Efficacy of Antimicrobial Therapy in Experimental Rat Pneumonia: Effects of Impaired Phagocytosis

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The importance of intact host defense mechanisms for successful antimicrobial therapy was investigated in an animal model. Recovery from lobar pneumococcal pneumonia as a result of penicillin therapy was studied in normal rats and in rats treated with cobra venom factor. This factor was used to selectively suppress the phagocytosis of pneumococci as a result of complement depletion. Although complete recovery from the infection occurred in normal rats after appropriate penicillin therapy, this was not the case in cobra venom factor-treated rats. Within the limitations of this study, evidence is presented for loss of antibiotic activity as a consequence of impaired phagocytosis.

Severe infections frequently occur in patients with hemoproliferative malignancies (5, 13, 15, 18, 19). They may be difficult to cure, as antimicrobial agents which are effective in normal hosts appear to have lost, at least in part, their efficacy in these patients. The high risk of infections in patients with malignancies may be due to an impairment of host defense mechanisms as a consequence of the malignant process itself or of the antineoplastic therapy (chemotherapy or radiotherapy), or both. It is known that both factors may suppress specific and nonspecific host defense mechanisms, including cellular immunity (7, 8, 17, 22), antibody formation (7, 17, 22, 36), and the number or function of granulocytes (6, 7, 17, 26). Usually several factors contribute simultaneously to the impairment of host defense in cancer patients. The critical role of individual host defense factors in the process of recovery during antimicrobial therapy can only be established in experimental infection. In the present study several aspects of antimicrobial therapy of experimental pneumococcal pneumonia in normal rats and in rats with impaired phagocytosis were examined. Phagocytosis was impaired by complement depletion by using injections of cobra venom factor (CVF). This model permits the study of the role of the host defense factors in the process of recovery during antimicrobial therapy.

MATERIALS AND METHODS

Animals. Female R-strain albino rats (specific pathogen free; 15 weeks old; weight, 180 to 210 g; bred in the Laboratory Animals Center of the Erasmus University Rotterdam) were used in all experiments.

Bacteria. A Streptococcus pneumoniae type III strain isolated from a clinical case was used in all experiments. The minimal inhibitory concentration for penicillin was 0.024 µg/ml (tube dilution test). The virulence of the bacteria was maintained by passage in rats every 2 months and storage at -70oC in Todd-Hewitt broth (Oxoid) with 10% glycerol (Merck).

Pneumonia. Experimental pneumonia was produced in the following manner. The rats were anesthetized with Hypnorm (Duphar) and pentobarbital. The left main stem bronchus was intubated, and the left lobe of the lung was inoculated with a suspension of 6 x 10³ S. pneumoniae type III cells in a volume of 0.02 ml. To estimate the severity of the resulting pneumonia, five animals were observed during the entire course of infection. In addition, dissections were made of groups of five animals at different intervals after inoculation, namely at 36, 84, and 132 h on rats who received no antibiotic therapy and at 36, 84, and 108 h on antibiotic-treated rats.

Parameters for the severity of the resulting pneumonia were as follows: roentgenogram of the chest, total body weight, rectal temperature, macroscopic examinations of the lesion in the left lobe of the lung, weight of the left lobe of the lung, quantitation of the number of viable bacteria in the left lobe and the four right lobes of the lung, and cultures of blood and pleural fluid.

Histology. Lungs were fixed by injecting the trachea with 10% Formalin under a constant pressure to reexpand the lungs. The specimens were then dehydrated in ethanol and toluol, imbedded in paraffin, sectioned, and stained with hematoxylin-eosin or by the Gram stain technique.

Antimicrobial treatment. The sodium salt of penicillin G in aqueous solution was injected intramuscularly into the thigh muscles of the rear legs. Every 12 h a dose of 2 mg/kg of body weight was administered. Nine injections were given, the first dose at 36 h after inoculation of the pneumococci.

Treatment with CVF. The venom of the cobra (Naja naja siamensis) was purified and kindly sup-
plied by I. A. Wilschut (Cell Tissue Kinet., in press). Rats were injected intraperitoneally 14 times with equal doses of CVF (7 µg/kg of body weight). The first dose was administered at the time of pneumococcal inoculation, followed by five doses every 8 h and eight doses every 12 h. To determine complement depletion in vivo, rats were bled at several times during CVF treatment by orbital puncture. Sera were tested by an assay of complement hemolytic activity and an assay for complement component C3.

Assay for complement hemolytic activity. The sera were tested in a mixture containing 0.2 ml of sheep erythrocytes (10⁵ cells, sensitized with hemolysin), 0.2 ml of serially diluted serum, and 0.8 ml of Veronal buffer (pH 7.2; Oxoid). After incubation for 60 min at 37°C, the mixture was centrifuged for 10 min at 1,300 × g, and the release of hemoglobin in the supernatant was recorded at 413 nm in a Zeiss spectrophotometer. Controls included 100 and 0% lysis (guinea pig complement and Veronal buffer, respectively, instead of rat serum). The data derived from this assay were presented as 50% complement hemolysis units per milliliter, corresponding to the reciprocal of the serum dilution that gave 50% hemolysis.

Assay for complement component C3. Serum C3 concentration was measured by the rocket immunoelectrophoresis technique of Laurell, with some modifications (Wilschut, in press). Briefly, to avoid blunt precipitate peaks, the serum samples were carbamylated with 2 M potassium cyanate for 1 h at 37°C. To give a more precise measurement of the C3 concentration, the area enclosed by the precipitation peak was determined. Rabbit anti-rat C3 serum was purchased from Nordic Immunological Laboratories, Tilburg, The Netherlands.

In vitro phagocytosis. A mixture of 7 × 10⁹ washed pneumococci and 1 ml of rat serum was tumbled gently at 14 rpm for 30 min at 37°C. After opsonization, the mixture was kept for 2 h at 4°C, which was followed by centrifugation of the pneumococci at 3,500 × g for 10 min at 4°C. A leukocyte suspension was obtained from rat peritoneal washings 24 h after intraperitoneal injection of 4.5 ml of thioglycolate (Difco Laboratories). The peritoneal cells were washed twice in Hank's bovine albumin–glucose solution (33%) and pelleted at 300 × g for 10 min at 4°C. Eighty-six percent of the leukocytes were polymorphonuclear leukocytes (PMN). In the phagocytic test, 7 × 10⁶ opsonized pneumococci and 7 × 10⁴ leukocytes were tumbled gently at 14 rpm and 37°C in a total volume of 0.3 ml of buffer in polypropylene tubes (Falcon Plastics). (The buffer contained 0.14 M NaCl, 5 mM KCl, 0.4 mM MgSO₄⋅7H₂O, 0.5 mM MgCl₂⋅6H₂O, 1.3 mM CaCl₂, 0.4 mM Na₂HPO₄⋅12H₂O, 0.4 mM KH₂PO₄, 5.6 mM glucose, and 4 mM NaHCO₃.) After 30 min phagocytosis was stopped by dilution in cold buffer (see above). The cells were centrifuged at 25 × g for 10 min at 4°C and washed to remove most of the extracellular bacteria. The leukocytes were then examined under a microscope in Gram-stained smears. A total of 400 leukocytes were observed, and the percentage of PMN containing pneumococci was calculated. Next, the average number of pneumococci per 100 PMN was estimated. The percent phagocytosis was calculated from the percentage of PMN containing pneumococci and the average number of pneumococci per cell. For instance, when 68% of the leukocytes contained pneumococci with an average of six pneumococci per cell, we calculated a total number of 1,632 pneumococci per 400 leukocytes. The estimation of the total number of pneumococci per 400 leukocytes, as seen after opsonization with normal rat serum, was called 100% phagocytosis.

RESULTS

Experimental pneumonia. After inoculation of the rats with 6 × 10⁷ pneumococci, pneumonia developed within 24 h. Initially the infection was confined to the left lobe of the lung. By 108 h the pulmonary lesion involved the entire left lobe, and the infection was spreading to the right lobes. No spontaneous recovery occurred in untreated rats. During the course of the infection total body weight decreased constantly (Fig. 1A), and rectal temperature decreased below normal after an initial rise (Fig. 1B). The presence of an inflammatory response in the left lobe of the lung can be seen in a roentgenogram of the chest (Fig. 2B). Within the duration of our experiments no animals died spontaneously from infection. The animals were sacrificed at 36, 84, and 108 h after initiation of infection, and data are presented in Fig. 1C through F. In these figures each point represents one animal. The involvement of the lung tissue in the infectious process was reflected by a proportional increase in weight (up to sixfold) of the left lobe of the lung (Fig. 1C). At 108 h the pulmonary lesion was very extensive. During the course of infection, the number of pneumococci cultured from the left lobe of the lung increased and reached an average number of 6 × 10⁹ bacteria at 108 h (Fig. 1D). Sooner or later the pneumonia caused a pneumococcal invasion of the bloodstream and pleural cavity. At 108 h this was reflected by positive cultures of blood and pleural fluid in all animals (Fig. 1E and F). A control experiment revealed that the technique of intubation itself did not influence total body weights or rectal temperatures.

Histology. The histological studies of the pulmonary lesion revealed that no lung abscesses were present. Neutrophils predominated in the lesion, and phagocytosis of pneumococci by PMN was observed in the alveoli (Fig. 3).

Antibiotic therapy. Penicillin therapy with repeated injections of 2 mg/kg per 12 h was begun 36 h after the initiation of infection. Figures 4A through F show that antibiotic therapy was successful in normal rats. After an initial decrease the total body weight increased again (Fig. 4A). Rectal temperature normalized after an initial increase (Fig. 4B). The spreading lesion was promptly arrested, as shown by the moder-
ate increase in weight of the left lobe of the lung (Fig. 4C). At 132 h all cultures of lungs, blood, and pleural fluid were sterile (Fig. 4D through F).

In vivo effects of CVF. Whole complement hemolytic activity in serum decreased to less than 2% of normal values after multiple injections of CVF (7 µg/kg). Figure 5 shows that the total levels of circulating C3 after the same CVF treatment fell to less than 3% of the pretreatment level.

Influence of CVF on phagocytosis in vitro. Pneumococci were effectively phagocytized by PMN after opsonization with normal rat serum (100%). The opsonic activity of the serum decreased to 18% upon heating at 56°C for 30 min and was reduced to 43% when rats were treated with CVF, as evidenced by an impaired phagocytosis of pneumococci (Table 1).

Pneumonia in CVF-treated rats. With the same number of pneumococci used in previous experiments pneumonia was produced in CVF-treated rats (Fig. 6A through F). The course of this infection was more severe in comparison with the infection in normal animals (Fig. 1A through F). As appeared from the roentgeno-
grams, the pneumonia spread more rapidly in the CVF-treated rats (Fig. 2C) than in the rats not treated with CVF (Fig. 2B). No spontaneous recovery occurred in CVF-treated rats, as evidenced by a continuous decrease in total body weights (Fig. 6A) and rectal temperatures. The values of the latter finally fell far below normal (Fig. 6B). Lungs taken from animals sacrificed at 36, 84, and 108 h after inoculation also revealed more severe pneumonia as a result of CVF treatment. The pulmonary lesions were very extensive, as seen in the increase in weight (up to sevenfold) of the left lobe of the lung (Fig. 6C). By 108 h an average of $3 \times 10^{10}$ pneumococci were cultured from the left lobe (Fig. 6D). In early stages of the infection pneumococci were seen in the blood and pleural fluid (Fig. 6 E and F), and by 108 h the average number of bacteria per milliliter of blood had increased to $2 \times 10^6$.

A control experiment revealed that the CVF treatment itself did not influence total body weights or rectal temperatures.

**Antibiotic therapy of CVF-treated rats.** Repeated injections of penicillin (2 mg/kg per 12 h starting 36 h after initiation of infection) was unsuccessful in eradicating the pneumococcal infection. There was also no increase in total body weights (Fig. 7A) and no normalization of rectal temperatures (Fig. 7B), and the cultures of lungs, blood, and pleural fluid remained positive (Fig. 7C through F). When the animals were sacrificed, the pneumonia was still actively spreading.

**DISCUSSION**

The purpose of this study was to examine the efficacy of antimicrobial therapy in animals with selectively impaired host defense mechanisms.

Pneumonia and septicemia are the most common types of infections in compromised hosts (5, 14, 30) and major problems in patients with severe neutropenia (23). An experimental pneu-
mococcal pneumonia in rats was chosen as an infection model, because the host-bacteria relationship in this model is relatively well understood (29, 33, 37, 39). The experimental pneumonia was characterized by a lobar consolidation as seen on the chest roentgenograms and at autopsy. Our histological studies of the pulmonary lesion revealed the same characteristics as described by Wood and Smith (42), except that there were no areas of abscess formation. In addition, bacteremia and extrapulmonary complications, such as pleurisy, were present as a rule. The pneumonia in the rats spread actively. Phagocytosis of pneumococci by PMN is the primary defense against this infection, and our histological results supported prior studies (42). However, phagocytosis alone was not sufficient for spontaneous recovery. To cure the infection, it was necessary to inject penicillin intramuscularly every 12 h at a dose of 2 mg/kg of body weight, starting 36 h after initiation of the infection. This dose was chosen because previous experiments with lower doses revealed that 2 mg/kg of body weight was the minimal effective dose required for the recovery of rats. Penicillin therapy was started 36 h after inoculation since at that time the pneumonia was developed. This permitted us to study the role of one host defense factor in the process of recovery during penicillin therapy. The influence of a selectively impaired host defense on the efficacy of antimicrobial therapy was examined by impairing phagocytosis in rats. Because phagocytosis of encapsulated pneumococci occurs after opsonization of the bacteria (28), impairment of phagocytosis was accomplished by removing the opsonins. In the early preantibody phase of infection, C3 is the principal component involved in the opsonization process (31, 38). C3 is directly activated by pneumococci (11, 34, 40), followed

Fig. 3. Sections of the left lobes of the lungs from rats inoculated with type III pneumococci. (A) Section stained with hematoxylin-eosin (×16). At the margin of the lesion in the edema zone there are few PMN in the alveoli. More to the center of the lesion are edema-filled alveoli containing many PMN. In the center of the lesion zone of advanced consolidation, alveoli are packed with PMN. (B) Section stained with Gram stain (×400). Alveoli contain PMN and pneumococci, many of which have already been phagocytized by PMN.
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Fig. 4. Action of penicillin (2 mg/kg per 12 h, starting at 36 h) upon the course of pneumonia in normal rats after inoculation with $6 \times 10^7$ type III pneumococci. (A) Total body weight loss (percentage) of five rats during infection. (B) Rectal temperature of five rats during infection (normal temperature, 37.4 ± 0.34°C). The following determinations were made after sacrificing 15 rats in groups of 5. (C) Weight of the left lobe of the lung (normal weight, 0.6 ± 0.15 g). (D) Number of viable pneumococci in the left lobe of the lung. (E) Number of viable pneumococci per milliliter of blood. (F) Culture of pleural fluid.

by fixation of C3b on bacterial surfaces. Through complement receptor sites on the surface of PMN (12, 21) binding of pneumococci to PMN occurs, resulting in phagocytosis.

These observations in animals explain why phagocytosis of the pneumococci was impaired by the depletion of plasma C3. This depletion was realized by repeated injections of the rats with CVF. It has been demonstrated that this factor may cause prolonged depletion of plasma C3 in several species (9, 20, 24). In the present study low levels of C3 were also observed in the sera of CVF-treated rats. The opsonic capacity of these sera was also grossly deficient, as measured by in vitro phagocytosis of pneumococci by

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<th>Preincubation medium</th>
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<td>Normal rat serum undiluted</td>
<td>100</td>
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<td>HBGb</td>
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<tr>
<td>Heated normal rat serum (56°C for 30 min)</td>
<td>18</td>
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<td>C3-deficient rat serume</td>
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* Type III pneumococci were preincubated in several media and added to the phagocytic system (see text).

**HBG, Hanks bovine albumin-glucose solution (32).**

' Obtained from CVF-treated rats (see text).
Fig. 5. Effect of CVF (7 µg/kg) on circulating C3 levels in serum. Each point represents the mean value of four rats (± standard deviation).

Fig. 6. Course of pneumonia in CVF-treated rats after inoculation with $6 \times 10^7$ type III pneumococci. (A) Total body weight loss (percentage) of five rats during infection. (B) Rectal temperature of five rats during infection (normal temperature, 37.4 ± 0.34°C). The following determinations were made after sacrificing 15 rats in groups of 5. (C) Weight of the left lobe of the lung (normal weight, 0.6 ± 0.15 g). (D) Number of viable pneumococci in the left lobe of the lung. (E) Number of viable pneumococci per milliliter of blood. (F) Culture of pleural fluid.
PMN. As expected from these in vitro experiments, CVF-treated rats appeared to be incapable of producing adequate phagocytosis of pneumococci in the alveoli. This was confirmed by the fact that the spreading of the pulmonary infection in CVF-treated rats was more rapid and extensive than in untreated rats. These data are in accordance with the observation that patients with abnormalities of the complement system, especially C3, suffer from recurrent pneumococcal infections (1, 2, 25). Gross et al. found that hypocomplementemia predisposes mice to bacterial pneumonia (16).

Despite therapy with penicillin, CVF-treated rats did not show signs of recovery. As the same therapy was successful in untreated animals, it must be concluded that penicillin shows a complete loss of efficacy during treatment of an infection caused by susceptible organisms in animals with impaired phagocytosis.

These data are in accordance with the findings of Smith and Wood, who reported that penicillin therapy of pneumococcal myositis lesions in irradiated mice was less effective than such therapy in normal mice (32). They concluded that the curative effect of penicillin was due to the combined effect of the drug and the cellular defenses of the host. The same conclusion also resulted from experiments of Dale et al. with irradiated dogs (10). However, the interpretation of these results is hindered by the fact that radiation not only induces granulocytopenia but
also damages other systems like erythropoiesis and thrombopoiesis, and so may cause untimely death of the animals (41). Experimental studies of Biro and Ivan (4), Trnka et al. (35), and Scott and Robson (27) revealed the effect of certain cancer chemotherapeutic agents upon the efficacy of antimicrobial therapy. However, interpretation of these results is difficult because most cancer chemotherapeutic agents have multiple immunosuppressive actions both on B and T cells as well as on neutrophils. In this study, we suppressed host defense against the infectious organisms by an indirect and consequently selective way. Therefore, we may conclude that loss of activity of this penicillin treatment schedule results from selectively impaired phagocytosis of the infectious organisms. The total effect of antimicrobial therapy during an infection is the combined effect of the curative action of the antibiotic (penicillin in this case) in combination with the activity of the cellular defenses of the host (PMN in this case). If this hypothesis is correct, a lack of phagocytic functions of PMN might be compensated partially by an improved antibiotic treatment schedule, i.e. by increasing the dose or frequency of injections, or both. In a companion paper (3) observations related to the dose and administration schedule of penicillin in rats with impaired phagocytosis are reported.

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LITERATURE CITED


