3 min by mouse peritoneal exudate cells. (a) Numbers of viable bacteria recovered from $(6-8) \times 10^6$ peritoneal exudate cells. (b) Percentage of viable intracellular bacteria (number of bacteria recovered at time 0 = 100%). Results are expressed as means, bar = SEM.

Fig. 4. Persistence of *E. coli* M14 (▲) and A-D M5 (■) and *P. mirabilis* M13 (●) in (a) liver, (b) spleen and (c) kidneys of mice killed at various times after i.v. injection of bacteria. The data are expressed as the mean total numbers of bacteria per tissue, bar = SEM. The dotted line indicates the lower limit of detection of the assay.

signs of abnormal pathology. A general observation was that *E. coli* M14 and A-D M5 were eliminated more efficiently from the reticulo-endothelial system than was *P. mirabilis* M13 (fig. 4). Mice given *P. mirabilis* M13 by i.v. injection looked ill and had ruffled fur 24 h after injection, but then appeared to recover. In contrast, mice given *E. coli* M14 or A-D M5 remained visibly healthy throughout the experiment. In preliminary experiments, 10^8 cfu of

E. coli M14 or A-D M5 or *P. mirabilis* M13 were injected i.p. into separate groups of 20 mice; 2 (10%) of 20 mice given *P. mirabilis* M13 died at 24 h in each of two experiments (a total of 4 deaths in 40 mice). None of 40 mice given *E. coli* M14 or A-D M5 died.

After i.v. injection, the number of viable *P. mirabilis* M13 in the liver decreased approximately 10-fold from 15 min to 48 h after injection; during

the same period, the number of viable *E. coli* M14 and A-D M5 in the liver decreased 1000-fold and 10 000-fold, respectively (fig. 4a). The number of viable *P. mirabilis* M13 was significantly greater ($p < 0.01$) than the number of *E. coli* M14 or A-D M5 at 1, 4, 24 and 48 h after injection. Furthermore, *E. coli* M14 appeared to be eliminated from the liver less readily than *E. coli* A-D M5, and this was significant ($p < 0.01$) at 4 and 24 h after injection. At 48 h, the numbers of viable *E. coli* in the liver were below the limit of detection of the assay in 6 (75%) of 8 mice that received strain A-D M5 but in none that had received strain M14, indicating that *E. coli* A-D M5 was cleared more efficiently than *E. coli* M14 by this time, although the numbers of viable bacteria were not significantly different. The relative persistence of bacteria in the liver was *P. mirabilis* M13 more than *E. coli* M14 more than *E. coli* A-D M5.

Bacterial elimination from the spleen paralleled that from the liver (fig. 4b). At all times from 1 h after bacterial injection, the numbers of *P. mirabilis* M13 cells in the spleen were greater than the numbers of *E. coli* M14 or A-D M5 ($p < 0.01$ compared with *E. coli* M14 at 1, 4, 24 and 48 h and *E. coli* A-D M5 at 24 and 48 h; $p < 0.05$ compared with *E. coli* A-D M5 at 1 and 4 h). Greater numbers of *E. coli* M14 than of *E. coli* A-D M5 were recovered from the spleen, mimicking the persistence of these strains in the liver. The numbers of viable *E. coli* M14 cells in the spleen were greater than those of *E. coli* A-D M5 ($p < 0.01$) at 24 h. At 48 h, the numbers of viable *E. coli* M14 and A-D M5 cells in the spleen were below the limit of detection of the assay in 3 (38%) of 8 and 7 (88%) of 8 mice, respectively, again indicating that *E. coli* M14 persisted to a greater extent than *E. coli* A-D M5.

Bacterial persistence in the kidneys was also monitored (fig. 4c) with the same overall results found with the spleen and liver, i.e., *P. mirabilis* M13 persisted more than *E. coli* M14, and *E. coli* M14 more than *E. coli* A-D M5. However, by the end of the 48-h assay, each of the three strains appeared to be eliminated from the kidneys, with viable bacteria detected in the kidneys of only one of eight mice given *P. mirabilis* M13.

In-vivo clearance of E. coli H40 and P. mirabilis H1 by the liver, spleen and kidneys

All livers, spleens and kidneys appeared grossly normal throughout this assay and there were no signs of abnormal pathology. Again, a general observation was that the *P. mirabilis* strain persisted in the reticulo-endothelial system to a greater extent

than the *E. coli* strain (fig. 5). Furthermore, mice given *P. mirabilis* H1 looked ill and had ruffled fur. In the first experiment, 20 mice were given *P. mirabilis* H1, with the intention of killing groups of four at 15 min and 1, 4, 24 and 48 h after injection. However, four mice were dead at 24 h, leaving only two mice available for assay at 24 and 48 h. Therefore, in the replicate experiment, 24 mice were given *P. mirabilis* H1, and again 4 mice were dead 24 h after injection, but four mice were still available for assay at 24 and 48 h after injection. Thus, of a total of 44 mice given *P. mirabilis* H1, 8 were dead at 24 h, i.e., 18% mortality. In contrast, mice given *E. coli* H40 appeared healthy throughout the 48 h experiment and none died.

After i.v. injection, the numbers of viable *P. mirabilis* H1 in the liver decreased approximately 100-fold from 15 min to 48 h after injection; during the same period, the numbers of viable *E. coli* H40 in the liver decreased 100-fold (fig. 5a). However, the numbers of viable *P. mirabilis* H1 were significantly greater ($p < 0.01$) than the numbers of *E. coli* H40 at 1 and 4 h after injection. Although the numbers of viable *P. mirabilis* H1 in the liver remained greater than the numbers of viable *E. coli* H40 for the remainder of the 48-h assay, these differences were not statistically significant.

Differences in the numbers of viable *E. coli* H40 and *P. mirabilis* H1 were more noticeable in the spleen than in the liver (fig. 5b). The numbers of *E. coli* H40 in the spleen decreased more than 1000-fold from 15 min to 48 h after injection, while the numbers of *P. mirabilis* H1 decreased over 10 000-fold. At all time points, the numbers of *P. mirabilis* H1 in the spleen were greater than those of *E. coli* H40 ($p < 0.01$ at 15 min and 1, 4 and 24 h, and $p < 0.05$ at 48 h). In six of eight mice given *E. coli* H40 bacteria were not detected in the spleen at 48 h, a phenomenon that was not observed with any of the six mice given *P. mirabilis* H1.

Persistence of *E. coli* H40 and *P. mirabilis* H1 in the kidneys was also monitored (fig. 5c) with the same overall result as noted with the spleen and liver, i.e., *P. mirabilis* H1 was eliminated less readily than *E. coli* H40. The numbers of *P. mirabilis* H1 in the kidneys were greater than those of *E. coli* H40 at nearly all time points throughout the assay ($p \leq 0.01$ at 15 min and 1, 4 and 24 h). At the end of the 48-h assay, viable bacteria were detected in the kidneys of only two of eight mice given *E. coli* H40 and in four of six mice given *P. mirabilis* H1.

Discussion

This study has analysed the interactions of mouse

Fig. 5. Persistence of *E. coli* H40 (▲) and *P. mirabilis* H1 (●) in (a) liver, (b) spleen and (c) kidneys of mice killed at various times after i.v. injection of bacteria. The data are expressed as the mean total numbers of bacteria per tissue, bar = SEM. The dotted line indicates the lower limit of detection of the assay.

peritoneal exudate cells with five strains of enterobacteria, three of *E. coli* and two of *P. mirabilis*. Initial experiments were with three mouse isolates, *E. coli* M14 and A-D M5 and *P. mirabilis* M13; although all three strains were killed intracellularly at similar rates, *P. mirabilis* M13 was ingested less readily than either *E. coli* strain. In subsequent *in vivo* experiments, *P. mirabilis* M13 was cleared less readily by liver and splenic phagocytes than *E. coli* M14 or A-D M5. Thus, the extent of phagocytic

uptake and not the rate of intracellular killing was associated with the relative inability of tissue phagocytes to eliminate *P. mirabilis* M13 from the liver and spleen. To expand and confirm these observations, two human isolates (*E. coli* H40 and *P. mirabilis* H1) were tested subsequently with similar results.

To study phagocytic uptake and intracellular killing, peritoneal exudate cells were allowed to ingest bacteria *in vivo*. The peritoneal exudate cells

were then harvested and stained with acridine orange to assess phagocytic uptake visually. Nearly all intracellular bacteria appeared viable at this time, although the bacteria were unevenly distributed among phagocytes. The total numbers of peritoneal exudate cells were similar for each mouse and the individual inocula for each of the five bacterial strains contained similar numbers of bacteria, but there were always fewer intracellular *P. mirabilis* cells visible than *E. coli* cells immediately after the peritoneal exudate cells were harvested. It was possible to distinguish blindly leucocyte suspensions containing *P. mirabilis* from those containing *E. coli* because there appeared to be 10- to 100-fold fewer intracellular *P. mirabilis* than *E. coli*. After 3 min for phagocytosis *in vivo*, calculations of the average number (obtained from bacterial cultures) of viable bacteria per leucocyte were reproducible for each strain; this ratio was significantly lower for the two *P. mirabilis* strains compared with each of the three *E. coli* strains (table). We concluded, as did Ofek and Sharon,⁹ that this ratio represented a numerical index of the extent of phagocytosis. Thus, the bacteria-to-leucocyte ratios obtained immediately after phagocytic uptake could be interpreted to indicate that *E. coli* was ingested more readily than *P. mirabilis*, an interpretation that was consistent with microscopic visualisation with acridine orange staining.

The phagocytic index for the human *E. coli* H40 strain was somewhat less than those for the two mouse *E. coli* strains suggesting that the human *E. coli* was not phagocytosed as readily as either mouse strains. The human *E. coli* strain also had a higher K_k value, indicating that it was not killed as readily as either of the two mouse strains. These observations might be explained by the hypothesis that mice respond differently to indigenous (mouse) and non-indigenous (human) isolates.¹¹ However, these differences were not statistically significant.

We cannot explain the lower phagocytic uptake of the two *P. mirabilis* strains compared to the three *E. coli* strains. Because *E. coli* M14 and *P. mirabilis* M13 were isolated originally from the intestinal flora of Swiss-Webster mice, it is likely that the mice had antibodies to both species; furthermore, because both species share the common enterobacterial antigen, antibody to one species could be expected to cross-react with the other species. Therefore, it is unlikely that the presence or absence of antibody played a major role in the differences in *in-vivo* phagocytic uptake between *E. coli* and *P. mirabilis*. There is also evidence that certain types of fimbriae on *E. coli* and *P. mirabilis* enhance susceptibility to phagocytosis by polymorphonu-

clear leucocytes, whereas other types of fimbriae have no opsonic activity.¹²⁻¹⁵ Although fimbriae were not typed in this study, the presence of fimbriae could not explain enhanced phagocytosis, because their presence on *P. mirabilis* M13 and H1 was associated with decreased phagocytic activity.

The rate of decrease in the numbers of viable intracellular bacteria appeared to be similar for all five strains tested (figs. 1a and 3a) and the K_k s were also similar, although the two human isolates appeared to be killed somewhat faster than the three mouse isolates. For the three mouse strains, an average of 14-17% of the intracellular bacteria remained viable at the end of the 2-h assay, reflecting an average of $\log_{10}$ 5.3 *E. coli* M14, $\log_{10}$ 5.6 *E. coli* A-D M5, and at least 10-fold fewer (i.e., $\log_{10}$ 4.0) viable *P. mirabilis* M13 per $(6-8) \times 10^6$ peritoneal exudate cells. A similar pattern was noted for *E. coli* H40 and *P. mirabilis* H1. The relatively fewer numbers of viable intracellular *P. mirabilis* M13 and H1 recovered at the end of this assay reflected their relatively lower numbers at the beginning of the assay. The lower numbers of *P. mirabilis* M13 and H1 at the beginning of the assay of *in-vitro* intracellular killing appeared to be due to the relative lack of phagocytic uptake of both strains compared with the three *E. coli* strains. Although the majority of intracellular bacteria were killed during the assay, substantial numbers of viable intracellular bacteria were recovered at its conclusion. Acridine orange staining of peritoneal exudate cells confirmed that, although the majority of the intracellular bacteria appeared dead, viable intracellular bacteria were easily detectable at this time.

We attempted to compare our results of intracellular killing of *E. coli* and *P. mirabilis* with results reported by others. There is evidence that the rate of intracellular killing of *S. typhimurium* by mononuclear phagocytes can vary depending upon the strain of mouse used and upon the conditions of phagocytosis, i.e. *in vivo* or *in vitro*. Using methodology similar to ours, van Dissel *et al.*⁸ reported that the average K_k for *S. typhimurium* was 0.031/min with C57BL/10 mice and 0.055/min with CBA mice. Also, after phagocytosis *in vivo* or *in vitro*, the average K_k for *S. typhimurium* varied from 0.055/min to 0.020/min with peritoneal exudate cells from CBA mice. This variability may not be typical because macrophages from C57BL/10 mice and CBA mice killed *L. monocytogenes* and *Staphylococcus aureus* with equal efficiency; here, the average K_k s for *L. monocytogenes* and *Staph. aureus* with macrophages from C57BL/10 and CBA mice were 0.022/min and 0.023/min for *Staph. aureus*, and

0.033/min and 0.032/min for *L. monocytogenes*. Similar comparisons of the effects of various test conditions on intracellular killing are not available for *E. coli* and *P. mirabilis*. The most germane comparison of our data with other information in the literature might be obtained from van Dissel *et al.*⁸ who reported that, after in-vivo phagocytosis in the peritoneal cavity of Swiss mice (the strain used in the present study), the average K_k for *S. typhimurium* was 0.049/min. K_k s for five less pathogenic enterobacterial strains in our study were 0.016–0.029/min. Therefore, as expected, each of these five strains appeared to be killed at a similar rate as has been reported for *S. typhimurium* or *L. monocytogenes*, two species known to survive and even replicate within macrophages.^{1–3} Of interest, each of the five enterobacterial strains used in this study appeared to be killed at a rate similar to that reported for *Staph. aureus*,⁸ a species considered to have some ability for intracellular survival within macrophages.^{16–18} Some investigators similarly claim that bacteria in the genus *Escherichia* also have an ability for facultative intracellular parasitism.²

To determine if our results for phagocytosis and in-vitro intracellular killing could be correlated with in-vivo killing by reticulo-endothelial phagocytes, the five test strains were injected i.v. into separate groups of mice and viable bacteria were counted in the liver, spleen and kidneys at 15 min and 1, 4, 24 and 48 h after injection. This was done because the kinetics of persistence of viable bacteria in the liver and spleen can be used to provide information about the functional state of macrophages from the liver (Kupffer cells) and, to a lesser extent, from the spleen.^{1,19} As a caveat, it should be mentioned that, after i.v. injection of bacteria, the initial phase of infection is associated primarily with phagocytosis by resident tissue macrophages but bacteria escaping initial clearance may be phagocytosed by polymorphonuclear leucocytes attracted to a site of infection.^{20–22}

The results of these in-vivo assays indicated that a greater number of *P. mirabilis* M13 than of the two mouse *E. coli* isolates persisted in the reticulo-endothelial system; similarly, a greater number of *P. mirabilis* H1 than the human *E. coli* H40 isolate persisted in the reticulo-endothelial system. These findings were evident in the liver and spleen, but were also supported by the results with the kidneys. Therefore, it was not surprising that the two *P. mirabilis* strains were associated with mortality in mice, whereas there were no deaths with *E. coli* strains. The comparatively slow rates of in-vivo elimination of *P. mirabilis* were associated with the

observation that the *P. mirabilis* strains were phagocytosed less readily by peritoneal macrophages. Because the K_k s of all five enterobacterial strains were similar, it seemed reasonable to speculate that the relative virulence of the *P. mirabilis* strains compared with the *E. coli* strains might be due to an antiphagocytic property of *P. mirabilis* and not to an increased ability of *P. mirabilis* to survive within mononuclear phagocytes. However, it should be noted that phagocytic uptake was calculated following phagocytosis by peritoneal macrophages that had been elicited with proteose peptone, and intracellular killing was assessed following in-vitro incubation of peritoneal exudate cells. We cannot be certain that similar kinetics of phagocytic uptake and intracellular killing occurred *in vivo* with splenic, hepatic or renal phagocytes. However, the in-vitro results were consistent with the in-vivo findings.

There is substantial evidence that tissue mononuclear phagocytes play a key role in the transport of normal intestinal bacteria and inert particles.^{3,6,23} The present experiments attempted to characterise the interactions of five strains of normal enterobacteria with mononuclear phagocytes. These strains appeared to have differing abilities for phagocytic uptake but similar rates of intracellular killing, and a small but noticeable proportion appeared capable of intracellular survival for at least 2 h within mononuclear phagocytes. This small incidence of intracellular survival may be critical. There is a repeated observation that, if a bacterial strain colonises the intestinal tract at a high concentration of $\log_{10}$ 10–11/g of intestinal contents, only *c.* $\log_{10}$ 1–2 viable bacteria translocate to the draining mesenteric lymph nodes.^{24–28} The results of this study also suggested that two *P. mirabilis* strains were more virulent than each of three *E. coli* strains. This relative virulence of *P. mirabilis* appeared to depend more on the rate of phagocytic uptake by mononuclear phagocytes than on the rate of intracellular survival within these phagocytes. These results should provide the basis for further studies designed to clarify the virulence factors associated with the in-vivo infectivity of various strains of enterobacteria.

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