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Limited Protective Effect of Rough Mutant Antisera in Murine Escherichia coli Bacteremia*

Summary: Previous studies in mice have demonstrated differing immunoprophylactic activity of antisera against rough mutants of *Enterobacteriaceae* in the prevention of lethal gram-negative bacteremia. In this study, in which CF_1 mice were made bacteremic with a serum-resistant *Escherichia coli* 06:K2:H1, the composite survival was significantly ($p < 0.001$) enhanced by i. v. pre-treatment one to two hours before injection with either normal rabbit sera or antisera to the J5 mutant of *E. coli* 0111. The protective efficacy of these preimmune and hyperimmune sera did not differ significantly. Since considerable variability in the mortality of control mice occurred in the 25 separate experiments, the results of individual experiments were grouped retrospectively according to survival in the individual control groups and compared for evidence of possible differences in the efficacy of these two sera. With the exception of a statistically significant difference in the efficacy in one group receiving an LD_{75-95} inoculum, no such differences were noted. Thus, the variable effects of a rough mutant antiserum were not explained by differences in the relative virulence in the inoculum. This study confirms earlier observations by others that the protective efficacy of the anti-J5 antisera in infected mice does not differ appreciably from that of normal rabbit sera, provided the same donor rabbits are the source of both preimmune and hyperimmune sera.

Zusammenfassung: *Begrenzte protektive Wirkung von Antiseren gegen Rauhmutanten bei der Escherichia coli Bakteriämie.* Versuche zur Wirksamkeit einer Immunprophylaxe gegen die tödliche gramnegative Bakteriämie bei Mäusen mit Antiseren gegen Rauhmutanten von *Enterobacteriaceae* brachten bisher unterschiedliche Ergebnisse. In der vorliegenden Studie wurde bei CF_1-Mäusen eine Bakteriämie durch einen serumresistenten *Escherichia coli* 06:K2:H1 Stamm hervorgerufen. Wenn die Tiere ein bis zwei Stunden vor Injektion der Bakterien mit normalem Kaninchenserum oder Antiserum gegen die J5-Mutante von *E. coli* 0111 i. v. vorbehandelt wurden, war die Gesamtüberlebenszeit signifikant erhöht ($p < 0{,}001$). Serum vor der Immunisierung und Hyperimmunserum unterschieden sich in ihrer protektiven Wirksamkeit nicht signifikant voneinander. In 25 getrennt durchgeführten Experimenten schwankte die Sterblichkeit der Mäuse in den Kontrollgruppen erheblich; daher wurden die Ergebnisse der einzelnen Experimente retrospektiv entsprechend den Überlebenszeiten der Tiere in den einzelnen Kontrollgruppen zusammengefaßt und nach möglichen Unterschieden in der Wirksamkeit der beiden Seren untersucht. Ein statistisch signifikanter Unterschied ließ sich lediglich in einer Gruppe nachweisen, die eine LD_{75-95} erhalten hatte. Die unterschiedliche Effektivität von Antiseren gegen Rauhmutanten läßt sich folglich nicht mit den Unterschieden in der relativen Virulenz des Inoculum erklären. Die vorliegende Studie bestätigt somit frühere Beobachtungen anderer Untersucher, daß die Wirksamkeit von Anti-J5-Serum und normalem Kaninchenserum nicht wesentlich verschieden ist, vorausgesetzt, daß das Serum vor der Immunisierung und das Hyperimmunserum von demselben Kaninchen gewonnen wurden.

Introduction

Previous studies have demonstrated that antisera against rough mutants of *Enterobacteriaceae* protect mice against lethal infection with smooth, heterologous strains of gram-negative enteric bacilli (1, 2). This protection has been attributed to cross-reacting antibodies to the common core glycolipid antigen which is a constituent of the cell wall outer membrane lipopolysaccharide of *Enterobacteriaceae,* and is comprised of lipid A bound to a trisaccharide consisting of 2-keto-deoxyoctonate residues (3). In the case of less rough mutants, such as the J5 mutant of *E. coli* 0111, additional polysaccharide antigenic determinants are present.

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Recent studies have challenged these observations (4, 5). *Greisman* et al. (5) found that protection from passively administered antisera against Rc, Rd, or Re chemotype mutants offered no greater protection than that afforded by sera from unimmunized rabbits, and this protection could not be correlated with agglutinating antibody titers to these rough mutants.

These conflicting results prompted the following study in which the protective activity of antisera against the J5 (Rc chemotype) rough mutant of *E. coli* 0111 was found to be similar to that of normal, preimmune rabbit sera in a murine model of gram-negative bacteremia.

Materials and Methods

Bacteria: For the preparation of rabbit antisera, the J5 mutant of *E. coli* 0111, a stable Rc chemotype, was provided by Dr. *Allen Woodhour* of Merck Research Laboratories, West Point, PA. The challenge strain in mice was an *E. coli* isolated from the blood culture of a patient at the Rhode Island Hospital and subsequently typed as 06:K2:H1 by Dr. *Fritz Ørskov* (International Escherichia and Klebsiella Centre, Statens Seruminstitut, Copenhagen, Denmark). The organism was serum-resistant (according to the method of *Goldman* et al. [6]) to pooled rabbit and mouse sera as well as to human sera. This strain was selected on the basis of its demonstrable surface reactivity with antisera against the J5 mutant of *E. coli* 0111, as determined by an indirect immunofluorescent antibody method (described below). This infecting organism was stored on blood agar plates, restreaked every ten days, and grown overnight in tryptic soy broth before injection into test mice. The challenge strain was passed bimonthly in CF_1 mice.

Rabbit sera: Hyperimmune antisera against the J5 mutant of *E. coli* 0111 were prepared in six adult albino New Zealand rabbits by multiple injections of a stock solution of 4 mg of acetone-dried bacteria (10^7–10^8) per ml of normal saline, according to the methods of *Johns* et al. (7). Twice weekly injections were given into the rabbit ear vein, beginning at 0.2 mg for three injections followed by doubling of the quantity approximately each week until a dose of 1.6 mg of acetone-dried bacteria had been given. This dose was given until high titers of hemagglutination and hemolytic antibody to the extracted core glycolipid of the J5 mutant (methods described below) had been obtained, at which time the rabbits were bled and sacrificed. Normal rabbit sera (NRS) had been obtained previously from these donor rabbits seven days before immunization was begun. Both normal and hyperimmune rabbit sera were separated by centrifugation, pooled respectively, and stored at − 20° C until use.

Antibody assays: To determine antibody titers in the hyperimmune and normal rabbit sera, J5 mutant cell wall core glycolipid was extracted and purified by the phenol-chloroform-petroleum ether method of *Galanos* et al. (8). The indirect passive hemagglutination (HA) and complement-dependent passive hemolysis (HE) assays were performed according to the methods of *Young* et al. (9), as modified by *Peter* and *Zinner* (10). Beginning at a 1:5 dilution in veronal buffered saline, serial twofold dilutions were made of heat inactivated (56° C for 30 min) rabbit sera which had been absorbed overnight with washed sheep red blood cells, and were tested in microtiter plates. For the HE assay, a 1:20 dilution of guinea pig complement (Gibco, Grand Island, NY) absorbed with zymosan to reduce the alternate pathway of complement activity, according to the method of *Pillemer* et al. (11), was added to each well. Anti-J5 core glycolipid HA and HE titers of the pooled hyperimmune rabbit sera were 1:10,240 and > 1:81,920, respectively, and those of pooled normal rabbit sera were 1:10 and 1:160, respectively.

E. coli strains were evaluated for cell wall binding by anti-J5 mutant antisera, using a modification of the indirect immunofluorescent antibody method of *Young* et al. (12). In this test, a dilute suspension of an *E. coli* strain was boiled for one hour in phosphate buffered saline, applied to a glass slide in a concentration to give approximately 10 bacteria per high power field, air dried and gently heat fixed. Serial twofold dilutions of an initial 1:10 dilution of the hyperimmune anti-J5 rabbit antisera of normal (preimmune) rabbit sera were added and slides were incubated for 30 min at room temperature in a humid chamber before washing with phosphate buffered saline. After the slide had been dried, fluorescein conjugated goat anti-rabbit globulin (Gibco, Grand Island, NY) in a 1:8 dilution was applied, and the slides were incubated for 30 min, as described previously. Duplicate slides were prepared and read independently by two observers for the highest serum dilution showing clear bright fluorescence. The recorded titer in this test is the geometric mean of these four readings. For the challenge *E. coli* strain chosen for the experiments reported here, the titers against normal rabbit sera and hyperimmune anti-J5 rabbit sera were 1:12 and 1:95, respectively.

Murine infections and passive protection experiments: CF_1 female mice (Charles River Breeding Company, Wilmington, MA), weighing 18 to 22 g, were injected intraperitoneally (i. p.) with 0.2 ml of the challenge *E. coli* suspension. This suspension was prepared by inoculating 10 cc of Mueller-Hinton broth with the challenge strain for overnight incubation at 37° C, and subsequent standardization to 10^9 colony forming units (CFU) per ml by spectrophotometric analysis (55% transmission at 610 nm). This standardized suspension was diluted to obtain an approximate LD_{95} inoculum for injection. Tail vein blood cultures yielded *E. coli* in two of the three mice sacrificed one hour after bacterial challenge.

In the individual passive protection experiments, 30 or 60 mice were randomly divided into three groups and given either hyperimmune anti-J5 *E. coli* 0111 antisera, normal rabbit sera or normal saline in a volume of 0.3 ml administered intravenously (i. v.) into the tail vein one to two hours before bacterial challenge. In later experiments, control mice did not receive normal saline, since mortality in mice given normal saline did not differ from that in non-injected control mice. The pooled rabbit sera for each experiment were heat inactivated for 30 min at 56° C and passed through a 45 micron Millipore filter before administration. Prior to the passive protection experiments, the pooled hyperimmune antisera were administered initially to eight uninfected mice without subsequent evidence of toxicity. Infected mice were observed at 18, 48, 72 and 96 hours after challenge. In most experiments, all deaths occurred within 48 hours of bacterial challenge. Survival for each experiment was determined by the number of mice alive at 96 hours. For statistical analysis, the Chi-square test with the Yates correction was used.

Results

Comparison of the total mortality in the three experimental groups of mice, depicted in Figure 1, demonstrated that survival of mice injected i. p. with the challenge strain of *E. coli* 06:K2:H1 was significantly ($p < 0.001$) enhanced by preceding i. v. administration of either

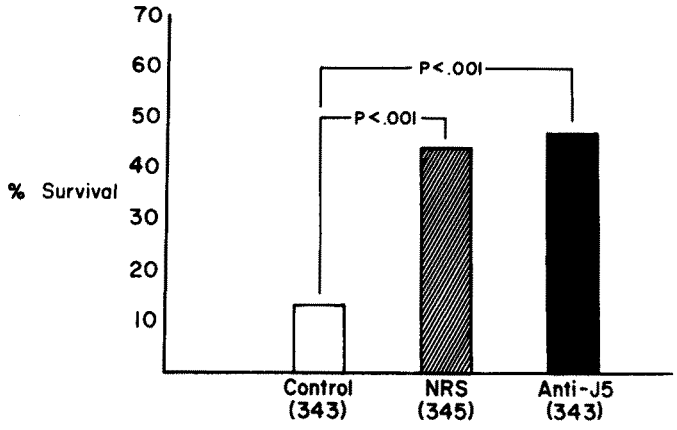

Figure 1: Composite survival of mice infected with *Escherichia coli* 06:K2:H1 and pretreated with normal rabbit sera (NRS) or anti-J5 rabbit sera. Controls received saline or no pretreatment.

hyperimmune anti-J5 sera or NRS. In 343 control mice, only 13% survived, whereas 47% of the 343 anti-J5 recipients and 44% of the 345 mice given NRS survived the *E. coli* challenge. The passive protection induced by antisera against the rough J5 mutant did not differ appreciably from that resulting from preimmune NRS.

These survival rates reflect the composite results of 25 separate experiments and do not demonstrate the considerable differences between individual experiments. Although an LD_{95} challenge inoculum in the control group in each experiment was desired, the observed mortality in the control mice varied from 60 to 100%. Similar variations in survival among both anti-J5 and NRS recipients occurred. This variability reflected decreasing virulence of the challenge *E. coli* strain during storage, even with bimonthly mouse passage. The inoculum administered ranged from 1.5×10^6 CFU to 4×10^7 CFU in these experiments.

In view of this variability, the results of individual experiments were grouped retrospectively according to survival in the individual control groups, and further analyzed for differences between the three experimental groups. In the composite results of the 11 experiments in which no control mice survived (Group A), both anti-J5 sera and NRS significantly ($p < 0.001$) enhanced survival, as shown in Table 1, but no difference in their respective protective efficacy occurred; 23% (36/158) of the NRS recipients and 20% (32/159) of the anti-J5 recipients survived. Similarly, in Group B, which includes the 14 other experiments in which some control mice survived (composite survival of 45/183 or 25%), pretreatment of mice with either NRS or anti-J5 sera also significantly ($p < 0.001$) enhanced survival, and to a similar extent. Sixty-three percent (117/187) in the NRS group and 69% (129/186) in the anti-J5 group survived.

The experiments in Group B were further divided into those in which less than 25% of the control mice (Group B-I) survived and those in which 25% or more survived

Table 1: Grouping of individual experiments according to the survival in the respective group of control mice. Group A experiments are those in which no control mice survived; Group B are those in which survival in some control mice occurred. All mice in each experiment received the same bacterial (*E. coli* 06:K2:H1) challenge inoculum. In both groups A and B, NRS and anti-J5 significantly, and to a similar extent, enhanced survival in comparison to that in the controls.

Group	Experiment #	Survival (no. survived/total no. infected) Controls	NRS recipients	Anti-J5 recipients
A	15	0/20	5/20	0/20
	16	0/10	0/10	0/10
	17	0/20	9/19	3/20
	19	0/20	2/20	1/20
	20	0/20	0/20	0/20
	21	0/20	7/20	5/20
	23	0/10	1/10	2/10
	25	0/10	6/10	6/10
	7A	0/10	1/10	7/10
	28	0/10	3/9	7/9
	6B	0/10	2/10	1/10
Total	11	0/160 (0%)	36/158 (23%)	32/159 (20%)
B	18	1/14	1/14	0/14
	24	2/10	3/10	2/10
	6A	1/10	5/10	8/10
	4	1/9	3/9	6/9
	26	2/10	4/14	8/13
	27	4/18	9/18	14/18
	9B	2/10	6/10	7/10
	11	2/10	5/10	8/10
	22	2/10	10/10	10/10
	13	5/20	18/20	17/20
	14	4/12	12/12	12/12
	12	7/20	19/20	16/20
	5	8/20	13/20	12/20
	9	4/10	9/10	9/10
Total	14	45/183 (25%)	117/187 (63%)	129/186 (69%)

Table 2: Analysis of Group B experiments according to survival in the control group of mice of ≥ 25% or < 25 %. Group B-I represents the composite results for those experiments in which less than 25% of the control mice survived; B-II represents the composite of those experiments in which 25% or more of the control mice survived. In both groups, survival in the NRS recipients and anti-J5 recipients was significantly ($p < .001$) enhanced in comparison to controls, but in Group B-I, survival in the anti-J5 recipients was significantly ($p = 0.022$) greater than that in the NRS recipients.

Group	No. of experiments	Survival (no. survived/total no. infected) (%) Controls	NRS recipients	Anti-J5 Recipients
B-I	9	17/101 (17%)	46/105 (44%)	63/104 (61%)
B-II	5	28/ 82 (34%)	71/ 82 (87%)	66/ 82 (80%)

(Group B-II). In this analysis, a difference between the protection resulting from pretreatment with anti-J5 sera and that with NRS was found. Both modalities of immunoprophylaxis significantly ($p < 0.001$) enhanced survival in these two subcategories, as shown in Table 2, but in Group B-I in which less than 25% of controls survived, the survival in the anti-J5 recipients of 63 of 104 mice (61%) was significantly ($p = 0.022$) greater than that of 46 of 105 mice (44%) in the NRS recipients. However, no significant difference in outcome between these two groups occurred when Group B was divided according to survival of more or less than 20% in the controls, although this subdivision resulted in the change of only one experiment (# 27) from the "high" to "low" survival group.

Discussion

In this study, both NRS and rabbit antisera against the J5 mutant of *E. coli* 0111 afforded protection against subsequent infection with a virulent serum-resistant strain of *E. coli* in CF_1 mice. This observation is consistent with that of *Greisman* et al. (5) who found that rabbit antisera against three rough *Enterobacteriaceae* mutants reduced mortality in outbred Swiss albino mice challenged with a heterologous gram-negative rod, but to a no greater extent than pretreatment with preimmune rabbit sera. These findings contrasted with earlier observations by others that passive immunization with either antisera to an Ra mutant of *Salmonella typhimurium* (1), antisera to the Re mutant of *S. minnesota* R595 (2), or that to its extractable core glycolipid (9) protected mice against death from induced gram-negative bacteremia. In addition, *Ziegler* et al. (13) demonstrated that anti-J5 antisera given to granulocytopenic rabbits at the onset of induced bacteremia due to *Klebsiella pneumoniae, E. coli* 017, or *E. coli* 04, significantly augmented the survival of these rabbits in comparison to that observed in recipients of nonimmune sera.

The reasons for these conflicting results are not clear. *Greisman* et al. (5) have suggested that the use of normal rabbit sera from different rabbits than those in which hyperimmune antisera is raised could explain these conflicting results, since the protective activity of normal rabbit sera varied widely in their studies. In the present study, NRS and anti-J5 antisera were obtained from the same rabbit donors. The protective effect of these two sera may also be dependent on the relative virulence of the challenge inoculum. However, in this study, grouping the experiments according to mortality in the control mice showed only slight differences in the efficacy of hyperimmune antisera in comparison to that of NRS (Table 2).

Other possible factors which might explain these conflicting results include variable sensitivity to endotoxin of different strains of mice, differing surface reactivity of the challenge organisms with antibody against rough mutant bacteria, the route of infection, and different methods of antisera preparation. Since CF_1 mice were used both by *McCabe* et al. (2) and in this study, whereas *Chedid* et al. (1) and *Greisman* et al. (5) used Swiss albino mice, the strain of experimental mice does not seemingly explain the divergent findings. Other investigators have used several different bacterial challenge organisms without evidence of a differing protective effect of hyperimmune and nonimmune sera (1, 2, 4, 5, 13). Furthermore, the selection of the challenge organism in this study on the basis of its demonstrable surface reactivity with anti-J5 antisera did not result in enhanced passive protection with hyperimmune sera. The possible importance of the route of infection is suggested by *Robson* (14) who found that active immunization of rats with a J5 mutant conferred significant protection against intraperitoneal but not intranasal challenge with one strain of *Pseudomonas aeruginosa,* whereas the reverse was observed with another *P. aeruginosa* strain. However, *Greisman* et al. (5) found that whereas the route of infection affected mortality, the passive immunoprophylactic activity of the different rabbit sera was similar in mice infected either i. v. or i. p. Finally, anti-rough mutant sera have been raised by other investigators with heat-killed rather than acetone-dried organisms (1, 2, 4, 5, 13), as was the case in the present studies. The effect of this difference is unknown and deserves further study, since acetone treatment of the rough mutant could affect the lipid content of the cell wall outer membrane.

The lack of enhanced protection from anti-J5 antisera in both this study and that of *Greisman* et al. (5) also contrasts with the recent finding of *Ziegler* et al. (15) in humans. In their study, human antisera against the J5 mutant administered to patients shortly after the onset of gram-negative bacteremia resulted in significantly reduced mortality in comparison to that of patients who received preimmune control human sera, indicating that these antisera may have an additive effect to that of antibiotics in the treatment of these infections. These conflicting results further indicate that the factors mediating humoral resistance to gram-negative infections are poorly understood, and suggest the need for further studies.

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