M. Trautmann, Y. Müller-Leutloff, T. Hofstaetter, F. R. Seiler, H. Hahn

Experimental Klebsiella Septicemia in Mice: Treatment with Specific Antibodies from the Rabbit Alone and in Combination with Gentamicin

Summary: Using a model of an experimental *Klebsiella pneumoniae* septicemia in mice, we examined the therapeutic effect of passively administered specific antibacterial antibodies from rabbits. Both specific IgM and IgG antibody proved to be therapeutically effective. However, the effect of IgG was markedly superior to that of IgM with regard both to the degree of protection and the time interval allowing efficient therapy after infection. The effect of IgG was due to a marked enhancement of *in vivo* phagocytosis, as demonstrated by monitoring bacterial numbers in the liver, spleen, lungs and kidneys. In mice immunocompromised with cyclophosphamide, treatment with IgG still exerted protection against low challenge inocula. When higher inocula were used, treatment with IgG ceased to influence the final mortality rates but delayed the course of the disease for several days by transient reduction of bacterial numbers in the parenchymal organs. In both normal and immunocompromised mice, concomitant treatment with gentamicin resulted in a marked synergistic enhancement of survival.

Zusammenfassung: *Experimentelle Klebsiellen-Sepsis bei Mäusen: Behandlung mit spezifischen Kaninchen-Antikörpern allein und in Kombination mit Gentamicin.* Am Modell einer experimentellen *Klebsiella pneumoniae*-Sepsis der Maus wurde der therapeutische Effekt spezifischer antibakterieller Antikörper vom Kaninchen untersucht. Sowohl IgG- als auch IgM-Antikörper erwiesen sich als therapeutisch effektiv, jedoch war der durch IgM vermittelte Schutzeffekt deutlich geringer als derjenige von IgG. Das nach der Infektion für eine effiziente Therapie zur Verfügung stehende Zeitintervall war bei Anwendung von IgM wesentlich kürzer als bei Therapie mit IgG. Wie Keimzahlbestimmungen in Leber, Milz, Lungen und Nieren zeigten, beruhte der Effekt von IgG auf einer ausgeprägten phagozytosefördernden Wirkung *in vivo*. Bei mit Cyclophosphamid immunsupprimierten Mäusen führte die Gabe von IgG nur dann zu einer Senkung der Letalität, wenn niedrige Infektionsdosen verabreicht wurden. Bei höheren Infektionsdosen führte die IgG-Behandlung lediglich zu einer passageren Keimzahlreduktion in den parenchymatösen Organen sowie zu einer Verzögerung des letalen Verlaufes der Sepsis um einige Tage. Die Kombinationstherapie mit IgG und Gentamicin hatte sowohl bei normalen als auch bei immunsupprimierten Mäusen einen ausgeprägten synergistischen Effekt.

Introduction

The high mortality of gram-negative infections in spite of appropriate antibiotic treatment (1, 2) has reactivated the interest in immunotherapeutic approaches to these diseases. *Klebsiella pneumoniae* is one of the organisms that are frequently isolated in hospital-acquired bacteremic infections (3). It has been shown by *Cryz* (4, 5) that serotype-specific anticapsular antibody may exert significant protection against experimental *Klebsiella* infection.

However, since *Klebsiella* infections often occur in severely immunocompromised hosts (6), the question remained whether specific antibodies would still exert protection under such conditions. In the present study we therefore examined the therapeutic effect of specific antibodies not only in normal, but also in markedly granulocytopenic animals. Since antibody treatment was found to be of limited value in immunocompromised mice, additional studies were carried out in order to detect the synergistic effects of antibody and gentamicin treatment.

Materials and Methods

Bacteria: The Caroli strain of *K. pneumoniae*, capsular serotype 2, was kindly provided by Dr. *Louis Chedid,* Institut Pasteur, France. The strain was maintained on Endo agar plates at 4°C with monthly transfer.

Mice: Eight to ten-week old NMRI mice of both sexes, weighing approximately 30g, were used for the experiments.

Production of antiserum and purification of immunoglobulins: Antibodies against the experimental bacterial strain were raised in rabbits by immunization with heat-inactivated bacteria. For this purpose, bacteria were grown overnight in trypicase soy broth, washed three times in physiological saline and adjusted to an approximate concentration of 10^8 bacteria/ml by measuring optical density at 365 nm and comparing it to a standard curve. Bacteria were then inactivated by heating in a water bath (60°C) for 1h. The sterility of the suspension was checked by plating on blood agar plates. The rabbits were immunized weekly by injecting 1 ml of the bacterial suspension i.m. into each animal. After four immunizations, collection of serum was started by taking 50 ml blood from the ear vein of each animal. Immunizations and bleedings were repeated weekly, and all serum specimens were frozen and stored at –20°C. After collecting a sufficient amount of serum, the specimens were thawed and pooled. The immunoglobulins were precipitated by ammonium sulfate, and the IgG and IgM fractions were separated by ion exchange chromatography on DE 52-cellulose (Whatman Ltd.). Both IgG

and IgM were adjusted to give a final concentration of 1 g protein per 100 ml physiological saline.

Determination of antibody titer: The antibody content of native serum and the purified immunoglobulin fractions were measured by passive hemagglutination using the technique of *Neter* (7), as modified by *Young* (8).

Experimental infections: For the infections, bacteria were grown in trypticase soy broth for 5 h (late log phase). The bacteria were washed three times in physiological saline and adjusted to the desired concentration by measuring optical density at 365 nm. In addition, the number of viable bacteria in each inoculum was determined by duplicate plating on blood agar plates after serial ten-fold dilutions. Mice were infected by injecting 0.2 ml of the bacterial suspension into the tail vein. The number of deaths was monitored daily until no further deaths occurred for five consecutive days. Treatment with immunoglobulins was performed by injecting 0.2 ml of the immunoglobulin solution into the tail vein.

Bacterial organ counts: In order to determine bacterial numbers in the liver, spleen, lungs and kidneys, mice were killed with carbon monoxide and the organs were removed aseptically. Each organ was placed in 10 ml of physiological saline and homogenized in a tissue homogenizer (Braun Ltd.). The organ suspensions were placed on ice and the number of viable bacteria in each suspension was determined by plating on blood agar plates in serial ten-fold dilutions. The lowest number of bacteria that could be detected by this technique was 10^2 per organ.

Immunosuppression: In the respective experiments, mice were immunosuppressed by i. p. injection of 300 mg/kg cyclophosphamide (Endoxan®, Asta Ltd.) 48h before infection. Before and during immunosuppression, white blood cell counts in each of 15 mice were determined using an automatic leukocyte counter (Coulter counter). Differential counts were performed on Pappenheim-stained smears.

Antibiotic treatment: Gentamicin (Refobacin®, Merck Inc.) was given in a dose of 3 mg/kg/day in two daily injections over a period of seven days. Each dose was injected in a volume of 1 ml i.p. The MIC of gentamicin for the experimental strain of *K. pneumoniae* was 0.06 mg/l.

Statistics: The one-way Chi-square method was used for statistical evaluation. The results were regarded as highly significant when p was less than 0.001, significant when p was less than 0.01, and weakly significant when p was less than 0.05.

Results

Antibody Titers

The hemagglutinating antibody titer of pooled rabbit antiserum against the experimental strain was 1:160,000, that of purified IgG (1% protein solution) was 1:10,000 and that of purified IgM (1% protein solution) 1:320,000. Thus, the IgM fraction contained the major part of the hemagglutinating activity.

Experimental Infections in Normal Mice

To establish a dose-response relationship and to characterize the course of the experimental disease, mice were infected with graded doses of bacteria. As shown in Figure 1, a challenge dose as low as 7×10^2 microorganisms proved to be lethal for 40% of the animals. However, higher doses of 7×10^4 to 6×10^5 bacteria were needed to achieve a 90%
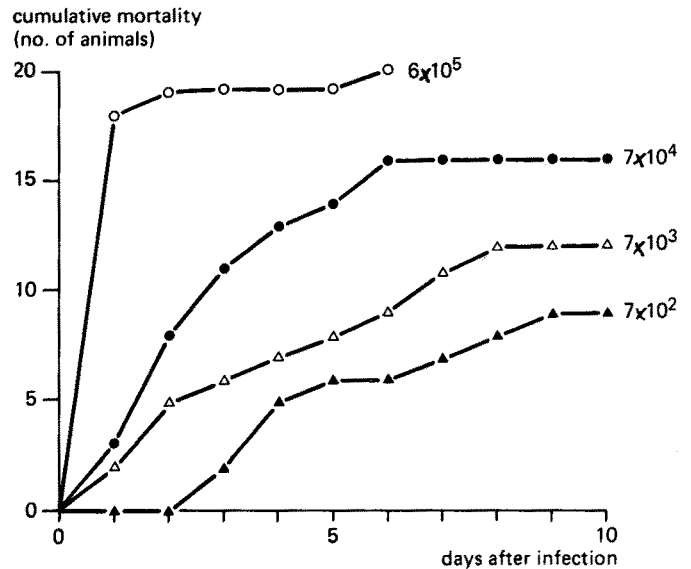

Figure 1: Dose-response relationship for *Klebsiella pneumoniae* type 2 in normal NMRI mice.

Table 1: Therapeutic effect of specific IgG and IgM antibody in experimental *Klebsiella* septicemia in mice*.

Time of immunoglobulin application (h after infection)	IgG		IgM
0	10/10**	hs	n. d.
2	7/10	hs	5/10 s
4	7/10	hs	0/10
6	1/10		0/10
8	1/10		0/10
10	0/10		n. d.
Control (no treatment)	0/10		

  * Challenge dose 1.2×10^5 cfu per animal;
** No. of survivors/total number challenged;
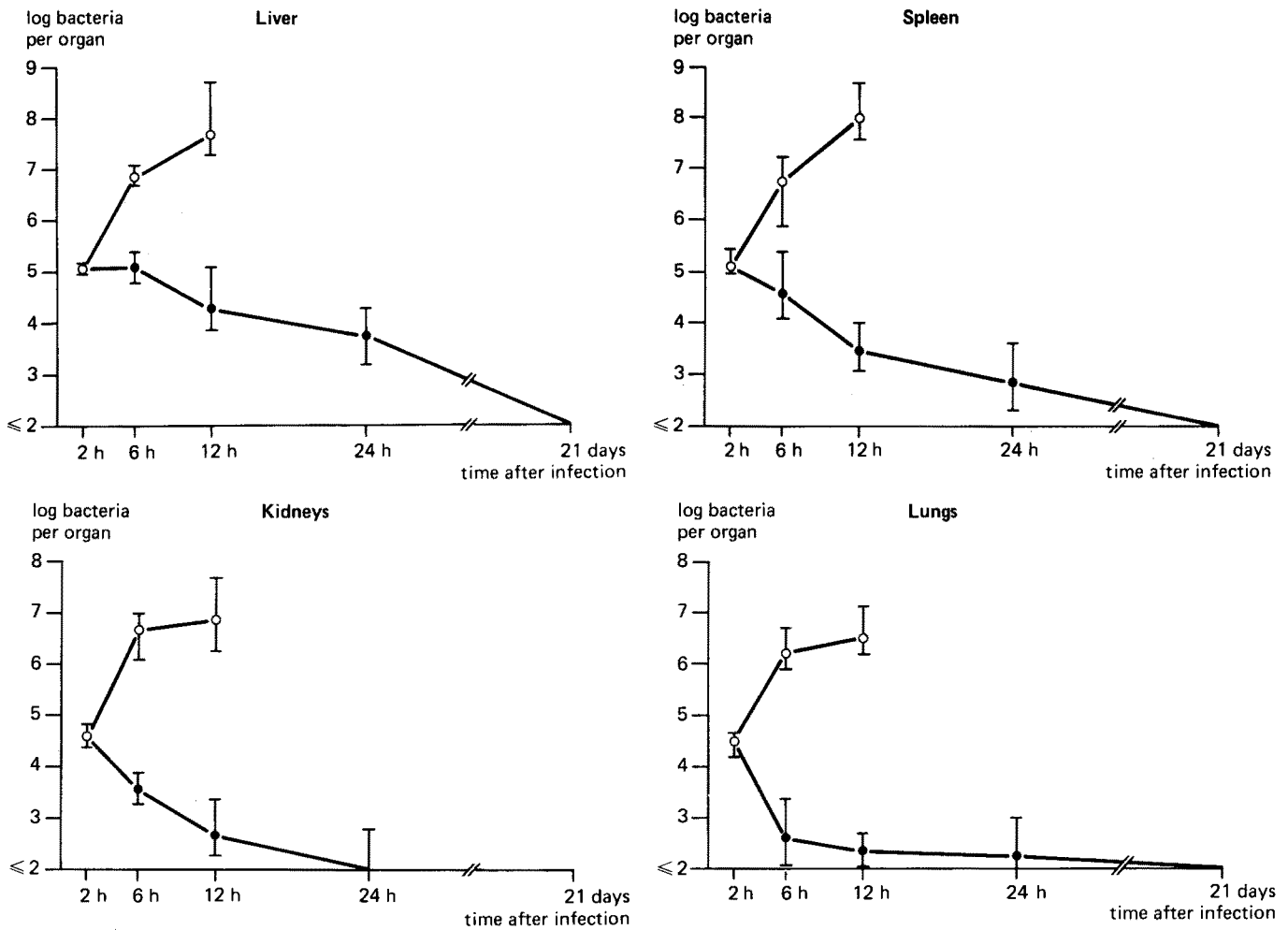n. d. = not done
hs = highly significant;
s = significant.

mortality. Such doses were generally used for therapeutic experiments with immunoglobulins. As can be seen from Figure 1, the course of the disease was rather slow, some deaths occurring as late as one week or more after infection.

Antibody Treatment in Normal Mice

Both IgG and IgM exerted significant protection when given early after infection (Table 1). However, in spite of its higher hemagglutinating activity, the IgM fraction was much less effective than IgG. In addition, IgM had no effect when given later than 2 h after infection, whereas the delayed administration of IgG still slightly enhanced survival (Table 1).

Bacterial Organ Counts in Normal Mice

Since IgG was more effective than IgM in the present septicemia model, further experiments were carried out with

Liver — log bacteria per organ vs. time after infection (2 h, 6 h, 12 h, 24 h, 21 days)

Spleen — log bacteria per organ vs. time after infection (2 h, 6 h, 12 h, 24 h, 21 days)

Kidneys — log bacteria per organ vs. time after infection (2 h, 6 h, 12 h, 24 h, 21 days)

Lungs — log bacteria per organ vs. time after infection (2 h, 6 h, 12 h, 24 h, 21 days)

Figures 2a–d: Bacterial organ counts in normal NMRI mice after infection with 1.3×10^5 cfu of *Klebsiella pneumoniae*. O = Untreated mice; ● = Mice treated with 0.2 ml IgG 2h after infection.

IgG to evaluate its influence on bacterial growth in the parenchymal organs. Mice were again infected with an approximate LD 90% (1.3×10^5 cfu). After infection, the animals were divided into two groups, one of which remained untreated whereas the other received IgG 2 h after infection. Five mice from the control group were killed 2 h after infection to determine the number of bacteria initially present in each organ. As shown in Figures 2a–d, nearly all of the bacterial inoculum could be recovered from the liver and spleen, whereas the lungs and kidneys contained lower amounts of bacteria. Further organ counts demonstrated that in untreated animals, rapid bacterial replication occurred in all the organs examined, leading to the death of the majority of animals after 12 h of infection. In contrast, a continuous decrease in viable bacteria was found in IgG-treated mice. The last group of these animals, which had survived until 21 days after infection, showed no detectable organ colonization (Figures 2a–d).

Antibody Treatment in Immunocompromised Mice

White blood cell counts in mice that had been treated with cyclophosphamide revealed a rapid decrease in total white blood cells during the first two days after injection of the drug. White blood cell counts were below $2 \times 10^3/mm^3$ from the second to the fifth day after injection, but returned to the initial values on the eighth day. Later, a strong rebound effect occurred. The total number of granulocytes fell below $5 \times 10^2/mm^3$ on the second day after injection of cyclophosphamide and remained at this level for three days.

Experimental infections were performed 48 h after the injection of cyclophosphamide, i.e. in the presence of marked granulocytopenia. As can be seen from Figure 3, even inocula as low as 2.3×10^1 microorganisms proved to be 100% lethal under these conditions. When immunosuppressed mice were infected with 1.5×10^5 bacteria, a dose that was generally used to challenge normal mice, the course of the experimental disease was strongly accelerated, leading to the death of all animals within two days.

The efficacy of treatment with IgG under these conditions was examined in a number of experiments which are summarized in Table 2. IgG still exerted significant protection when low challenge inocula were used. However, when
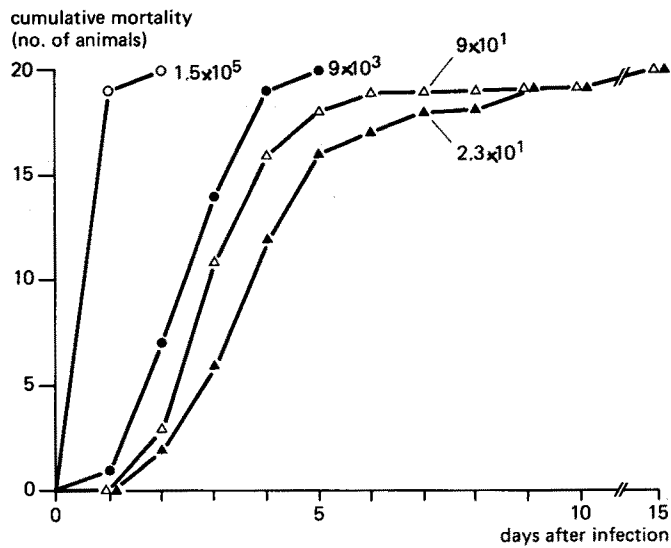
Figure 3: Dose-response relationship for *Klebsiella pneumoniae* Type 2 in immunocompromised mice.

Table 2: Effect of IgG treatment in cyclophosphamide-treated mice.

Time of immuno-globulin application (h after infection)	Challenge dose (cfu)			
	1.5×10^5	0.9×10^4	1×10^3	0.9×10^2
2	0/20*	4/20	12/20 hs	14/20 hs
4	0/20	0/20	4/20	6/20 s
6	0/20	1/20	1/20	4/20 ws
8	0/20	1/20	0/20	1/20
10	0/20	0/20	1/20	2/20
Control (no treatment)	0/20	0/20	1/20	0/20

* Number of survivors/total number challenged;
hs = highly significant;
s = significant;
ws = weakly significant.

the challenge dose was raised to 1.5×10^5 microorganisms, treatment with IgG failed to lower the final mortality. A more detailed description of this experiment, how-ever, reveals that treatment with IgG was able to slow down the course of the infection, postponing the steep increase in mortality for two to three days (Table 3). With regard to this finding, it was of interest to establish whether repeated injections of IgG, given daily for four days and thus covering the whole period of granulocytopenia, would be able to lower the final mortality. However, the
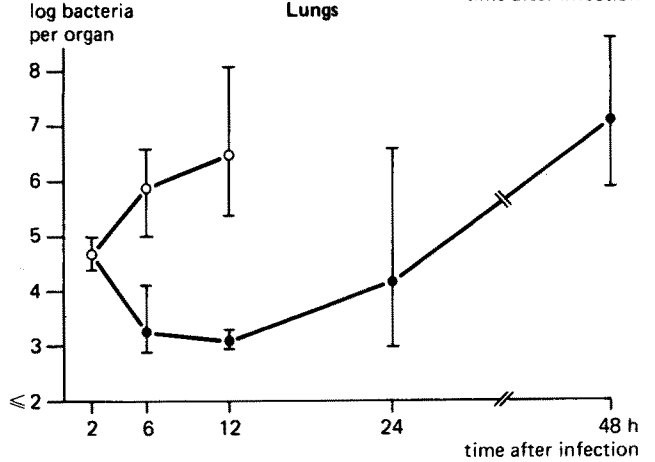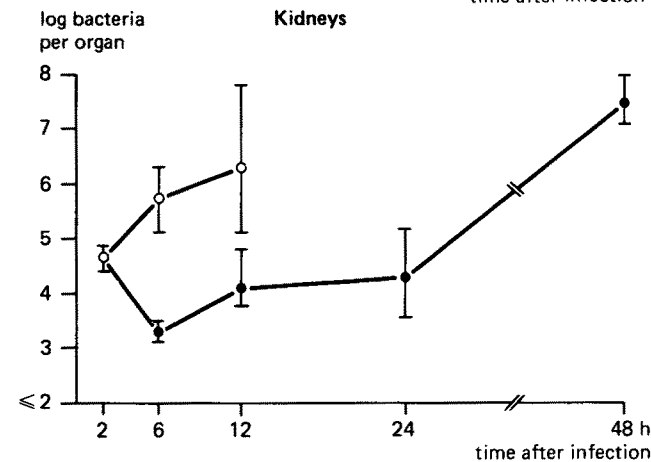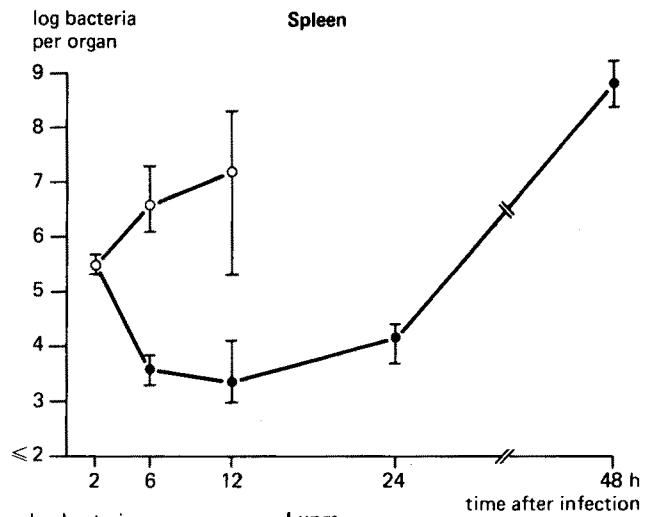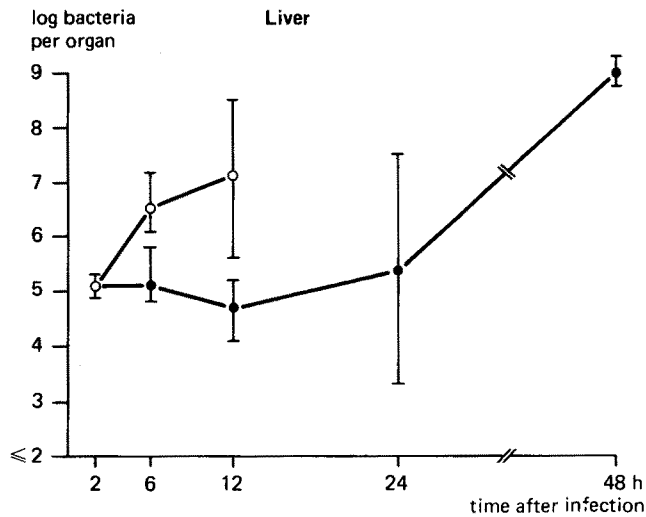
Figures 4a–d: Bacterial organ counts in immunocompromised NMRI mice after infection with 1.5×10^5 cfu *Klebsiella pneumoniae*, ○ = Untreated mice; ● = Mice treated with 0.2 ml IgG 2h after infection.

Table 3: Effect of IgG treatment on the course of infection in immunocompromised mice*.

Time of immuno-globulin application (h after infection)	Day after infection				
	0	1	2	3	9
2	20**	20	19	3	0
4	20	20	15	4	0
6	20	20	17	4	0
8	20	15	7	1	0
10	20	10	4	1	0
Control (no treatment)	20	1	0		

* Challenge dose 1.5×10^5 cfu;
** Survivors (groups of 20 mice were challenged).

effect of repeated injections of IgG was not superior to that of a single initial injection (data not shown).

Bacterial Organ Counts in Immunosuppressed Mice

As the organ counts revealed, treatment with IgG initially led to a decrease in bacterial numbers similar to that in normal mice. However, beyond 12 h after infection, bacteria again replicated in all the organs examined. Animals that were still alive 48 h after infection were found to be in a moribund state. Examination of their organs revealed extremely high numbers of bacteria (Figures 4a–d).

Combination of IgG and Gentamicin Treatment

In preliminary experiments that were conducted with different doses and administration schedules of gentamicin, it was found that a dose of 3 mg/kg/day given in two daily injections protected 70% of the infected animals against lethal *Klebsiella* infection when antibiotic treatment was started 2 h after infection (data not shown). However, when the beginning of treatment was postponed, antibiotic treatment gradually became less effective. Similarly, gentamicin treatment was of little effect in cyclophosphamide-treated mice, even if the first dose of the antibiotic was given as early as 2 h after infection.
In order to study synergism with IgG, antibiotic treatment was started 6 h after infection in normal mice and 2 h after infection in immunocompromised mice. In both cases, gentamicin treatment alone lowered mortality only slightly from 100% to about 80% (Table 4). A single injection of IgG, given at the beginning of antibiotic treatment, was of little effect only in normal mice and completely ineffective in immunosuppressed mice. However, when both treatment regimes were combined, there was a marked synergistic enhancement of survival in both normal and immunocompromised animals (Table 4).

Discussion

Several authors have previously demonstrated the protective effects of specific antisera against experimental *K. pneumoniae* infections (9–11). However, in most of these studies only prophylactic effects were achieved, and

Table 4: Combination treatment of experimental *Klebsiella* septicemia with IgG and gentamicin.

Treatment	Survivors/no. challenged			
	Normal mice		Immunocompromised mice	
IgG alone	4/40		0/40	
Gentamicin alone	8/40	hs	7/40	hs
Gentamicin + IgG	25/40		20/40	
Control	0/20		0/20	

Challenge doses were 1.2×10^5 cfu for normal mice and 1.4×10^5 cfu for immunocompromised mice. See text for treatment schedules; hs = highly significant.

no data on the therapeutic use of passively administered specific antibody were reported. *Greisman* et al. (11) found specific antiserum to be highly protective against lethal *Klebsiella* infection in mice when injected prior to infection. However, they even saw severe adverse reactions (acute anaphylaxis) when the antiserum was given several hours after infection.

Using a bacterial strain identical to that previously used by *Greisman* et al. (11), *Chedid* et al. (12) and *McCabe* (13), we were able to demonstrate significant *therapeutic* effects of passively administered rabbit antibody. The marked therapeutic potential of our antiserum as compared to that of *Greisman* et al. (11) might be due to a longer immunization procedure yielding a higher content of specific antibody. In addition, a more protracted immunization schedule might yield higher amounts of specific IgG antibody which could be shown, in the present experiments, to be of higher therapeutic effectiveness than specific IgM with regard both to the degree of protection and the time interval allowing efficient therapy after infection.

Since IgG proved to be superior to IgM in the present model, further therapeutic studies were performed with IgG. It was found that the effect of IgG correlated to an enhanced clearance of bacteria from the parenchymal organs, suggesting that IgG promoted *in vivo* phagocytosis. Further experiments in immunocompromised animals supported this view by yielding evidence of the fact that, in the presence of only low numbers of circulating phagocytes, the effect of IgG was only transient and was followed by a rapid recurrence of bacterial replication. Since repeated administrations of IgG were unable to prevent a fatal outcome in these animals, it can be assumed that the recrudescence of the infection was not due to a diminished antibody response of the animals (induced by cyclophosphamide), but to an exhaustion of the limited pool of circulating phagocytes.

This observation made it evident that antibody treatment alone might be of little value in immunocompromised hosts lacking adequate numbers of phagocytes. Since different authors have reported synergistic effects between IgG and antibiotics in experimental models of gram-negative infection (14–16), we tested such a combination in both normal and immunocompromised mice. Marked

synergism was detected between suboptimal gentamicin treatment and a single injection of specific IgG. In granulocytopenic animals, this effect could be attributed to an inhibition of recurrence of bacterial replication after the initial drop in bacterial organ counts effected by IgG. In normal mice, the reason for such synergism remains to be determined. It may be possible, however, that physicochemical alterations of the bacterial cell wall after opsonization by IgG (17) facilitate the penetration of antibiotics that act within the bacterial cell. It has recently been shown *in vitro* that the exposure of gram-negative bacteria to IgG may cause a disintegration of outer bacterial membrane structures, which enhances the action of antibiotics (18, 19).

The present report on a positive immunological action of gentamicin is in contrast to previous findings of other authc,s, who observed a negative influence of this aminoglycoside antibiotic on polymorphonuclear leukocyte function (20, 21) and on the phagocytic activity of alveolar macrophages (22). However, this detrimental immunological action of gentamicin usually occurred in the presence of antibiotic concentrations that exceeded therapeutic serum levels. When very low antibiotic concentrations were used, aminoglycosides were even shown to enhance phagocytosis by macrophages (23). The present study shows that under therapeutic conditions *in vivo*, the detrimental action of gentamicin on cellular host defense factors may play a minor role in comparison to its modulating effect on gram-negative bacteria, which seems to act synergistically with the humoral immune defense.

Finally, it should be stressed that synergistic effects between antibiotics and IgG may depend on the bacterial strains and the antibiotics used. In a previous study (24), in which specific rabbit antibody was used to treat an experimental *Pseudomonas aeruginosa* septicemia in mice, we were not able to detect any synergism between specific IgG and piperacillin. Therefore, further studies are needed to examine in detail the mechanism of interaction between antibiotics and IgG.

Acknowledgement

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