Killing of *Escherichia coli* in the peritoneal cavity of convalescent mice; role of specific and non-specific immune mechanisms

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Summary. Mice surviving a sublethal *E. coli* O18:K1 infection possess a greatly increased resistance to a subsequent lethal *E. coli* O18:K1 peritonitis. A similar increase in resistance can also be achieved by LPS pretreatment. The early course of the infection in the convalescent mice at day 1 was identical to that in LPS-pretreated mice. At this time, the convalescent mice were also able to restrict the growth of the heterologous *E. coli* O78(ColV) strain, suggesting that non-specific phagocyte activation was responsible for the increased destruction of the bacteria. At day 4, the kinetics of infection in convalescent mice were identical to those in mice passively immunised with specific anti-K1 capsule antiserum. A rapid decline in the numbers of viable homologous, but not heterologous, bacteria took place in the peritoneal cavity suggesting effective antibody-mediated opsonisation followed by phagocytosis and killing of the bacteria.

Introduction

An intraperitoneal injection of *E. coli* O18:K1 in mice caused a rapidly developing peritonitis and septicaemia, and death within 24–48 h (Vuopio-Varkila *et al.*, 1987a). However, if the challenge dose was small (c. 0.1 LD₅₀; 4×10^4 cfu), the mice survived and were able to eradicate the bacteria from the peritoneal cavity within 24 h. At this time the convalescent mice also showed an increased resistance to an otherwise lethal dose of *E. coli* (Vuopio-Varkila *et al.*, 1987b). A similar increase in resistance to infection could be achieved by subcutaneous injection of a small dose of heterologous rough-type lipopolysaccharide (LPS) (Vuopio-Varkila *et al.*, 1987b). In the LPS-pretreated mice, however, the destruction of bacteria started 3–4 h after challenge, i.e., earlier than in the convalescent mice. As resistance to the new challenge could be observed as early as 24 h after the primary infection or LPS treatment, we concluded that it was most probably due to a non-specific activation of phagocytic cells (Parant, 1983; Vuopio-Varkila *et al.*, 1987b).

The present study was undertaken to characterise the development of resistance in mice convalescing from *E. coli* O18:K1 infection and the roles of non-specific macrophage activation and the specific immune response.

Materials and methods

Bacterial strains

For the standard mouse challenge we used *E. coli* strain IH 3080 (serotype O18:K1:H7) (Vuopio-Varkila *et al.*, 1987a), isolated from a case of human neonatal meningitis. In some experiments, other challenge strains were used (table I). All strains were stored at -70°C in skimmed milk in small volumes until used. For injection, the bacteria were grown overnight in Luria broth (Miller, 1972) at 37°C on a rotatory shaker, the culture was diluted 10-fold with fresh medium and grown for a further 120 min in similar conditions.

Experimental infection

(CBA $\times$ C57BL/6) F1 hybrid mice (8–10 weeks old) bred at this Institute were used in all assays. The bacterial challenge (10-fold dilutions of exponential-phase bacteria in 0.2 ml of saline) was injected intraperitoneally (ip). The LD₅₀ was determined from the day 5 survival data for groups of 6 mice (Reed and Muench, 1938).

Tissue counts of bacteria

Samples were taken from the bacterial inoculum as well as from the peritoneal cavity for measuring the numbers of viable bacteria by plating out a series of 10-fold dilutions. Mice were killed by cervical dislocation, and the peritoneal fluid was harvested by injecting 2 ml of saline ip and withdrawing 0.5 ml after gentle massaging. The dilution factor was included in all calculations.

Table I. *E. coli* strains used in this study

Strain	Description	Isolated from	Used for	LD50 (cfu/mouse)
IH3080	O18:K1:H7	neonatal meningitis	ip challenge	4×10^5
IH23265	O18:K5	neonatal meningitis	ip challenge	3.6×10^6
IH3136	O1:K1	septicaemia following pyelonephritis of a 1 month old boy	ip challenge	2×10^6
94141/78	O78(CoIV)	faeces of a septicaemic patient in Hungary; received from Dr. H. Milch, National Institute of Hygiene, Budapest, Hungary.	ip challenge	$c. 3 \times 10^6$

Each point in the figures represents the number of bacteria (cfu)/total volume of peritoneal fluid and is the geometric mean of results from three mice.

Radiolabelling of the bacteria

The bacteria were grown in Luria broth (Miller, 1972) overnight at 37°C with aeration, centrifuged (3000 rpm, 10 min) and diluted 40-fold in Davis's minimal medium (Miller, 1972) containing glucose 0.1% w/v and Luria broth 0.001% v/v. They were grown in the same conditions to early logarithmic phase (20 Klett units, Klett-Summerson colorimeter, red filter), when 100 µCi of 35 S-methionine (*c.* 1200 Ci/mmol; Amersham Laboratories, Buckinghamshire) was added and growth continued to 50 Klett units. The bacteria were then pelleted (Eppendorf centrifuge, 2 min), washed twice and suspended in phosphate-buffered saline, pH 7.4 (PBS). The specific activity determined in a liquid scintillation counter was approximately 5×10^7 cpm/ 5×10^8 bacteria present in 1 ml of the suspension.

In-vivo phagocytosis of radiolabelled bacteria

Mice were inoculated ip with 1×10^6 live bacteria (1×10^5 cpm/mouse) in 0.2 ml of PBS. A sample (1 ml) of the peritoneal contents was then collected with PBS at different times, as described above. From a sample of 0.1 ml, the number of viable bacteria was determined by plating 10-fold dilutions on Luria agar. The total radioactivity recovered from the peritoneal cavity was determined from a further 0.1-ml sample. A third sample (0.8 ml) was centrifuged (550 rpm for 5 min) to pellet the peritoneal cells and bacteria associated with them but to leave the free bacteria in the supernate. The specific activity of the samples was determined with a liquid scintillation counter. Each point shown represents the mean of the results from three mice.

LPS pretreatment

E. coli K-12 LPS, the same preparation used previously (Vuopio-Varkila *et al.*, 1987b), was isolated by the phenol-chloroform-petroleum-ether method (Galanos *et al.*, 1969) and diluted in water, stored at -70°C in small

volumes and briefly sonicated before use. Mice were given subcutaneous (sc) injections of 5 µg of *E. coli* K-12 LPS in 0.1 ml, 24 h before bacterial challenge.

Passive immunisation

The K1 capsule-specific hyperimmune serum from a horse (H46) immunised with *Neisseria meningitidis* group B was a gift from Dr J. Robbins, NIH, Bethesda, MD, USA. Mice were given an ip injection of 0.2 µl of inactivated (56°C, 30 min) anti-K1 antiserum in 0.2 ml of saline 2 h before challenge.

Results

Kinetics of *E. coli* O18:K1 infection in convalescent mice

Mice that had recovered from a sublethal *E. coli* O18:K1 infection were resistant to subsequent challenge with large doses of *E. coli*, up to 50 LD50 (2×10^7 cfu) (table II). This increase in resistance was observed as early as 24 h after the sublethal challenge (Vuopio-Varkila *et al.*, 1987b) and persisted for at least 14 days. The resistance to infection in the convalescent mice was thus of longer duration than in LPS-pretreated mice, in which it did not

Table II. Mortality of convalescent mice from *E. coli* O18:K1 infection

Pretreatment	Challenge dose (day 0)	Number of dead mice/ total number challenged
Sublethal <i>E. coli</i> O18:K1 challenge on day -2	5 LD50	1/12
	50 LD50	3/9
day -4	10 LD50	0/6
day -7	10 LD50	0/6
day -14	10 LD50	0/6
no pretreatment	10 LD50	6/6

last for more than 4 days (Vuopio-Varkila *et al.*, 1987b).

To study whether this difference was due to different mechanisms of bacterial elimination, the course of the infection in these mice was followed. When the convalescent mice were challenged with a 5 LD₅₀ dose of homologous *E. coli* O18:K1 one day after the initial infection, bacterial growth in the peritoneal cavity was very similar to that in mice pretreated with LPS (5 µg of *E. coli* K-12 LPS given sc 24 h before challenge) (fig. 1). One hour

was seen in the numbers of bacteria recovered from the peritoneal cavities of the convalescent mice, showing that only c. 1% of the inoculum was viable (fig. 1). This rapid decline in live bacteria suggested effective opsonisation followed by killing of the bacteria. Indeed, the early course of infection in the day-4 convalescent mice was similar to that in mice passively immunised with a protective amount of opsonising anti-K1 capsule antibodies (Vuopio-Varkila *et al.*, 1987a) (fig. 1). A similar rapid elimination of the injected bacteria was seen in mice tested on day 14 or even day 52 of their convalescence (data not shown).

Fig. 1. Early kinetics of *E. coli* O18:K1 infection in the mouse peritoneal cavity. The challenge dose (5 LD₅₀) is indicated by an arrow. Each point represents the geometric mean of results from three mice; the vertical lines show the scatter between the results. Normal mice ●, day-1 convalescent mice □, day-4 convalescent mice ■, K-12 LPS-treated mice (5 µg of LPS sc 24 h before) ○, antiserum-treated mice (0.2 µl of anti-K1 antiserum 2 h before) △.

after injection, the numbers of bacteria recovered from the peritoneal cavities of these mice were similar to those in control mice, whereas at 4.5 h, both the day 1 convalescent and the LPS-pretreated mice had c. 100-fold fewer viable *E. coli* in their peritoneal cavities than did the controls.

If the time interval between the sublethal *E. coli* O18:K1 infection and the challenge was increased to 4 days, the course of infection was completely different. By 1 h after challenge, a dramatic drop

Killing of bacteria in the peritoneal cavity

To show that the reduction in bacterial numbers seen in the day-4 convalescent mice was due to intraperitoneal killing of the bacteria, ³⁵S-methionine-radiolabelled *E. coli* O18:K1 was used as the challenge (fig. 2). The viable bacteria were determined as colony forming units (cfu) and the total bacteria as radioactivity (total cpm) in peritoneal washings taken 30 and 60 min after challenge. The percentage of radiolabel found in the cell-free supernate of the peritoneal fluid (supernate cpm) was also measured. In a peritoneal sample drawn immediately after challenge, we could account for 98% of the radioactivity. This count was taken as the initial cpm (= 100%).

Most of the viable bacteria were eliminated from the peritoneal cavity by 30 min, both in the mice pretreated with the anti-K1 serum and in the day-4 convalescent mice (fig. 2A). The numbers of viable bacteria were reduced in the antiserum-treated and day-4 convalescent mice to 4 and 12%, respectively after 60 min, while the total radioactivity was reduced much less, to 46–69% (fig. 2B). Most of the label was found to be associated with the phagocytic cells (fig. 2C) suggesting that the bacteria were rapidly opsonised and taken up by phagocytic cells capable of killing them. The slow reduction of radioactivity in the peritoneal cavity (to c. 55% in 60 min) was most probably due to two factors, the escape of bacteria from the peritoneal cavity via lymphatic absorption (Dunn *et al.*, 1985) and their adherence to the walls of the peritoneal cavity.

In untreated as well as day-1 convalescent and LPS-treated mice the numbers of viable bacteria decreased only slowly, at the same rate as radioactivity, during the first hour of infection, and at 30 min most of the label was found in the supernate of the peritoneal fluid, i.e., the bacteria had not been engulfed by the phagocytes (fig. 2A and 2C).

From the above data we tentatively conclude

Fig. 2. The fate of ^{35}S -methionine-labelled *E. coli* O18:K1 in the peritoneal cavity after a 2.5 LD₅₀ challenge. Numbers of viable bacteria (A), total radioactivity (B) and radioactivity found in the supernate (C) were measured from the peritoneal washings drawn from three mice; the points shown are means of the values. A sample drawn immediately after inoculation was taken to represent 100% for calculations. Normal mice ●, day-1 convalescent mice □, day-4 convalescent mice ■, LPS-pretreated mice (5 μg of K-12 LPS sc 24 h before) ○, antiserum-treated mice (0.2 μl of anti-K1 antiserum 2 h before) ▲.

that the resistance to infection in the day-1 convalescent mice was due to macrophage activation, whereas in the day-4 convalescent mice, antibody-mediated opsonisation was prominent.

Specificity of infection resistance in convalescent mice

Because the two possible mechanisms of resistance, macrophage activation or antibody-mediated opsonisation, differ in specificity, an attempt was made to distinguish between them by challenging the convalescent mice with heterologous *E. coli* strains that shared either the O18 or the K1 antigen with the O18:K1 strain, or had neither of these antigens. Three mouse-virulent *E. coli* strains were used, all of which cause peritonitis and septicaemia in mice when injected ip at moderate (4×10^6 – 10^7 bacteria/mouse) doses.

Day-4 convalescent mice were resistant to the O18 and the K1, but not to the O78 bacteria. By contrast, the resistance in day-1 convalescent mice was non-specific, also encompassing the O78 strain. In the day-4 convalescent mice, both *E. coli* O18:K5 (fig. 3A) and *E. coli* O1:K1 (fig. 3B) were rapidly eliminated from the peritoneal cavity during the first hour of infection (a drop of 1.5–2.1 log₁₀ in cfu). Growth of the heterologous, antigenically unrelated *E. coli* O78 (fig. 3C) strain was uninhibited

for the first hour after challenge in both day-1 and day-4 convalescent mice, and was subsequently eliminated from the day 1 but not from the day-4 convalescent animals.

Discussion

Mice that had recovered from a sublethal *E. coli* infection were shown to be very resistant to a subsequent, otherwise lethal *E. coli* challenge. A similar increase in resistance could also be induced by LPS-pretreatment (Vuopio-Varkila *et al.*, 1987b). When the course of *E. coli* infection was studied in the convalescent mice at different times after sublethal infection, we found that the infection kinetics of day-1 convalescent mice were identical to those of LPS-pretreated mice. At this time the convalescent mice were also able to restrict the ip growth of an unrelated, heterologous *E. coli* strain. Both of these observations suggest that non-specific phagocyte activation was responsible for the enhanced bacterial destruction. At day 4 the convalescent mice were able to rapidly reduce the numbers of viable challenge bacteria in the peritoneal cavities. A similar, efficient elimination of challenge organisms was observed in mice treated with anti-capsular antibody, suggesting that effective antibody-mediated opsonisation, phagocytosis

Fig. 3. Kinetics of *E. coli* O18:K5 (A), O1:K1 (B) and O78(ColV) (C) infections in the peritoneal cavity of normal mice ●, day-1 convalescent mice □, and day-4 convalescent mice ■. The challenge dose is indicated by an arrow. Each point represents the geometric mean of results from three mice; the vertical lines show the scatter between the values.

and killing of the bacteria occurred in the peritoneal cavities of day-4 convalescent mice. Only antigenically-related bacteria, which shared a major cell-surface antigen (either O or K), were eliminated in these mice.

Since the studies of Rowley (1955), Landy (1956)

and Abernathy (1957) it has been evident that pretreatment with bacterial cell-wall components, in particular with LPS, can increase resistance to infection. The LPS-induced enhanced elimination of bacteria is due to non-specific systemic activation of phagocytes, especially macrophages (Galelli *et*

al., 1977) and activation is of short duration, 4–8 days after the last LPS injection (Rowley, 1964; Tegtmeier and Andersen, 1983; Vuopio-Varkila *et al.*, 1987b). In convalescent mice, however, the enhanced resistance lasted much longer. This suggested that either a sublethal *E. coli* infection was a stronger inducer of non-specific immunity or that additional defence mechanisms (e.g., antibody-mediated killing) were involved.

For studying the early kinetics of *E. coli* infection in these mice we chose two times—day 1 and day 4 after the sublethal infection. On day 1 the increased resistance was most probably due to non-specific cell activation, because the specific antibody response had hardly had time to develop, whereas the LPS-mediated increase in resistance developed in 24 h (Vuopio-Varkila *et al.*, 1987b). In day-4 convalescent mice the role of specific opsonising antibodies in promoting phagocytosis was evident. The kinetics of infection in the day-4 convalescent mice were identical to those in antibody-treated mice. The observation that only antigenically-related *E. coli* strains were eliminated from the day-4 convalescent mice also suggested the importance of specific antibodies at this time. Consistent with this, we also found that the day-4 convalescent mice were not resistant to lethal, systemic *Salmonella typhimurium* or *Klebsiella pneumoniae* infections (unpublished observation).

The peritoneal cavity of day-4 convalescent mice was shown to be the site of the enhanced killing of the bacteria. The phagocytosis and killing of the capsulate *E. coli* O18:K1 bacteria, that are highly

resistant to complement-mediated killing (Horwitz and Silverstein, 1980; Vaara *et al.*, 1984), were most probably due to effective in-situ opsonisation of the bacteria by antibodies directed to their cell-surface structures. At 30 min after bacterial injection, most of the radiolabel was found in the cell fraction of the peritoneal washings in both the day-4 convalescent and the antibody-treated mice. Our attempts to show with EIA the presence of anti-capsular or anti-O18 LPS antibodies in the peritoneal washing of convalescent mice on days 4 or 7 have, however, failed (unpublished observations), probably because of low concentrations of antibodies present.

The mechanism by which the day-1 convalescent and the LPS-pretreated mice killed the injected bacteria is still obscure. The bacteria remained for several hours in the peritoneal fluid without association with phagocytic cells (fig. 2C). The total number of peritoneal exudate cells in the peritoneal washings of both day-1 and day-4 convalescent mice before challenge was increased, and the cells showed signs of activation (greater size, more granules), but the same was not true of LPS-pretreated animals (Vuopio-Varkila, unpublished observations). The lag period between the challenge and killing of the bacteria suggests that recruitment of other effector cells might be required for efficient destruction of the bacteria in LPS-treated mice and we are currently trying to characterise these cells.

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