Folia Microbiol. **48** (5), 665–669 (2003) **http://www.biomed.cas.cz/mbu/folia/**

Establishment of a Sepsis Model Following Implantation of *Klebsiella pneumoniae*-Infected Fibrin Clot into the Peritoneal Cavity of Mice

V. TOKY[a], S. SHARMA[a], B.B. ARORA[b], S. CHHIBBER[a]*

[a]*Department of Microbiology, Panjab University, Chandigarh, India*
[b]*Department of Pathology, Medical College, Maharshi Dayanand University, Rohtak, India*

Received 25 March 2003

ABSTRACT. Successful establishment of sepsis by entrapping a dose of 150 colony forming units of *Klebsiella pneumoniae* in a fibrin clot following implantation into the peritoneal cavity of mice is reported. The dose in the fibrin clot gave 50 % mortality in mice, spread over a period of one week. All the infected mice showed positive blood culture up to 6 d post-infection; histopathology revealed inflammatory changes in both liver and spleen. Introduction of *K. pneumoniae* into experimental mice without entrapment in fibrin clot caused no mortality and blood culture remained positive only up to 2 d; histopathology of liver and spleen throughout the period of study showed relatively mild inflammatory changes, which almost cleared during 14 d post-infection. The use of the fibrin-clot model may thus be considered to be useful in studying both the initial and the persisting stage of infection in the peritoneum, whence a slow release of bacteria into the blood takes place which finally leads to sepsis and septicemia.

Septic shock that results from a Gram-negative bacterial infection is a major cause of morbidity and mortality, especially in elderly and hospitalized patients, and current therapies remain largely supportive (Mayeus 1997). New ways of therapy require validation using well-controlled clinical trials to assess the acceptability of the drugs and devices before use in humans. Therefore it is necessary to have a suitable, reproducible and affordable animal model for initial experiments.

The available sepsis models are not representative of the clinical septic syndrome since pathophysiological responses they produce are not similar to those seen in septic syndrome in humans (Wichterman *et al*. 1980). Only few animal models are available which closely simulate the septic syndrome in humans. In this context the use of the fibrin clot model reported in large animals such as dogs and guinea pigs has been found to be superior to others as this model produces an evolving septic process from the point of view of infection that closely mimics the human septic syndrome (Natanson *et al*. 1989). It has been successfully employed in the same model in rats by DeMarsh *et al.* (1996). Recent reports highlighting the genetic similarity between mice and humans (Kondo *et al*. 2001; Mural *et al*. 2002) draw attention to the fact that these small, affordable animals can serve as useful experimental models.

Here we report the suitability of mice for a septic-shock model established with *Klebsiella pneumoniae* entrapped in a fibrin clot.

MATERIAL AND METHODS

Bacterial strains. Fifty blood isolates of *K. pneumoniae* were obtained from the *Department of Microbiology, Post Graduate Institute of Medical Education and Research* (PGIMER) (Chandigarh, India). Eight standard strains of *K. pneumoniae* were obtained from Dr. M. Trautmann (*Department of Medical Microbiology and Hygiene, University of Ulm*, Germany); *K. pneumoniae* ATCC 43816 was obtained from Dr. D.P. Speert *(Department of Pediatrics, University of British Columbia*, Vancouver, Canada). These were identified biochemically according to Ørskov (1984). Both blood isolates and standard strains were evaluated for their ability to cause death in the mouse fibrin–thrombin clot model. Two of the standard strains, ATCC 43816 and B5055, were found to cause in this model a significant decrease in the survival of mice; based on a preliminary study, an isolate of *K. pneumoniae* ATCC 43816 was selected for further experiments. The strain was maintained on nutrient agar at 4 °C.

*Corresponding author.

Mice. LACA female mice (body mass 20–25 g) were used. The mice were housed in standard plastic cages and fed laboratory chow and water *ad libitum*.

Animal model. To seed the fibrin clot, microorganisms were grown overnight in 20 mL nutrient broth at 37 °C. After centrifugation, they were twice washed in isotonic sterile saline and finally resuspended in normal saline. The concentration was estimated as absorbance at 600 nm. Inoculum size was assayed based on viable counts (determined on MacConkey's agar plates by scoring colony forming units, CFU).

Infected fibrin clots were prepared according to DeMarsh *et al.* (1996). Briefly, clots were made from commercial citrated bovine fibrinogen (*Sigma*, USA), which was dissolved in saline solution to a final concentration of 1 %. The fibrinogen solution was sterilized by passing through 0.22 μm *Millipore* filter. *K. pneumoniae* cells were added to the fibrinogen solution before coagulation. Simultaneously, 10 units of bovine thrombin (*Sigma*) were added and the resulting mixture was incubated at room temperature for 30 min before implantation. Operative anesthesia was obtained with diethyl ether and the abdomen prepped with 70 % ethanol. All procedures were carried out through 5 mm midline incision and closed with continuous nonabsorbable sutures. The fibrin clot containing the bacteria was inserted in the peritoneal cavity before suturing. Equal number of animals served as controls. The mortality rate was registered daily for a minimum of 2 weeks.

Bacterial dose. For the preliminary dose standardization, different doses of bacteria were entrapped in a fibrin clot and inoculated into different groups of mice (each group consisted of 8 mice); mortality was then followed. Finally, the dose which gave 50 % mortality in mice over a period of 7 d was selected for inducing sepsis; mice receiving a sterile clot served as control. Establishment of sepsis was also checked with bacteria not entrapped in the clot.

Culture studies. For peripheral blood culture studies, whole blood samples were collected from the tail vein of mice every day starting 1 d after implanting the bacterial clot. In organ culture studies, spleen and liver were removed aseptically from the mice on different days after infection and separately placed in sterile pre-weighed tubes. The organs were weighed and homogenized in 1 mL of sterile normal saline. For both blood culture and organ culture studies, serial dilutions were made in saline and the number of organisms was determined by plating on MacConkey's agar plates subsequently incubated for 1 d at 37 °C.

For *histopathological examination* a portion of liver and spleen was fixed in 10 % buffered aqueous formaldehyde, processed according to the standard method (Junqueira *et al*. 1989) and assessed for inflammatory response after hematoxylin–eosin staining; the inflammatory response was classed as mild, moderate and severe inflammation.

RESULTS

Bacterial dose standardization. The strain of *K. pneumoniae* ATCC 43816 when tested in a sepsis model was able to induce sepsis and gave a good inflammatory response. A dose of 150 CFU (established in preliminary dose standardization experiments; Fig. 1) if given in fibrin clot, gave 50 % mortality spread over a period of 7 d. High doses resulted in 100 % mortality; doses 2×10^3–6×10^6 CFU killed all animals within 2–3 d. Thus LD_{50} dose was selected for developing a sepsis model in which mortality would be spread over a 1-week period (considered as a sufficient time to produce actual disease process resembling

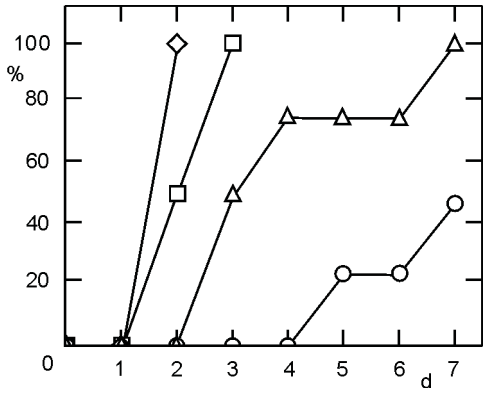

Fig. 1. Relationship of the dose response of *Klebsiella pneumoniae* ATCC 43816 with mortality (%) in LACA/female mice during 7-d experiments; doses (all in CFU): 6×10^6 – *diamonds*, 6000 – *squares*, 2000 – *triangles*, 150 – *circles*.

human sepsis). However, no mortality was observed with this dose interval when bacteria were not entrapped in a fibrin clot.

Blood culture was found to be positive in both experimental groups (entrapped in the fibrin clot or without the clot) of mice within 1 d. The blood counts in mice receiving bacteria in the fibrin clot increased and reached a peak of 4.04 log units before the death of the animals; in non-clot group, blood counts became negative 2 d after infection.

Organ culture of liver and spleen. Bacteria recovered from the liver and spleen of mice infected with fibrin–thrombin clot were in the range of 8.0–8.25 and 6.8–7.4 log units, respectively. As there was no mortality in mice receiving bacteria without fibrin clot the mice were sacrificed and the organ culture of liver and spleen was done after 2, 4 and 14 d after infection; after 2 weeks the organs were sterile (blood counts became negative).

Histopathological changes were observed in both liver and spleen of mice receiving bacteria in fibrin clot. Moderate inflammatory response was characterized by a marked congestion, organ surface covered with inflammatory exudates, focal collection of acute inflammatory cells, necrotic foci and mild microvesicular fatty changes; it was found in both liver and spleen when 150 CFU in fibrin clot were given (Fig. 2). Very mild inflammatory response could be seen in both liver and spleen when animals were challenged with a dose of 200 CFU not entrapped in a fibrin clot.

DISCUSSION

In earlier studies with a similar model in larger animals such as dogs, guinea pigs and rabbits, very high doses (10^8–10^9 CFU) were preferred for the establishment of sepsis (Fink *et al*. 1984; Alexander *et al*. 1989; Asheg *et al*. 2001). However, the use of very high doses in live organisms reflects more appropriately an acute intoxication rather than a true sepsis. In the case of a large bolus of bacteria (10^{11}–10^{12}) an enormous amount of endotoxin is released which has been strongly implicated for its capacity to initiate generalized inflammatory response (Bucklin and Morrison 1995). Therefore, an ideal situation of a more realistic animal model would be the use of a low dose of an appropriate pathogen so that its initial colonization progresses to an evolving sepsis. Melissen *et al.* (1994) could establish sepsis in mice with a low dose of *K. pneumoniae* (10^4 CFU) by immunomodulating the immune response with injections of cyclophosphamide. However, such a model is not acceptable because it alters the immune status of the host.

We were successful in establishing sepsis within 1 d with a low dose (150 CFU) of *K. pneumoniae* ATCC 43816 without using immunomodulators. DeMarsh *et al.* (1996) studied the hematoregulatory activity of SK&F 107647 peptide during conventional antibiotic therapy with the same rat model and found the blood culture to be positive within 18–25 h after infection establishment. It has also been reported that Gram-negative facultative bacteria translocate more easily than anaerobes and Gram-positive bacteria (Wells 1990). This may explain the early positive blood culture in both our study and the study by DeMarsh *et al*. (1996) (the organisms under study were *K. pneumoniae* and *E. coli*, respectively).

The success of a septicemia model requires that the infecting organism becomes localized in distant organs (cf. Waldon *et al*. 2002). Nabber *et al*. (1998) have shown the translocation of *Clostridium difficile* infection from a localized infection site to the intestinal mucosa and then to different organs. We suppose this mechanism could function in the transfer of bacteria from the peritoneal cavity to different organs as the cultures of liver and spleen were found to be positive. The highest counts were found in liver followed by spleen and blood. Direct comparison of earlier results with the present data is not possible as no quantitative measurements of bacterial load in different organs have been done.

The presence of bacteria in different organs is not sufficient to assess the septicemia and thus there is a need to evaluate the extent of tissue damage following translocation of bacteria. Nabber *et al*. (2000) found that during the development of sepsis continuous liberation of endotoxin induces the release of pro- and anti-inflammatory mediators; the suppression of the immune system enables the bacteria present in the organs to cause more pronounced tissue damage seen in late sepsis. Our observations were similar and confirm the earlier explanation.

The fibrin-clot model in mice, which can be used for infectious Gram-negative bacteria implantation in a very small dose entrapped in the peritoneal cavity, successfully simulates septic shock in its characteristic parameters. The use of mice has a special advantage in the light of the recent observation by Mural *et al.* (2002) that some data from mice can be extrapolated to humans.

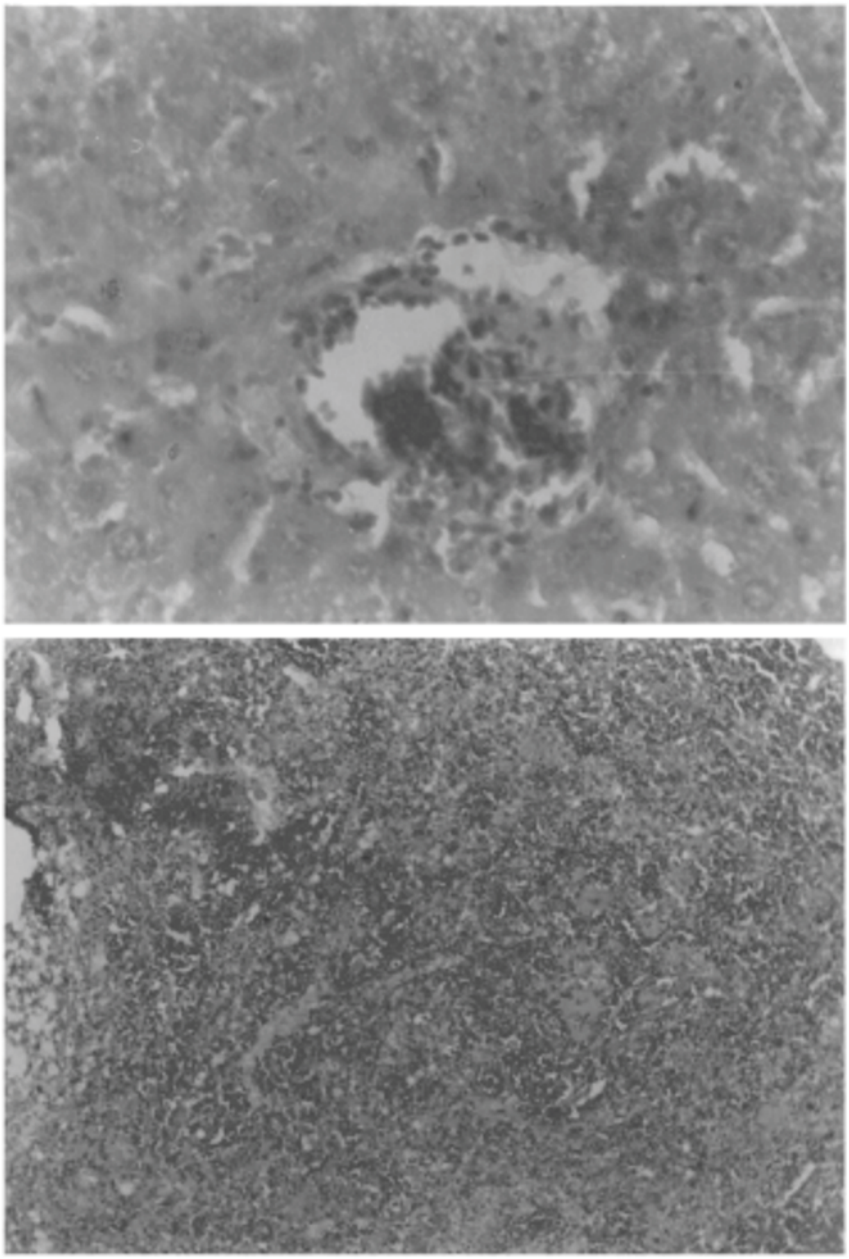

Fig. 2. Microphotograph of a moderate inflammatory response shown in transverse section of liver (*above*; ×400) and spleen (*below*; ×100); both hematoxylin–eosin stained.

REFERENCES

ALEXANDER J.W., GONCE S.J., MISKELL P.W., PECK M.D., SAW H.: A new model for studying nutrition in peritonitis: the adverse effect of overfeeding. *Ann.Surg*. **209**, 334–338 (1989).

ASHEG A.A., FEDOROVÁ V., PISTL J., LEVKUT M., REVAJOVÁ V., KOLODZIEYSKI L., ŠEVČÍKOVÁ Z., PILIPČINEC E.: Effect of low and high doses of *Salmonella enteritidis* PT4 on experimentally infected chicks. *Folia Microbiol.* **46**, 459–462 (2001).

BUCKLIN S.E., MORRISON D.C.: Differences in therapeutic efficacy among cell wall-active antibiotics in a mouse model of Gram-negative sepsis. *J.Infect.Dis*. **172**, 1519–1527 (1995).

DEMARSH P.L., WELLS G.I., THOMAS F., LEWAMDOWSKI T.F., FREY C.L., BHATNAGAR P.K., OSTOVIE E.J.R.: Treatment of experimental Gram-negative and Gram-positive bacterial sepsis with the hematoregulatory peptide SK&F 107647. *J.Infect.Dis.* **173**, 203–211 (1996).

FINK M.P., MACVITTIE T.J., CASEY L.C.: Inhibition of prostaglandin synthesis restores normal hemodynamics in canine hyperdynamic sepsis. *Ann.Surg*. **200**, 619–626 (1984).

KONDO S., SHINAGAWA A., SAITO T., KIYOSAWA H., YAMANAKA I., AIZAWA K., FUKUDA S., HARA A., ITOH A., KAWAI J., SHIBATA K., HAYASHIZAKI Y.: Computational analysis of full length mouse cDNAs compared with human genome sequences. *Mamm.Genome* **12**, 673–677 (2001).

MAYEUS P.R.: Pathobiology of lipopolysaccharide. *J.Toxicol.* **51**, 415–435 (1997).

MELISSEN P.M.B., VIANEN W.V., BAKKER-WOUDEN BERG I.A.J.M.: Treatment of *Klebsiella pneumoniae* septicemia in normal and leukopenic mice by liposome-encapsulated muramyl tripeptide phosphatidylethanolamide. *Antimicrob.Agents Chemother*. **38**, 147–150 (1994).

MURAL R.J., ADAMS M.D., MYERS E.W., SMITH H.O., MIKLOS G.L., WIDES R., HALPERN A., LI P.W., SUTTON G.G., NADEAU J., SALZBERG S.L., HOLT R.A., KODIRA C.D., LU F., CHEN L., DENG Z., EVANGELISTA C.G., GAN W., HEIMAN T.J., LI J., LI Z., MERKULOV G.V., MILSHINA N.V., NAIK A.K., QI R., SHUE B.C., WANG A., WANG J., WANG X., YAN X., YE J., YOOSEPH S., ZHAO Q., ZHENG L., ZHU S.C., BIDDICK K., BOLANOS R., DELCHER A.L., DEW I.M., FASULO D., FLANIGAN M.J., HUSON D.H., KRAVITZ S.A., MILLER J.R., MOBARRY C.M., REINERT K., REMINGTON K.A., ZHANG Q., ZHENG X.H., NUSSKEN D.R., LAI Z., LEI Y., ZHONG W.: A comparison of whole-genome shotgun-derived mouse chromosome 16 and human genome. *Science* **296**, 166–6171 (2002).

NABBER P., MIKELSAAR R.H., SALMINEN S., MIKELSAAR M.: Bacterial translocation, intestinal microflora and morphological changes of intestinal mucosa in experimental models of *Clostridium difficile* infection. *J.Med.Microbiol.* **47**, 591–598 (1998).

NAABER P., SMIDT I., TAMME K., LIIGANT A., TAPFER H., MIKELSAAR M., TALVIK R.: Translocation of indigenous microflora in an experimental model of sepsis. *J.Med.Microbiol*. **49**, 431–439 (2000).

NATANSON C., DANNER R.L., ELIN R.J.: Role of endotoxemia in cardiovascular dysfunction and mortality. *Escherichia coli* and *Staphylococcus aureus* challenges in a canine model of human septic shock. *J.Clin.Invest*. **83**, 243–251 (1989).

ØRSKOV I.: Genus *Klebsiella*, pp. 461–465 in N.R. Kreig, J.G. Holt (Eds): *Bergey's Manual of Systematic Bacteriology, Vol. 1*. Williams and Wilkins, Baltimore (USA) 1984.

WALDON E., SOBIŚ-GLINKOWSKA M., SZEWCZYK E.M.: Evaluation of selected features of *Staphylococcus cohnii* enabling colonization of humans. *Folia Microbiol.* **47**, 565–572 (2002).

WELLS C.L.: Relationship between intestinal microecology and the translocation of intestinal bacteria. *Antonie van Leeuwenhoek* **58**, 87–93 (1990).

WICHTERMAN K.A., BAUE A.E., CHAUDRY I.H.: Sepsis and septic shock – a review of laboratory models and a proposal. *J.Surg.Res*. **29**, 189–201 (1980).