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Pathogenesis of Respiratory *Klebsiella pneumoniae* Infection in Rats: Bacteriological and Histological Findings and Metabolic Alterations

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Gram-negative bacterial pneumonias have become increasingly important as nosocomial infections. The following model was developed to study the pathogenesis and evaluate therapy of such infections. Intranasal instillation of rats with a suspension of 5×10^6 *Klebsiella pneumoniae* caused bronchopneumonia with 24 h. Bacteria were isolated from the lungs in large numbers ($>10^5$ colony-forming units [CFU]) for at least 13 days after inoculation. Thereafter, the viable concentration decreased to about 10^3 CFU at 21 days but increased to 10^4 CFU at 28 days. Mortality rarely exceeded 25%. Plasma zinc concentration decreased, and plasma seromucoid, lysozyme, and α_2-macrofetoprotein increased during respiratory *K. pneumoniae* infection in rats. There seemed to be a linear relationship between seromucoid concentration and the concentration of *K. pneumoniae* in the lung expressed in $\log_{10}$ units. Plasma zinc, α_2-macrofetoprotein, or lysozyme levels, however, did not change until the concentration of bacteria retrieved from lungs exceeded 4 to 5 logs. Analysis of blood samples obtained serially from the orbital sinuses revealed that rats that succumbed to infection had significantly higher levels of seromucoid, α_2-macrofetoprotein, and lysozyme and lower levels of plasma zinc than infected rats that survived. Progressive increases in seromucoid and particularly in lysozyme and α_2-macrofetoprotein appeared to be predicative of death. It is postulated that the threshold effect observed for α_2-macrofetoprotein and lysozyme reflect significant damage to lung tissue, and thus these two variables are good indexes of the severity of this infection. We propose that this model may be of value in elucidating the pathogenesis of respiratory *K. pneumoniae* as well as in assessing various modes of therapy.

In recent years, the medical literature has contained numerous reports of pneumonias of gram-negative etiology, particularly of nosocomial origin (6, 13, 23, 27). *Klebsiella pneumoniae* has frequently been reported as the cause of these pneumonias (6, 28, 29). In view of the serious nature of these clinical illnesses, we have begun laboratory studies of the pathogenesis of respiratory infections caused by *K. pneumoniae*. *Klebsiella* pneumonia differs from many other pneumonias in that lung tissue is often destroyed (7). We have thus attempted to relate the concentration of bacteria in the lung to histological findings and systemic metabolic alterations. This approach was based upon previous studies that demonstrated that alterations in plasma zinc, seromucoid, and α_2-macrofetoprotein concentrations appeared to correlate with the severity of tissue damage (19), whereas serum lysozyme activity seemed to reflect the appearance, frequency, and severity of pyogranulomatous lesions (3) during tularemia in rats. The objective of this study was to ascertain whether alterations in plasma zinc, seromucoid, α_2-macrofetoprotein, and lysozyme would also serve as useful indexes of the severity of acute respiratory *K. pneumoniae* infection and thus be of potential prognostic value, as well as providing information relevant to the pathogenesis of this important respiratory disease.

MATERIALS AND METHODS

Test organism. The techniques for growing, storing, and enhancing the virulence of the A-D strain of type 1 *K. penumoniae* have been reported previously (1). Cultures for inoculation of rats were grown at 3°C for 18 h in Trypticase soy broth (TSB; BBL, Cockeysville, Md.) and then diluted 1:10 in fresh medium. The resulting suspension contained approximately 5×10 viable cells per ml.

Test animals. The animals used in most of the studies were male F-344 rats (F-344/MaifBR),

weighing from 150 to 250 g. They were obtained from commercial sources, housed in plastic cages, and fed commercial rat chow and water ad libitum. In a preliminary experiment several additional strains of rats were used: B.U.F. (BUF/MaifBR), Wistar-Lewis (LEW/MaifBR), Sprague-Dawley (Crl:COBS CD[SD]BR), and Long-Evans (Crl: COBS[LE]fBR).

Inoculation technique. Rats were lightly anesthetized with ether, and 0.1 ml of the organism suspension was delivered to the nares with a 100-μl Oxford sampler (Oxford Laboratories, Foster City, Calif.). The inoculum delivered contained approximately 5×10^6 cells.

Tissue sampling and preparation. Rats were anesthetized with halothane (Ayerst Laboratories, Ottawa, Ontario), and the abdominal and thoracic cavities were opened. Blood was obtained by cardiac puncture, and selected organs were then aseptically removed. Organs were weighed and homogenized in 2 ml of TSB in a Brinkmann polytron (Brinkmann Instruments, Westbury, N.Y.). The number of bacteria in the entire organ was estimated by the routine plating procedures given below.

Viable organism estimation. Serial decimal dilutions of various tissues were made in TSB, and 0.1 ml of selected dilutions was spread onto the surface of three Trypticase soy agar plates. After overnight incubation at 37°C, the number of colonies was determined and concentrations were calculated in terms of colony-forming units.

Histological procedures. Samples of lung, liver, spleen, and kidneys were taken for histological examination. Tissues were fixed in 10% buffered neutral formalin, cut at 6 μm, and stained with hematoxylin and eosin on Brown and Haup stains. The sections were then examined microscopically.

Deposition analysis. The pattern of distribution of microorganisms within the respiratory tract after intranasal challenge was determined with spores of the strain of *Bacillus subtilis* var. *niger* (*B. globigii*). *B. globigii* was used rather than *K. pneumoniae* because host defenses were considered less likely to affect the relatively inert spore than the metabolically active *Klebsiella*. An aqueous suspension of spores was incubated at 65°C for 20 min to kill vegetative forms and then diluted in gelatin-phosphate solution (0.2% gelatin in 0.03 M phosphate buffer, pH 7.0) to a final concentration of 3.5×10^5 spores per ml. The viable concentration of spores was determined by dilution in gelatin-phosphate medium and spreading 0.1 ml of selected dilutions on blood agar base. Colonies were counted after overnight incubation at 3°C.

Rats were lightly anesthetized with ether and inoculated intranasally with 0.1 ml of spore suspension. Five minutes later they were killed by cervical dislocation, and selected tissues were obtained, homogenized, and assayed for bacteria. The tissue specimens obtained were (i) as much of the upper-respiratory epithelium, from nares to larynx, as could be dissected out, including the mouth, oropharynx, tongue, and nasopharynx; (ii) the trachea, main bronchi, and as much of the secondary bronchi as extended externally from the pulmonary hilus; (iii) both lungs; and (iv) the esophagus and stomach.

Biochemical studies. In the first of the studies reported, rats were lightly anesthetized with halothane, the thoracic and abdominal cavities were opened, and the thoracic inferior vena cava was transected. Blood accumulating within the pleural cavity was collected and transferred to tubes containing 100 U of heparin. Plasma was collected after centrifuging at 1,000 $\times$ *g* for 20 min. In the second study, infected and sham-inoculated rats were bled from the orbital sinus at selected intervals over a 30-day period, and plasma was collected for analyses.

Plasma zinc concentration was determined by atomic absorption spectrophotometry (20). Lysozyme levels were measured by the method of Osserman and Lawlor (16). α_2-Macrofetoprotein was determined by the immunoprecipitin assay of Weimer and Benjamin (32). The seromucoid fraction of plasma was extracted and measured by the method of Neuhaus et al. (15).

RESULTS

On the basis of a preliminary experiment, the F-344 rat strain seemed to be more susceptible to infection than the other strains, and consequently was chosen for further studies. Using *B. globigii*, it was shown that although only 10% of the bacteria inoculated could be recovered, at least two-thirds of the recovered cells were found below the larynx and about 20% could be isolated from the lung. Thus, infection of the lower-respiratory tract can be established after intranasal instillation of a suspension of bacteria. Preliminary studies of bacterial distribution in rats showed that the mean concentration of *K. pneumoniae* in the lungs, expressed in $\log_{10}$ units, reached 6 logs within 24 h and persisted at this concentration for 2 to 5 days. Despite the uniformity of the mean concentraton over this period, there was a great range in the concentration found in the lungs of individual rats. Bacteria were also found in blood, spleen, and liver tissue, but not with the same high degree of frequency and in a considerably lower concentration (2 to 4 logs) than in lung tissue.

Histological analysis revealed that at day 1 after inoculation of *K. pneumoniae*, the rats had moderate to severe multifocal bronchopneumonia characterized by a cellular infiltrate composed almost exclusively of neutrophils and a few macrophages. These cells filled alveolar and bronchiolar spaces of affected areas (Fig. 1A). Alveolar spaces at the margin of a lesion were filled with eosinophilic proteinaceous edema fluid (Fig. 1B). Exudate in the center of a lesion had a vacuolated appearance associated with numerous gram-negative bacilli (Fig. 1C). The bacteria were most commonly seen in the vacuoles that were composed of mucinous material, probably from a capsular product of the bacteria.

Similar lesions were present in the lungs of

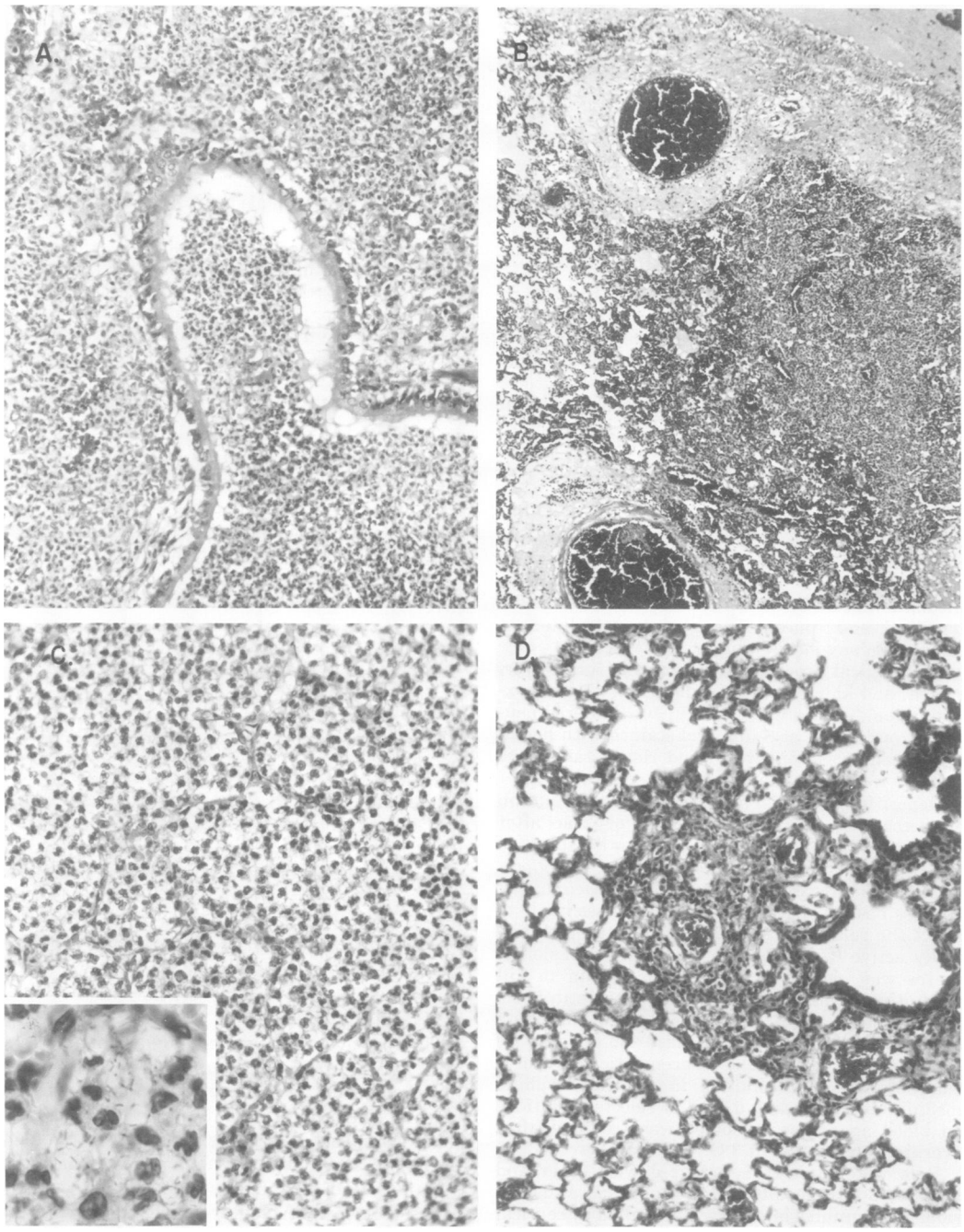

FIG. 1. *(A) Bronchiole with neutrophilic plug and adjacent alveoli containing similar infiltrate (day 1). ×136. (B) Section of lung showing perivascular edema and alveolar cellular infiltrate with the fluid-filled alveoli at the margin of the lesion (day 1). ×53. (C) Alveoli in the center of a large lesion. Alveolar spaces are distended with polymorphonuclear leukocytes and clear vacuolar spaces (day 1). ×326. Inset: A higher magnification of alveolar infiltrate showing polymorphonuclear leukocytes, a few macrophages, and numerous gram-negative bacterial rods (Brown & Haup stain) (day 1). ×814. (D) Day 6 postexposure showing early resolution of the pneumonia with an infiltrate composed primarily of macrophages within alveoli. ×136.*

rats 2 and 3 days postexposure. An acute splenitis was present in some animals at 1, 2, and 3 days (not shown). Gram-negative bacilli were observed in the splenic red pulp within pale vacuolar spaces in one rat at 3 days, indicating systemic infection. Microscopic changes were not observed in liver and kidney.

Rats examined on day 6 postexposure had a resolving pneumonia with macrophages predominating in the reactive areas (Fig. 1D). Complete resolution occurred by day 8, and rats sacrificed on days 8 and 10 appeared to be normal.

Biochemical studies based upon sequential killing were initially concentrated on the acute phase of the illness, 1 to 6 days postinoculation, but additional animals were killed at 13, 21, and 28 days to assess whether a relapse or chronic infection might occur in this model in a manner similar to that observed in *K. pneumoniae*-infected patients (30). Figure 2 summarizes the data on the concentration of bacteria found in the lungs, blood, and spleen of these animals during this period. The change in spleen weight observed during infection is also depicted. Figure 3 displays the alterations in plasma lysozyme, zinc, α_2-macrofetoprotein, and seromucoid concentrations in these animals. Plasma lysozyme activity was significantly elevated ($P < 0.005$) above control levels within 24 h after intranasal inoculation of *K. pneumoniae* and was double control levels by day 2. Lysozyme values in infected rats returned to control levels on day 6 but rebounded upward on days 13 and 21, returning toward control values on day 28. Plasma zinc concentration was significantly decreased on day 1 ($P < 0.005$), further depressed on days 2 and 3, and then slowly returned toward, but did not achieve, control values even as late as 28 days postexposure. α_2-Macrofetoprotein was detectable in 6 of 16 rats by day 1 and 11 of 16 rats on days 2 and 3 and returned toward control levels after 6 days. Low but detectable levels were found on days 13 and 21. Seromucoid concentration was twice that of control values on days 2 and 3 ($P < 0.005$), returned to control levels on day 6, and rebounded upward on days 13 and 21.

Because the mean values of several of the biochemical parameters seemed to vary with the bacterial concentration in the lung, the individual values obtained for rats on day 3 were compared with ranked lung titers (Fig. 4). Not until the concentration of *K. pneumoniae* isolated from lungs exceeded 4 to 5 logs did α_2-macrofetoprotein, zinc, or lysozyme levels change. In contrast, there seemed to be a linear

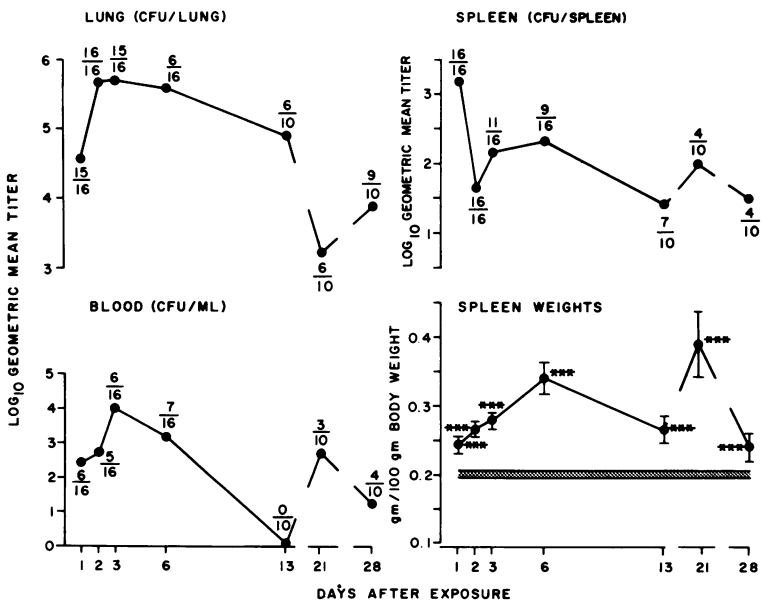

FIG. 2. *Concentration of viable K. pneumoniae in selected tissues over a 28-day period. Spleen weights of infected animals were significantly different from controls at every period ($P < 0.005$ [***]). Fractions represent the number of rats from which organisms were isolated over total number tested. CFU, Colony-forming units.*

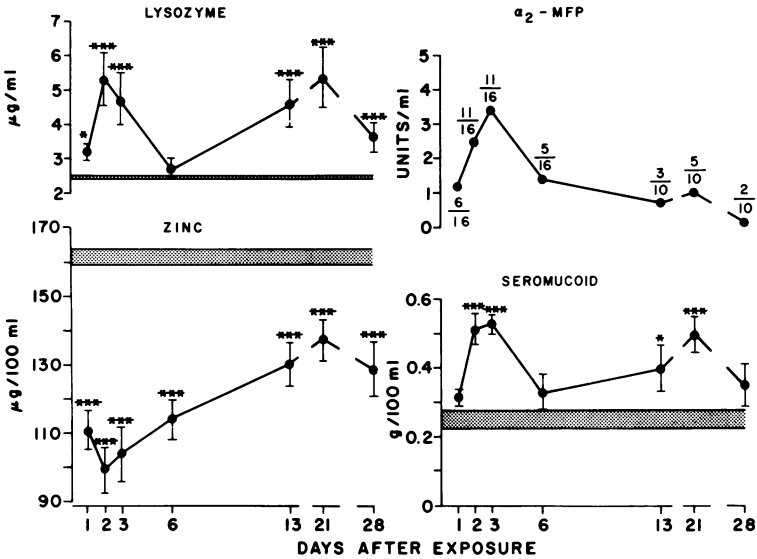

FIG. 3. *Metabolic sequelae of Klebsiella pneumoniae in acute and recrudescent phases of disease. Statistical analysis (Mann-Whitney test): (*) $P < 0.025$ and (***) $P < 0.005$. Shaded bar represents the mean + standard error of the mean of value for 32 control animals. Sixteen infected and 8 controls were sacrificed on days 1, 2, 3, and 6, and 10 controls were infected on days 13, 21, and 28. The fractions over the points in the α_2-macrofetoprotein (MFP) figure represent frequency of detection.*

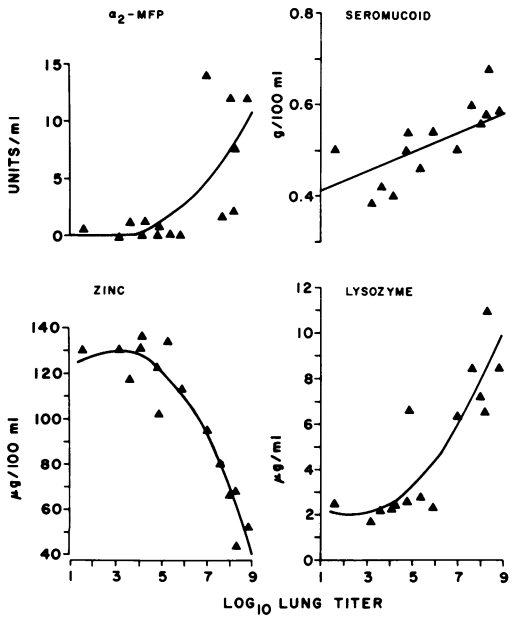

FIG. 4. *Relationship of viable organism concentration in lungs and induced metabolic sequelae 3 days after intranasal instillation of Klebsiella pneumoniae. (MFP) Macrofetoprotein. (Data fitted by method of least squares.)*

relationship between seromucoid concentration and the log of the lung concentration of microorganisms.

The results of the second set of studies wherein the same animals were serially bled from the orbital sinuses instead of being killed, are summarized in Fig. 5. The data from the infected rats were grouped according to whether the animals survived or died during the month-long study. Only the mean values are shown. Not included in this figure are the leukocyte count and hematocrit values. Rats that ultimately died from the infection had marked leukopenia, whereas the leukocyte counts of surviving infected rats did not differ significantly from those of controls. A difference in bacteremia also was observed: seven of the eight rats that died had persistent bacteremia, in contrast to the transient bacteremia found in only three of the eight survivors. The effect of frequent sampling, manifested by depressed hematocrit values, was similar in all groups.

Repeated blood sampling provoked a decrease in plasma zinc concentration in all groups; however, infected rats that died from infection had significantly lower zinc values ($P < 0.005$) than either control or infected survivors on days 2 and 3.

The plasma seromucoid concentration of the controls did not display a significant change, indicating that repeated orbital bleeding had no effect on this parameter. Infected survivors had significantly higher levels of seromucoid than controls ($P < 0.005$). Infected rats that died had even higher plasma seromucoid concentrations than infected survivors.

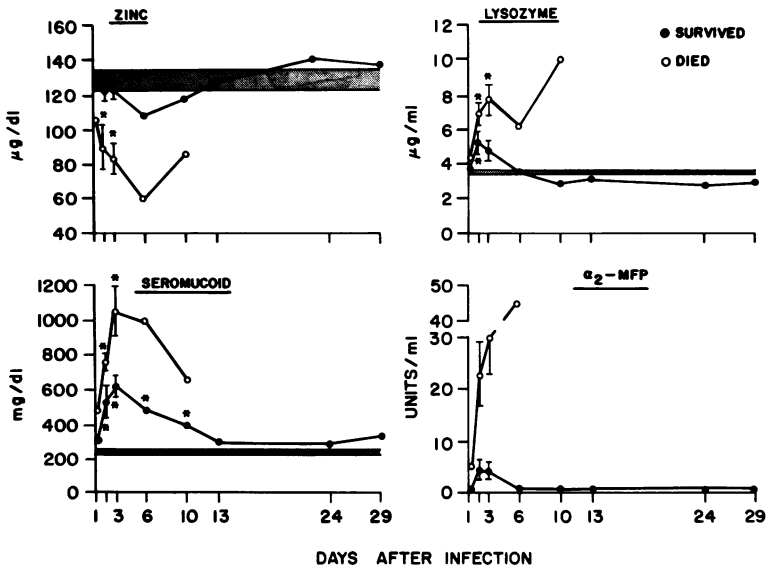

FIG. 5. *Alterations in selected biochemical constituents of plasma after intranasal instillation of Klebsiella pneumoniae. Shaded bar represents the mean ± standard error of the mean of value for all of the control animals. Controls for α_2-macrofetoprotein (MFP) showed no response and are omitted.*

No α_2-macrofetoprotein was detectable in the plasma of control rats over the first 10 days, indicating that repeated orbital bleeding was an insufficient stress or trauma to trigger this host response. Infected survivors showed transient detectable amounts of this fetal globulin in their plasma. Infected rats that died showed marked, generally progressive amounts of α_2-macrofetoprotein until they died.

Plasma lysozyme concentration also did not change as a consequence of repeated blood sampling. Transient increases in lysozyme were detectable in the plasma from some of the infected surviving rats. Infected nonsurvivors displayed higher levels of lysozyme than the survivors and these elevated concentrations generally persisted until death, often peaking just before death.

DISCUSSION

The majority of laboratory studies of infection with *K. pneumoniae* have been carried out in mice. Such a model is convenient for testing the efficacy of drugs but is inconvenient for studies of pathogenesis and metabolism. The present model, though exhibiting markedly lower mortality than mice (1), permitted us to carry out determinations of tissue bacterial content, histological analysis, and selected metabolic parameters of plasma on the same animal, thereby allowing us to assess whether the extent of metabolic alterations correlated with the severity of illness. The use of rats also allowed us to take serial samples from animals so that we could test whether the correlations between specific metabolic parameters and degree of illness would in fact be of prognostic value.

The search for relevant prognostic indexes for bacterial infection continues to be of value because the use of antibiotics may at times serve merely to conceal rather than to cure an infectious illness. Our choice of metabolic indexes was based on evidence of prior usefulness in this regard and/or the likelihood that knowledge of alterations in these parameters would yield information as to the pathogenesis of respiratory *K. pneumoniae* and the nature and extent of the host-parasite interactions.

We measured plasma lysozyme activity as an index of phagocytosis. Lysozyme, most likely, is released by neutrophils and monocytes (5, 12), either during phagocytosis or as a consequence of the degradation of senescent phagocytes (1). Recent studies indicate that increased serum lysozyme activity correlates with the appearance, frequency, and severity of pyogranulomatous lesions during tularemia in rats (3) and appears to be a useful prognostic indicator in experimental *Bacteroides* peritonitis (2). In our study, mean plasma lysozyme values were elevated early in the course of the illness, returned to control values and again increased during what appeared to be a milder recrudescence of the disease, days 13 to 28. Analysis of the data from day 3 indicated that lysozyme concentration exhibited a threshold in regard to concentration of bacteria in the lungs. In the serial

sampling study, most of the infected rats that died were found to have progressively increasing lysozyme levels which were significantly higher than those found in infected survivors.

Plasma zinc concentrations have been shown to decrease in bacterial (19, 31) and viral (18) illnesses. The sensitivity of this variable to trauma, as indicated in our serial bleeding study, would seem to preclude it from being a useful prognostic indicator unless used in conjunction with other indicators. However, Lindeman et al. (9) described a patient with acute suppurative *Klebsiella* pneumonia with extremely low plasma zinc levels that increased with clinical improvement.

Seromucoid is the designation for a class of so-called acute-phase globulins, which are rich in carbohydrate. The precise composition of this class of acidic glycoproteins varies from species to species. In humans this designation encompasses α_1-acid glycoprotein (orosomucoid), tryptophan-poor α_1-glycoprotein, haptoglobin, zinc α_2-glycoprotein, and perhaps α_1-antitrypsin (25). Many of these acute-phase globulins have been shown to be markedly elevated during trauma, inflammation, and infection (2, 8, 19, 33, 35). Measurement of seromucoid thus provides an approximation of alterations in acute-phase globulin concentrations. In our studies increased seromucoid concentration appeared to parallel the concentration of bacteria in the lung, and higher levels of seromucoid were detectable in infected animals that eventually died than in infected animals that survived. Measurement of seromucoid or perhaps of a specific protein from that class, such as haptoglobin or orosomucoid, may thus have merit as a prognostic indicator.

α_2-Macrofetoprotein is a macroglobulin that appears in the plasma of adult rats during inflammation and infection (19, 32). The appearance of α_2-macrofetoprotein in plasma during inflammation is due to de novo synthesis and secretion by the liver (24). α_2-Macrofetoprotein well fulfills the requirements for use as an indicator, albeit nonspecific, of the inflammatory state in the rat, in that it is normally absent, has a broad response range, increases rapidly, and disappears quickly. Repeated orbital sinus bleeding elicited little or no detectable α_2-macrofetoprotein response. Also, α_2-macrofetoprotein did not appear until the lung content of bacteria reached 4 to 5 logs. Both of these findings indicate that a threshold in regard to the extent of host-parasite interaction or degree of tissue damage must be reached before the liver synthesizes α_2-macrofetoprotein. Infected nonsurvivors usually had progressively increasing quantities of α_2-macrofetoprotein in their plasma which were significantly higher than those found in the plasma of infected survivors. In fact, the last sample taken from the nonsurvivors before death often had three to five times as much α_2-macrofetoprotein as the survivors.

That alterations in plasma zinc, lysozyme, and α_2-macrofetoprotein appeared to exhibit thresholds suggested that the disease process itself exhibited a threshold above which death was likely to ensue. This threshold seems to be related to the mass of microorganisms since the increased concentration of lysozyme and α_2-macrofetoprotein in the plasma correlated positively with the number of organisms detectable in the lung, and persistent increases in these two variables were predicative of death. A similar relationship between microorganism dose and the onset of metabolic sequelae and death or both has been observed in experimental gas gangrene in goats (10, 11) and in tularemia in the rat (3, 19, 34).

Although small numbers of bacteria were found in the spleen and liver, the organ primarily affected during *Klebsiella* pneumonia was the lung. Our initial observations indicated that decreases in plasma zinc and increases in seromucoid, lysozyme, and α_2-macrofetoprotein correlated with the concentration of bacteria in the lung. Our later study provided evidence that the extent of change in these variables was related to the severity of disease and in fact could be used to predict whether infected rats would succumb to the illness. *K. pneumoniae* are not readily phagocytized due to the presence of a polysaccharide capsule (14). *Klebsiella* pneumonia is associated with considerable amounts of lung tissue damage (7). During tularemia in the rat, a disease caused by an organism that is also resistant to phagocytosis (21), alterations in serum enzymes and trace metal and protein metabolisms appeared to correlate with the severity of liver damage (3, 19). It is thus tempting to speculate that the threshold effect we observe, particularly for α_2-macrofetoprotein and lysozyme, may reflect the onset of tissue damage, rather than merely the presence of bacteria.

Taken together, the data in this paper yield some further insight into the host-parasite interactions during acute *Klebsiella* pneumonia in rats and indicate that selected metabolic sequelae may have value as prognostic indicators.

Subsequent studies will be directed at evaluating selected metabolic sequelae as valid indexes of the efficacy of antibiotic therapy during acute *Klebsiella* pneumonia. The reasons for the recrudescence of the infection at 3 to 4 weeks remains to be determined. It may be that

a pulmonary abscess or pleuritis harbored the bacteria; both have been reported in patients (4, 22). It would thus appear that rat models may also be of value in studying chronic respiratory infection.

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