Experimental *Escherichia coli* peritonitis in immunosuppressed mice: the role of specific and non-specific immunity

J. VUOPIO-VARKILA

National Public Health Institute, Mannerheimintie 166, SF-00280 Helsinki, Finland

Summary. An experimental *Escherichia coli* septicaemia-peritonitis model was adapted to immunosuppressed mice. The mice were made neutropenic by a sublethal dose of cyclophosphamide, which resulted in a 100-fold increase in their susceptibility to intraperitoneal injection of *E. coli* O18:K1. A lethal infection could be prevented by passive immunisation with anti-K1 capsular or anti-O18 LPS antibodies but not with anti-J5 bacterial antibodies. The anti-K1 and anti-O18 antisera were able to increase the LD₅₀ of the *E. coli* challenge by factors of 50 and 5, respectively. The role of non-specific, lipopolysaccharide (LPS)-mediated resistance to infection was also investigated in this model, in which only long-living phagocytic cells such as macrophages are believed to be functional. Pretreatment of mice with LPS was shown to prevent growth of the bacterial challenge in the peritoneal cavity and blood and to result in a five-fold increase in the LD₅₀ of the challenge strain. These findings suggest an important role for macrophages as effector cells in defence against *E. coli* infection.

Introduction

Systemic infections with gram-negative bacilli are a major risk for hospitalised patients with serious underlying disease. Immunosuppressive chemotherapy further increases this risk (Weinstein *et al.*, 1983*a* and *b*). To study these infections, their treatment and prevention, a recently described systemic mouse *E. coli* O18:K1 infection model (Nowicki *et al.*, 1986; Vuopio-Varkila *et al.*, 1987*a*) has been modified. Susceptibility to *E. coli* was increased by inducing neutropenia with an injection of cyclophosphamide. This is an alkylating agent, also used in human cancer chemotherapy, that acts primarily on short-lived cell types such as polymorphonuclear leukocytes (Petursson and Chervenick, 1982) and also on B-cells and, to a smaller extent, on T-cells or macrophages (Turk and Parker, 1982; Hunninghake and Fauci, 1976). Similar immunosuppression models have been reported for infections with *Klebsiella* spp. and *Pseudomonas aeruginosa* in dogs (Harvath and Andersen, 1976), rats (Lumish and Norden, 1976) and mice (Buhles and Shifrine, 1977; Trautmann *et al.*, 1985).

Two different approaches to preventing this lethal *E. coli* infection are reported here, one by specific immunity induced by passive transfer of antisera, the other by induction of non-specific

resistance. Passive immunisation of normal, immunocompetent mice with anti-K1 or anti-O18 antiserum protects against lethal *E. coli* O18:K1 peritonitis (Vuopio-Varkila *et al.*, 1987*a*). It was, however, uncertain whether these antibodies could also protect neutropenic immunosuppressed mice.

Natural resistance to infection can also be increased by an injection of bacterial lipopolysaccharide (LPS) (Landy, 1956; Abernathy, 1957). Pretreatment of normal mice with *E. coli* K-12 LPS protects them from systemic infections caused by *E. coli* strains (Vuopio-Varkila *et al.*, 1987*b*). This non-specific increase in resistance is primarily due to macrophage activation and to augmentation of their bactericidal capacity (Galelli *et al.*, 1977). Whether or not such non-specific resistance to *E. coli* infection could also be induced in immunosuppressed mice was of great interest because of the high degree and wide spectrum of resistance achieved in normal mice.

Materials and methods

Bacterial strains and culture conditions

For mouse challenge, *E. coli* strain IH 3080 (serotype O18ac:K1:H7), isolated from human neonatal meningitis (Nowicki *et al.*, 1986), was used. It was stored at -70°C in skimmed milk until use. The log-phase inoculum for injection was prepared by growing the

bacteria overnight in Luria broth (Miller, 1972) at 37°C on a rotary shaker, then diluting the culture 1 in 10 and growing for a further 120 min in similar conditions.

Strain J5 (Elbein and Heath, 1965) is a rough, UDP-galactose epimerase-deficient mutant of *E. coli* O111 obtained from Dr E. Ziegler, University of California, San Diego, CA, USA. *E. coli* strain DM 3219-54 (serotype O18ac:K5:H7) was from Drs F. and I. Orskov, Statens Serum Institut, Copenhagen, Denmark, and strain W 3350 was *E. coli* K-12 from Dr W. Brammar, University of Leicester, UK.

Immunosuppression model

Mice were given an intraperitoneal (ip) injection of a sublethal dose (300 mg/kg mouse body weight) of cyclophosphamide (CY) (Syklofosfamid, Lääke-Farmos, Turku, Finland) in a volume of 0.3 ml, 48 h before infection. Blood leukocyte counts were determined in a haemocytometer, from blood samples drawn from the retro-orbital plexus of mice anaesthetised with ether at different times, to follow the immunosuppressive activity of CY treatment.

Experimental infection

(CBA × C57B1/6) F1 hybrid mice bred at this Institute (8–10 weeks old) were used in all assays. The bacterial challenge doses were 10-fold dilutions of exponentially-growing *E. coli* IH 3080 in 0.2 ml of saline injected ip into groups of six mice. The LD50 was determined by the method of Reed and Muench (1938) from the day 5 survival data.

Viable counts

Samples were taken from the bacterial inoculum as well as from the peritoneal cavity and blood at different times (three mice at each time) for measuring the numbers of viable bacteria by plating series of dilutions on solid medium. The blood samples (100 µl) were obtained from the retro-orbital plexuses of ether-anaesthetised mice. Thereafter the mice were killed by cervical dislocation and the peritoneal fluid harvested by injecting 2 ml of saline ip and withdrawing a 500 µl sample after gentle massage. The counts are given as cfu/ml of fluid.

Antisera

Rabbit hyperimmune sera were obtained after immunisation of New Zealand White rabbits—four injections subcutaneously (sc) over a period of 6 weeks—with 10⁸ heat-killed bacteria per injection (Saxén and Mäkelä, 1982) and without adjuvant. The rabbits were bled 10 days after the last injection. The K1 capsule-specific hyperimmune serum of a horse (H46) immunised with *Neisseria meningitidis* group-B bacteria was a gift from Dr J. Robbins, NIH, Bethesda, MD, USA.

Lipopolysaccharide preparation

E. coli K-12 LPS was extracted by the phenol-chloroform-petroleum ether method of Galanos *et al.* (1969), diluted in water and briefly sonicated before use.

Passive immunisation

Mice were passively immunised ip with 0.2 ml of different dilutions of heat-inactivated (56°C, 30 min) antisera, 2 h before bacterial challenge. Control animals were given saline.

Lipopolysaccharide pretreatment

Mice were given sc injections of *E. coli* K-12 LPS in a volume of 0.1 ml, 24 h before bacterial challenge.

Statistical method

The significance of differences between the survival rates of controls and antiserum-treated mice was determined by Fisher's exact probability test (Swinscow, 1978).

Results

The experimental infection in immunosuppressed mice

A single ip injection of CY resulted in a severe leukopenia in the peripheral blood of the mice (fig. 1). The total blood leukocyte counts fell in 48 h from 6.9×10^6 cells/ml in normal mice to 1.3×10^6 cells/ml in the CY-treated mice, and the number of polymorphonuclear cells fell from 3.4×10^6 cells/ml to $<0.5 \times 10^6$ cells/ml, a value corresponding to profound neutropenia in man. These low levels of leukocytes persisted from day 2 to day 7. The total leukocyte number harvested from the peritoneal cavity also decreased from 6.0×10^6 cells/ml to 2.7×10^6 cells/ml by day 2 (data not shown). The experimental infection was, therefore, started on day 2 after injection of CY.

The LD50 of the challenge strain *E. coli* O18:K1 in the CY-treated mice was 100-fold lower than in normal mice (4×10^3 versus 4×10^5) (fig. 2). Most of the deaths in the CY-treated mice occurred 24–72 h after challenge.

The kinetics of *E. coli* O18:K1 infection in the immunosuppressed mice resembled those in normal mice (Vuopio-Varkila *et al.*, 1987a) (figs. 3 and 4). However, 100-fold smaller inocula given to CY-treated mice resulted in rapid growth of the bacteria in the peritoneal cavity and in subsequent appearance of viable bacteria in the blood. Bacteria were

Fig. 1. The effect of CY treatment (300 mg/kg ip at time 0) on total peripheral blood leukocyte counts. The values at each time are means of at least five mice; the vertical lines show the ranges of values.

Fig. 3. The numbers of viable *E. coli* O18:K1 in the peritoneal cavity (—) and blood (---) of immunosuppressed mice after ip challenge. The challenge dose, indicated by an arrow, was 5 LD₅₀. Each point represents the geometric mean of results with three mice; the vertical lines show the ranges of values.

Fig. 2. The mortality dose-response curves in groups of six normal (—) or immunosuppressed (---) mice, 5 days after challenge. The data for normal mice are taken from Vuopio-Varkila *et al.* (1987a).

Fig. 4. The numbers of viable *E. coli* O18:K1 in the peritoneal cavity (—) and blood (---) in normal mice after ip challenge. The challenge dose, indicated by an arrow, was 5 LD₅₀. Each point represents the geometric mean of results with three mice; the vertical lines show the ranges of values.

found in the liver and spleen at the same time as bacteraemia was detected; the counts in these organs by 24 h were *c.* 100 times higher than in the blood (data not shown). With a dose of 2×10^4 bacteria (5 LD50) the generation time of the bacteria in the peritoneal cavity of the CY-treated mice during the early hours of infection was 45 min, similar to the generation time in normal mice. Low inocula (<1 LD50) were cleared from the mice within 24 h of injection, with a reduction in the numbers of viable bacteria in the peritoneal cavity already apparent by 10 h (data not shown). When normal mice were given a challenge dose of 2×10^4 bacteria (only 0.05 LD50 for these mice), the bacteria were cleared from the peritoneal cavity within 24–36 h (data not shown).

Passive immunisation

Graded doses of antisera were injected into the immunosuppressed mice before challenge. Significant protection was observed with anti-K1 capsular antiserum ($p < 0.001$) and, to a lesser extent, with anti-O18 LPS antiserum ($p < 0.02$). The anti-K1 serum ($\geq 0.2 \mu\text{l}/\text{mouse}$) raised the LD50 by a factor of 50 and the anti-O18 serum ($\geq 20 \mu\text{l}/\text{mouse}$) by a factor of 5. Three-fold smaller amounts of either serum did not give significant protection. Also, the kinetics of the infection in the serum-treated animals were similar in normal and immunosuppressed mice; the numbers of viable bacteria in the peritoneal cavities of mice that had received anti-K1 serum decreased, within 60 min of challenge with 5 LD50, to 1% of its initial value in normal mice and to 4% of the initial value in immunosuppressed mice (data not shown). This enhanced clearance was apparently enough to prevent the spread of the bacteria to the blood and other organs. The corresponding effect of the anti-O18 serum on bacterial numbers was somewhat less in both infection models (data not shown).

The anti-J5 serum did not protect the immunosuppressed mice from a lethal *E. coli* challenge (table I). This lack of effect corresponds to our previous studies with the *E. coli* infection model in normal mice in which antibodies to deeply-situated outer-membrane structures, such as the core glycolipid part of LPS, were not protective (Vuopio-Varkila *et al.*, 1987a).

Effect of LPS pretreatment

When the mice were given sc injections of graded doses of *E. coli* K-12 LPS 24 h after CY treatment and challenged 24 h thereafter with *E. coli* O18:K1,

Table I. Protective capacity of immune sera against *E. coli* peritonitis in immunosuppressed mice

Type of antiserum	Amount of serum (μl)	Number of mice dead/total challenged (and percentage) with dose	
		5 LD50	50 LD50
anti-K1-capsule	0.2	2/15 (13.3)	3/6 (50.0)
anti-O18-LPS	20	7/15 (46.6)	4/6 (66.7)
anti-J5-bacteria	66.7	9/12 (75.0)	NT
saline	200	11/12 (91.7)	6/6 (100)

an increase in resistance to infection was observed (table II). A dose of LPS $0.5 \mu\text{g}/\text{mouse}$ resulted in a five-fold increase in LD50. A similar, although more effective, increase of non-specific resistance to *E. coli* O18:K1 challenge has been reported previously in normal mice (Vuopio-Varkila *et al.*, 1987b). The bacterial growth in the peritoneal cavity decreased by 2.5 h after an inoculum of 5 LD50 (fig. 5a), and by 5 h the numbers were below the detection limit of 10^2 cfu/ml of fluid. This decline in the number of viable bacteria in the peritoneal cavity was accompanied by decreased blood (fig. 5b) and liver counts (data not shown).

Discussion

An experimental *E. coli* peritonitis-septicaemia model was adapted for use with neutropenic, immunosuppressed mice. Compared to the previously described systemic *E. coli* O18:K1 infection (Nowicki *et al.*, 1986; Vuopio-Varkila *et al.*, 1987a) 100-fold smaller inocula (4×10^3 instead of 4×10^5 bacteria/mouse) were required to induce lethal infection. The immunosuppressed state of the animals resembled that seen in hospitalised patients receiving cancer chemotherapy. In both normal and immunosuppressed mice, the infection progressed rapidly without the need for adjuvants to increase the virulence of the *E. coli*.

A sublethal dose (300 mg/kg) of CY was chosen to induce neutropenia in the mice. The leukopenia

Table II. The effect of LPS-treatment on survival of immunosuppressed mice subjected to a lethal (5 LD50) *E. coli* O18:K1 challenge

LPS dose (μg)	Number of dead mice/total challenged (and percentage)
5	3/12 (25)
0.5	11/24 (46)
0.05	8/12 (67)
none	15/15 (100)

Fig. 5. The effect of LPS treatment on the kinetics of *E. coli* O18:K1 infection in immunosuppressed mice. (A) peritoneal cavity, (B) blood; (---) mice treated with 5 µg of LPS, (—) controls without LPS treatment. The challenge dose (5 LD₅₀) is indicated by an arrow. Each point represents the geometric mean of results for three mice; the vertical lines show the ranges of values.

reached its nadir in the peripheral blood by days 2–3 and the leukocyte levels remained low until day 7, which was the final observation day in the experiments. In the study of Petursson and Chervenick (1982), a dose of CY 200 mg/kg ip markedly decreased the total blood leukocyte levels, including neutrophils, and the number of megakaryocytes and granulocyte progenitors in the bone marrow and spleen for the same period. A high dose of CY, as used in the present study, also suppressed B and T cell proliferation (Turk and Parker, 1982), but had less effect on macrophage function. It is assumed generally that the main line of defence against extracellular pathogens such as *E. coli* or *Klebsiella* spp. is phagocytosis by polymorphonuclear leukocytes (Tatsukawa *et al.*, 1979; Tsuru *et al.*, 1981).

When the immunosuppressed mice were challenged with *E. coli* O18:K1 they were markedly more susceptible than normal mice, as evidenced by a 100-fold decrease in LD₅₀. A similar decrease in LD₅₀ of *E. coli* O18:K1 is also seen after whole body irradiation (700 rad, 3 days before challenge) (unpublished data). Despite this increase in susceptibility, the immunosuppressed mice were still resistant to infection by a non-capsulated mutant of the *E. coli* O18:K1 strain even when doses of 1×10^7 or 1×10^8 bacteria were injected ip (unpublished data). This is consistent with the role of K1 capsule as a virulence factor of *E. coli* (Kim *et al.*, 1986). In

Klebsiella spp. (Trautmann *et al.*, 1985) and *P. aeruginosa* (Tegtmeier and Andersen, 1983) peritonitis models, decreases in resistance of 1000- and 10-fold, respectively, have been reported after a similar, single ip injection of CY.

In this infection model, a high inoculum (5 LD₅₀) resulted in rapid bacterial growth in the peritoneal cavity. Bacterial counts in the blood followed the bacterial concentrations in the peritoneal cavity. If the inocula were smaller, the mice were able to restrict the growth of the bacteria and to clear them by 24 h. In normal mice a similar restriction of bacterial growth seems to be a consequence of macrophage activation (Vuopio-Varkila *et al.*, 1987b). Thus, even in the immunosuppressed mice, some defence mechanisms, e.g., the complement-mediated killing of non-capsulate bacteria, capable of responding to the bacterial challenge, were functional despite an otherwise great reduction in resistance to infection.

Passive immunisation of the immunosuppressed mice with anti-capsular or anti-O18 antibodies resulted in increases in resistance to *E. coli* O18:K1 infection of 50- and 5-fold, respectively. Both immune sera were as protective in the immunosuppressed mice as in the normal mice, when estimated by the volume of antiserum (0.2 µl of anti-K1 or 20 µl of anti-O18 per mouse) needed for maximal protection (Vuopio-Varkila *et al.*, 1987a). In the study of Trautmann *et al.* (1985) with a *klebsiella*

peritonitis model in CY-treated mice, a large amount (200 μ l) of specific anti-klebsiella IgG antiserum produced a 100-fold increase in LD50 when administered shortly after bacterial challenge. In a pseudomonas infection model in granulocytopenic mice (Tegtmeier and Andersen, 1983) a serotype-specific anti-LPS antiserum only transiently delayed the otherwise lethal infection. However, Pierson *et al.* (1976) protected immunosuppressed mice against a 10 LD50 pseudomonas challenge by the transfer of a large amount (200 μ l) of pseudomonas immune serum.

The role of anti-K1 and anti-O18-LPS antibodies in protection against experimental *E. coli* O18:K1 infection in normal mice has been established by several studies (Bortolussi and Ferrieri, 1980; Cross *et al.*, 1983; Vuopio-Varkila *et al.*, 1987a), even by using monoclonal antibodies to these cell surface components (Pluschke and Achtman, 1985; Kaufman *et al.*, 1986). In the klebsiella and pseudomonas models the antibody-mediated immunity is based on serotype-specific O-antigen antibodies (Pierson *et al.*, 1976; Tegtmeier and Andersen, 1983; Trautmann *et al.*, 1985).

Anti-J5-antiserum directed against the coreglycolipid of LPS was no more protective against *E. coli* O18:K1 challenge in the immunosuppressed mice than it was in normal mice (Vuopio-Varkila *et al.*, 1987a). The reason for its lack of efficacy is probably the same in both cases—the shielding effect of the capsule and smooth-type LPS, which prevent the binding of antibodies to deeper epitopes (Van der Ley *et al.*, 1986; Vuopio-Varkila *et al.*, 1987a).

LPS is an efficient modifier of the immune system. Since the early work of Landy (1956) and Abernathy (1957), it has become clear that host

resistance to infection can be non-specifically increased by an injection of LPS. In a recent study with granulocytopenic mice, Tegtmeier and Andersen (1983) showed that active immunisation with whole *P. aeruginosa* increased non-serotype-specific resistance to a subsequent pseudomonas challenge which lasted for 8 days after immunisation. This early-phase resistance was probably due to LPS-induced macrophage activation that increased their bactericidal capacity, whereas specific opsonising antibodies were shown to be important later, 12 days after the end of the immunisation. In the present study, a definite resistance-increasing effect of a subcutaneous K-12 LPS injection was shown in the neutropenic mice. The non-specific resistance was good, causing a five-fold increase in the LD50 of *E. coli* O18:K1. In normal, immunocompetent mice the LPS-induced resistance was even more effective (Vuopio-Varkila *et al.*, 1987b). Because the CY treatment used to induce the immunosuppression has little effect on long-living cells, the increased resistance to *E. coli* O18:K1 was probably the result of macrophage activation. The LPS-treatment did not increase the number of neutrophils in the blood or in the peritoneal cavity (data not shown), and their possible role as the effector cell responsible for bacterial killing is unlikely.

This observed LPS-mediated increase in resistance is interesting in view of the possibility of using a similar non-specific stimulation to prevent serious infections in immunosuppressed patients. Several problems are, however, inherent in the clinical use of LPS, otherwise generally known as endotoxin.

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