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Experimental Gram-Negative Bacterial Sepsis: Prevention of Mortality Not Preventable by Antibiotics Alone

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Outbred Swiss mice were inoculated intraperitoneally or intravenously with one 90 to 100% lethal dose of *Escherichia coli* O:18, *Proteus mirabilis*, or *Klebsiella pneumoniae*. After carefully timed intervals, aminoglycoside antibiotics were begun at dosages and intervals predetermined to constitute optimal therapy. With progressive increases in delay of antibiotic therapy, mortality rates increased progressively from 0% to 90 to 100%. Standardized models of infection were developed by selecting delay periods before initiating antibiotic therapy such that 50 to 70% mortalities resulted. Utilizing these models, agents with reputed anti-endotoxin activity were administered concomitantly with the delayed antibiotic therapy to determine if any could prevent gram-negative septic mortality no longer preventable by the antibiotics alone. The following were observed: (i) adrenal corticosteroids prevented mortality that was no longer preventable by optimal aminoglycoside antibiotic therapy alone; (ii) specific antisera also did so, provided anaphylaxis was circumvented; (iii) in one model (*P. mirabilis*), such protection by adrenal corticosteroids and specific antiserum could be additive; (iv) adrenal corticosteroids and specific antiserum acted synergistically with the aminoglycoside antibiotics—no protection was achieved by delayed administration of the steroids or antiserum alone; (v) timing was crucial—the synergistic protective activity of adrenal corticosteroids and of specific antiserum with aminoglycosides declined rapidly as infection progressed; (vi) cyclophosphamide pretreatment markedly impaired the synergistic protective activity of specific antiserum and of adrenal corticosteroids with aminoglycosides; (vii) no reputed anti-endotoxin agents other than adrenal corticosteroids and specific antiserum proved capable of preventing mortality not preventable by aminoglycoside antibiotics alone. These included antisera to rough mutant *Enterobacteriaceae* of Rc, Rd, and Re chemotypes, anticoagulants (heparin), ascorbic acid, antiproteolytic agents (aprotinin), alpha adrenergic blockers (phenoxybenzamine), prostaglandin synthetase inhibitors (acetylsalicylic acid, sodium salicylate, indomethacin), nicotinamide, glucose, and insulin-glucose-potassium mixtures.

During the past several decades, a number of agents have been identified that are capable of mitigating the pyrogenic and toxic activities of gram-negative bacterial endotoxins (1, 3-6, 9, 12-18, 20, 21, 23-25, 29-31, 33, 36, 39, 42, 43, 46, 47). Some of these agents have also been found capable, when tested in experimental models, of reducing mortality from gram-negative bacterial infection. Thus, antisera raised against certain rough mutant gram-negative bacteria, possessing the ability to mitigate the toxicity of endotoxins from heterologous bacterial species, significantly reduced mortality from *Klebsiella pneumoniae*, *Escherichia coli*, and *Pseudomonas aeruginosa* in agranulocytic rabbits when given early in the course of bacteremia (7, 48). Another agent with anti-endotoxin activity, cortisone, reduced mortality when given 1 h before challenge of mice with *Salmonella typhi* or *Neisseria meningitidis*, although it was not protective against challenge with several other gram-negative bacterial species (9). Although studies such as these indicate that some anti-endotoxin agents can reduce mortality from gram-negative sepsis, given the current availability of potent antibiotics, the more crucial issue is whether agents possessing anti-endotoxin activity can prevent mortality that is not preventable by appropriate antibiotic therapy alone. The present studies, utilizing a critically controlled experimental murine model, were designed to answer this question. The findings indicate that of

all the reputed anti-endotoxin agents tested, only specific antiserum and adrenal corticosteroids proved capable of reducing mortality during antibiotic-treated, gram-negative bacterial sepsis. The conditions under which this protection was achieved and the potential hazard of specific antiserum therapy are described.

MATERIALS AND METHODS

Infecting agents. Three bacterial species were used for the study of gram-negative sepsis: *E. coli* O:18, isolated from the urine of a patient with acute pyelonephritis; *Proteus mirabilis*, also isolated from the urine of a patient with acute pyelonephritis; and *K. pneumoniae*, Caroli strain, type II, obtained from Louis Chedid of the Pasteur Institute and kindly provided by William McCabe of Boston University Medical Center. The organisms were maintained at 4°C on Trypticase soy agar (BBL) slants and subcultured every 4 to 6 weeks. In vitro sensitivities of each of these microbes to aminoglycoside antibiotics, determined by the minimal inhibitory concentrations of the antibiotic in Trypticase soy broth (BBL) at pH 7.4 and 37°C were as follows: *E. coli* O:18, 3.1 μg of gentamicin per ml; *P. mirabilis*, 6.25 μg of kanamycin per ml; *K. pneumoniae*, 1.0 μg of gentamicin per ml. In each case, bactericidal levels of antibiotic were achieved with twofold-higher concentrations.

Experimental infections. Outbred Swiss albino mice of mixed sexes, 20 to 25 g, were housed 10 per cage and fed antibiotic-free Purina Lab Chow during an acclimatization period of 5 to 7 days. Immediately before each study, each colony of 10 animals was randomly divided into test and control groups and inoculated intraperitoneally (i.p.) or intravenously (i.v.) with 16- to 18-hour cultures of *E. coli*, *P. mirabilis*, or *K. pneumoniae*. These organisms had been grown in Trypticase soy broth at 37°C, harvested by centrifugation, washed with pyrogen-free sterile physiological saline at room temperature, and suspended in the saline at concentrations determined turbidometrically at 580 nm. One-milliliter suspensions were used for i.p. challenge and 0.25 ml was used for i.v. challenge. Control and test animals were always challenged with the bacterial suspensions in an alternate manner to minimize possible effects of changing bacterial numbers and viability during the total injection period. Immediately after i.p. inoculation, each animal was examined for external leakage. Small differences (<10) in numbers of control and test animals (see Results) reflect the rejection of animals exhibiting leakage of more than 3 drops of the inoculum. In a few studies, larger differences in numbers of control and test animals are reported (controls numbering approximately twice the test group). This is due to use of three groups (one control and two test groups), of which one test group was injected with an agent not pertinent to the present report. The chi-square test was used to determine statistical significance for comparisons of mortality. In a few instances, the same control group was involved in a series of such comparisons. In these instances, Scheffe's multiple comparisons test (8) was applied when $P < 0.05$ by the chi-square test.

Therapeutic agents. Antisera. Antisera were prepared to each of the smooth challenge strains described above as well as to the following rough gram-negative bacterial mutants: *Salmonella minnesota* 595, chemotype Re, obtained through the courtesy of Siegfried Schlecht, Max Planck Institut für Immunbiologie, Freiburg; *Salmonella typhimurium* SL 1032, chemotype Rd, generously supplied by Lawrence Rothfield, University of Connecticut; and a rough J5 mutant of *E. coli* O:111, chemotype Rc, obtained from Edward Heath, University of Iowa, and kindly provided by Rhona Stein, University of Pittsburgh. For immunization, the smooth organisms were grown overnight in Trypticase soy broth at 37°C; for the rough mutants, six to ten typical rough colonies of a given bacterial species were selected from blood agar plates and collectively grown overnight at 37°C in Trypticase soy broth. All broth cultures were washed three times in sterile pyrogen-free physiological saline, heated in saline at 100°C for 10 min, and suspended in the saline at a concentration of 10^8 per ml. Immediately before the rough mutant broth cultures were heated, aliquots were inoculated on blood agar to confirm the absence of significant reversion to smooth forms. Antiserum was obtained by injecting albino New Zealand rabbits i.v. with 1.0-ml aliquots of the heat-killed washed bacterial suspension for 3 consecutive days of each week for 2 weeks and bleeding 7 days after the sixth dose of antigen. Preimmune serum from the corresponding donors was obtained 5 to 7 days before immunization and pooled in the same proportions as the immune sera. All sera were stored at −20°C until use. Aliquots, 0.1 ml, of serial dilutions of the sera in physiological saline, starting at 1:10, were mixed with 0.1 ml of suspensions (10^8 per ml) of heat-killed organisms previously washed three times in physiological sterile saline; the mixtures were agitated for 60 min on a Boerner-type rotating machine at approximately 120 rotations per min at room temperature, and agglutination was read at ×100 magnification. Known negative and positive serum controls were always run concurrently. The "fine" pattern of autoagglutination that occurred with the dilute suspensions of heat-killed rough mutants in 0.9% saline could be readily distinguished from the coarse clumping that occurred in the presence of antibody by simultaneously comparing the antiserum-induced agglutination patterns with those produced in saline or preimmune serum diluted in saline. The final antibody titers were taken as the highest dilution of sera producing an agglutination pattern that could be consistently differentiated from that occurring in saline with the readings performed in "blind" fashion. Postimmunization agglutination titers to the challenge organisms, *E. coli* O:18, *P. mirabilis*, and *K. pneumoniae*, determined by this microagglutination technique, were 1:5,120, 1:2,560, and 1:640, respectively. Postimmunization agglutinating antibody titers to the rough mutants ranged between 1:2,560 and 1:10,240. The preimmunization pooled sera exhibited no detectable (<1:10) agglutinating antibody titers to the smooth challenge strains of bacteria or to the rough mutants used in these studies. The antisera to the Re 595 *S. minnesota* and J5 *E. coli* rough mutants were also titrated for antibody against their respective lipopolysaccharides. For purposes of check-

ing the antigenic composition of the currently used rough mutants, these purified lipopolysaccharides were obtained from other investigators. The lipopolysaccharide from the J5 mutant of *E. coli* O:111 (extracted with phenol-water) was kindly provided by Abraham Braude, University of California. This was treated with sodium hydroxide before absorption onto human O erythrocytes for hemagglutination (HA) titration as described by Neter and co-workers (34). The Re 595 *S. minnesota* lipopolysaccharide (extracted with phenol-chloroform-petroleum ether and treated with sodium hydroxide for use in the HA titration) was a generous gift from Otto Luderitz, Max Planck Institut für Immunbiologie. The pooled antisera to the Re 595 *S. minnesota* mutant exhibited an HA titer of 1:640, and the pooled antisera to the J5 mutant of *E. coli* exhibited an HA titer of 1:2,560. The preimmune pooled sera possessed HA titers less than 1:10. Antibody to these rough mutant lipopolysaccharides were also titrated by using the bentonite flocculation technique (45). (Of interest, unlike smooth lipopolysaccharides, the J5 *E. coli* rough mutant lipopolysaccharide required sodium hydroxide treatment as described for passive HA [34] to permit sufficient absorption to bentonite to detect flocculating antibody. The Re 595 *S. minnesota* lipopolysaccharide was provided to us already treated with sodium hydroxide; the non-alkali-treated antigen was therefore not tested for bentonite absorption.) The relationship of antibody titers obtained by the bentonite flocculation technique to those obtained by HA and bacterial agglutination techniques in individual rough mutant antisera is shown in Table 1. The pooled antisera to the J5 *E. coli* and to the Re 595 *S. minnesota* rough mutants used for protection studies each exhibited bentonite flocculation titers of 1:640, compared to less than 1:10 in the preimmune pooled sera. These rough mutant antisera were also examined for antibody to lipopolysaccharides extracted from their respective smooth wild-type parent organisms. The pooled Re 595 *S. minnesota* antiserum exhibited a bentonite flocculating titer of less than 1:10 (the smooth lipopolysaccharide was obtained from Difco Laboratories), and the J5 *E. coli* antiserum exhibited a titer of 1:160 (the smooth lipopolysaccharide was kindly provided by Abraham Braude). This contrasted with titers of 1:640 and 1:1,280, respectively, in comparably prepared antisera against the smooth wild-type parent strains. For protection studies, all antisera were injected i.v. in 0.25-ml amounts at times specified in Results; control animals were given 0.25 ml of normal sera consisting of comparably pooled aliquots of preimmune sera from the corresponding donors.

Agents other than antisera. Unless otherwise specified, each of the following were diluted in sterile pyrogen-free physiological saline immediately before use and administered intramuscularly (i.m.) in 0.20-ml volumes via 26-gauge needles in dosages and at times specified in Results: antibiotics—gentamicin sulfate (Garamycin, Schering Corp.) and kanamycin sulfate (Kantrex, Bristol Laboratories); adrenal corticosteroids—methylprednisolone sodium succinate (Solumedrol, The Upjohn Co.) and dexamethasone sodium phosphate (Decadron, Merck, Sharp & Dohme); other reagents—sodium heparin (The Upjohn Co.), ascorbic acid (Cevalin, Eli Lilly & Co.), aprotinin (Trasylol, FBA Pharmaceuticals), phenoxybenzamine (Dibenzyline, Smith, Kline & French Laboratories), glucagon (Eli Lilly & Co.), niacinamide (Eli Lilly & Co.), nicotinic acid (Niacin, Eli Lilly & Co.), glucose (Dextrose, McGaw Laboratories), and insulin (Iletin, Eli Lilly & Co.). Three prostaglandin synthetase inhibitors were also studied: (i) buffered acetylsalicylic acid, prepared by adding Alka-Seltzer tablets (Miles Laboratories) to sterile pyrogen-free water as described by Hinshaw and co-workers (25)—control animals received comparable quantities of the inert ingredients (sodium bicarbonate and citric acid) in sterile pyrogen-free water; (ii) sodium salicylate (Fisher Scientific Co.), diluted in sterile pyrogen-free physiological saline; and (iii) indomethacin (Indocin, Merck, Sharp & Dohme), dissolved in sterile pyrogen-free phosphate buffer, pH 8.0, and administered i.v. in 0.20-ml volumes; control animals were given buffer alone. All the prostaglandin synthetase inhibitors were membrane filtered (Millipore Corp.) and shown to be bacteriologically sterile.

RESULTS

Development of antibiotic-treated models of gram-negative bacterial sepsis. (i) Quantitation of mortality in non-antibiotic-treated mice. Mortality dose-responses after *E. coli* O:18, *P. mirabilis*, and *K. pneumoniae* challenge were initially defined without antibiotic treatment and are shown in Fig. 1. Within the sensitive dose-response ranges, i.p. challenge

TABLE 1. *Comparative antibody titrations in individual rabbit antisera to rough mutant Enterobacteriaceae*

Technique	Reciprocal antibody titer[a]								
	Anti-J5 *E. coli* O:111				Anti-595 Re *S. minnesota*				
	1	2	3	4	5	6	7	8	9
Bentonite flocculation	320	640	640	1,280	320	320	320	640	640
Passive HA	640	2,560	2,560	10,240	320	320	320	640	1,280
Bacterial agglutination	2,560	5,120	5,120	10,240	1,280	2,560	2,560	2,560	2,560

[a] Antibody titers of each antiserum were assessed against their respective antigens. Preimmune sera possessed antibody titers ≤10.

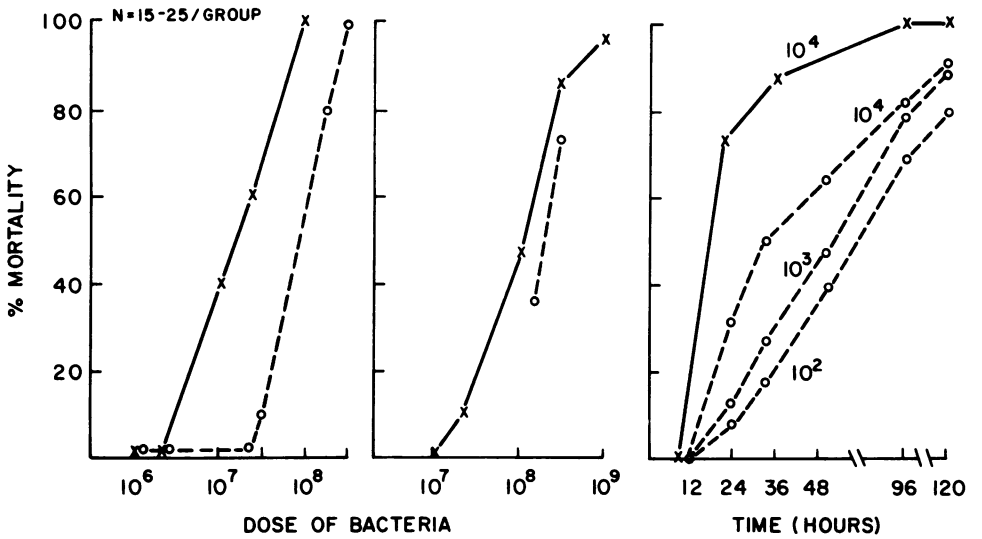

FIG. 1. *Dose-response relationships for E. coli O:18 (left), P. mirabilis (center) and K. pneumoniae type II (right) mortality in outbred Swiss mice. E. coli O:18 and P. mirabilis mortality were recorded at 96 h. Symbols:* (×) *i.p.;* (○) *i.v.*

consistently resulted in higher mortality rates than i.v. challenge. With either route, mortality from *E. coli* and *P. mirabilis* increased minimally after 36 h, and no further deaths occurred after 96 h. In all subsequent studies, mortality rates after challenge with these organisms were therefore recorded at 96 h. In contrast, *K. pneumoniae* proved lethal regardless of inoculum size, the latter determining only the rate of mortality as reported previously by McCabe (32). In the subsequent studies, mortality rates after *K. pneumoniae* challenge were therefore recorded daily. For all three challenge species, 5×10^8 heat-killed suspensions administered i.v. or i.p. produced no mortality, suggesting that bacterial replication was required for lethality when inocula of 90 to 100% lethal doses (LD_{90-100}), or less, were used.

(ii) Definition of "optimal" antibiotic schedules and of standard delay periods. In preliminary studies, 150 mice were inoculated i.p. with one LD_{100} of *E. coli* O:18 (10^8), and at hourly intervals 25 animals were selected sequentially and given a single i.m. injection of 0.1 mg of gentamicin. Mortality increased progressively with delay in antibiotic administration; an 80% mortality occurred after a 2-h delay. Using this 2-h delay, additional studies were carried out with various dose schedules of gentamicin (Table 2). A schedule of 0.1 mg of gentamicin every 2 h for 32 h (omitting the 2 a.m. to 6 a.m. doses) yielded the lowest mortality rate, and i.m. administration was virtually as effective as that via the i.v. route. Continued gentamicin therapy beyond 32 h was found in other studies not to further reduce mortality significantly, and the above schedule was therefore selected as optimal. That optimal therapy should require such

TABLE 2. *Effect of dosage schedule of gentamicin on mortality of Swiss mice from E. coli O:18 (10^8 i.p.)*

Expt	Gentamicin			Mortality	
	Dose (mg/mouse)	Route	Schedule[a]	*n*	%
1	0	i.m.	A	40	100
	0.01	i.m.	A	29	97
	0.10	i.m.	A	61	77
	1.0	i.m.	A	95	66
2	0.10	i.m.	A	26	81
	0.10	i.v.	A	27	74
3	0.10	i.m.	A	33	79
	0.10	i.m.	B	39	62
	1.0	i.m.	B	39	62
	0.01	i.m.	C	39	80
	0.10	i.m.	C	40	53
	1.0	i.m.	C	33	61
4	0.10	i.m.	C	35	57
	0.10	i.v.	C	35	54

[a] A = gentamicin, single dose, 2 h after *E. coli* inoculation; B = same as A, but then repeated every 8 h for 72 h; C = same as A, but then repeated every 2 h for 32 h, omitting 2 a.m. to 6 a.m. doses.

closely spaced injections of gentamicin was anticipated from its reported rapid elimination from the circulation of mice (44), as well as from the rapid decline of its protective activity as determined by administering single doses of the antibiotic at varying intervals before, rather than after, challenge (Table 3).

Using the above-determined optimal gentamicin dosage schedule, the effect of delay in institution of such therapy was titrated in a single large group of infected animals (Fig. 2). The ability of gentamicin to completely prevent mortality when begun concomitantly with i.p. inoculation of *E. coli* O:18 and the progressive refractoriness to the antibiotic therapy with progressive delay in initiation of such therapy are apparent. A delay of 2 h before initiation of the optimal therapy yielded a mortality within the 50 to 70% range, and this was selected as the "standard delay period" for the subsequent studies of *E. coli* O:18 sepsis.

Using the optimal gentamicin dose schedule determined for *E. coli* O:18 sepsis, the effect of delay in initiation of such therapy was then determined for *K. pneumoniae* sepsis, and these results are also depicted in Fig. 2. Again, the ability of gentamicin to completely prevent mortality when begun concomitantly with i.p. inoculation of *K. pneumoniae* and the progressive increase in refractoriness to the antibiotic as therapy was delayed are apparent. Unlike *E. coli* O:18 sepsis, it was found necessary to continue the gentamicin beyond 32 h at a dose of 0.1 mg i.m. every 6 to 8 h, or else mortality, which tended to plateau by 48 h, would again begin to increase progressively. To ensure a "steady state," mortality rates for *K. pneumoniae* were recorded at 96 h, while the animals were still receiving antibiotic. A delay of 5.5 h in initiating such gentamicin therapy yielded mortality rates within the 50 to 70% range, and this was selected as the standard delay period for the subsequent studies of *K. pneumoniae* sepsis.

TABLE 3. *Effect of pretreatment of Swiss mice with a single dose of aminoglycoside antibiotics on mortality from E. coli O:18 or P. mirabilis*

Time antibiotic[a] given (h before challenge)	Mortality			
	E. coli O:18 (10^8 i.p.)		*P. mirabilis* (5×10^6 i.p.)	
	n	%	*n*	%
−2	10	100	22	82
−1.5	10	80	21	90
−1.0	18	67	23	61
0	20	5	30	0

[a] Gentamicin, 0.10 mg/mouse i.m., for *E. coli* O:18 sepsis. Kanamycin, 0.5 mg/mouse i.m., for *P. mirabilis* sepsis.

The optimal antibiotic dosage schedule for *P. mirabilis* sepsis, using kanamycin as the antibiotic, was determined in a manner comparable to that outlined above in the gentamicin studies. Thus, in a preliminary study, 150 mice were inoculated i.p. with one LD_{90-100} of *P. mirabilis* (5×10^8), and at hourly intervals 25 animals were selected sequentially and given single i.m. injections of 0.5 mg of kanamycin. Mortality increased progressively with delay in antibiotic administration; an 84% mortality occurred after a 3-h delay. Using this 3-h delay, additional studies were carried out to define the optimal dosage schedule of kanamycin (Table 4). A kanamycin schedule of 0.5 mg i.m. every 2 h for 32 h (omitting the 2 a.m. to 6 a.m. doses) produced the lowest mortality rate, and i.m. administration was as effective as the i.v. route. Continued kanamycin therapy beyond 32 h was found in other studies not to further reduce mortality significantly, and the above schedule was therefore selected as optimal. As with gentamicin, the requirement for closely spaced injections of kanamycin for optimal therapy was anticipated from its reported rapid elimination from the circulation of mice (26), as well as from the rapid decline in its protective activity as determined from studies with single doses of the antibiotic given at varying intervals before challenge (Table 3).

Using the above-determined optimal kanamycin dosage schedule for *P. mirabilis* sepsis, the effect of delay in initiation of such antibiotic therapy was titrated, and the results are depicted in Fig. 2. The ability of kanamycin to completely eliminate mortality when begun concomitantly with, or up to 1.5 h after, i.p. inoculation of *P. mirabilis* and the progressive refractoriness to antibiotic therapy after relatively short further delays in initiation of such therapy are apparent. A delay in kanamycin therapy of 3.5 ± 0.5 h yielded mortalities within the 50 to 70% range, and this interval was selected as the standard delay period for the subsequent studies of *P. mirabilis* sepsis.

Prevention of mortality not preventable by optimal antibiotic therapy alone. In the previous section, three models of gram-negative bacterial sepsis were developed which defined a standard delay period after which time, despite initiating optimal aminoglycoside antibiotic therapy, mortality rates approximated 50 to 70%. In the subsequent studies the following agents, reputed to possess anti-endotoxin activity, were administered concomitantly with the optimal antibiotic therapy after the standard delay pe-

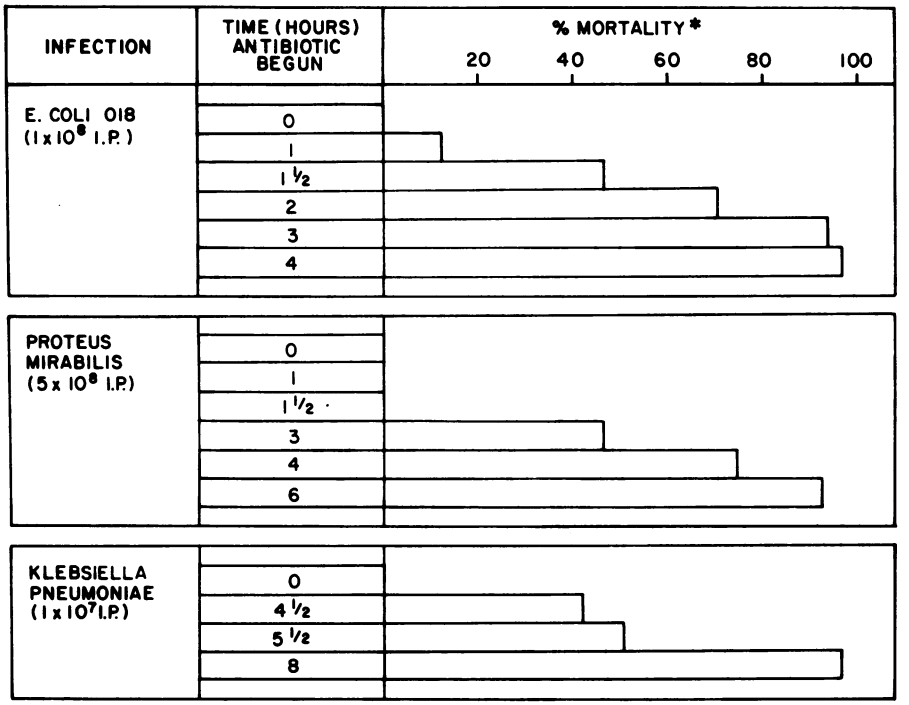

FIG. 2. *Effect of delay in initiating optimal aminoglycoside antibiotic therapy on mortality after challenge of Swiss mice with E. coli O:18, P. mirabilis, or K. pneumoniae. Gentamicin was given i.m. for E. coli and K. pneumoniae infections; kanamycin was given i.m. for P. mirabilis infection. All mortality rates were recorded at 96 h. * n = 25 to 30 per group. (See text for dosage schedules of antibiotics.)*

riod to determine if any could prevent mortality no longer preventable by the antibiotic alone.

(i) Antiserum. The effect of i.v. administration of rabbit antisera to a variety of gram-negative bacterial antigens was evaluated during *E. coli* O:18 sepsis (Fig. 3). No significant protection was discernible with normal rabbit serum or with antisera against the Re chemotype (595 mutant) of *S. minnesota*, the Rc chemotype (J5 mutant) of *E. coli* O:111, or the Rd chemotype (SL 1032 mutant) of *S. typhimurium*. Nor was protection observed with antisera against a smooth heterologous strain of *E. coli* (*E. coli* O: 111). In contrast, specific antiserum to the *E. coli* O:18 conferred highly significant protection ($P < 0.001$). This protection by the specific antiserum required the presence of the concomitant antibiotic activity since comparably delayed administration of the antiserum alone gave no protection. When treatment with antibiotic and specific antiserum were both postponed beyond the standard delay period by 1 additional h (total of 3 h after challenge), the antiserum no longer potentiated the antibiotic protection.

The effect of i.v. administration of rabbit antisera to a variety of gram-negative bacterial antigens was similarly evaluated after the standard delay period during *P. mirabilis* sepsis (Fig. 4). As with *E. coli* O:18 sepsis, no protection was conferred by normal rabbit serum or by antisera against the Rc chemotype (J5 mutant) of *E. coli* O:111 or the Re chemotype (595 mutant) of *S. minnesota*. In contrast, specific antiserum to *P. mirabilis* exerted a highly significant protective effect ($P < 0.001$). Moreover, as with *E. coli* O: 18 sepsis, this protection by specific antiserum required the presence of the concomitant antibiotic activity since comparably delayed administration of specific antiserum alone gave no significant protection. Similarly, the potentiation of protection by specific antiserum was no longer evident when the antibiotic and antiserum therapy were both postponed 1 h beyond the standard delay period (total of 4.5 h after challenge). The ability of specific antiserum to prevent mortality not preventable by aminoglycoside antibiotic alone extended to sepsis induced by i.v. *P. mirabilis* challenge (Fig. 4), and again, postponement of therapy by 1 h beyond the standard delay period led to an appreciable

decline in effectiveness of the antiserum protection.

The effect of i.v. administration of rabbit antisera to various gram-negative bacterial antigens was also evaluated after the standard delay period during *K. pneumoniae* sepsis. As in the *E. coli* O:18 and *P. mirabilis* studies, utilizing control and test groups of 40 animals each, no protection was observed with normal rabbit sera or with rabbit antisera to the Re chemotype (595 mutant) of *S. minnesota* or the Rc (J5) mutant of *E. coli* O:111, regardless of whether the *K. pneumoniae* sepsis was induced by i.p. or i.v. challenge. However, in contrast to the *E. coli* O:18 and *P. mirabilis* sepsis, specific antisera to the *K. pneumoniae* given after the standard delay period consistently provoked immediate (<10 min) mortality in approximately 50% of the treated animals. Since such immediate deaths were never seen when aliquots of the same anti-*K. pneumoniae* serum were given during *E. coli* O:18 or *P. mirabilis* sepsis, and since the same specific antiserum conferred high levels of protection against *K. pneumoniae* when given as pretreatment, the acute mortality appeared to represent "reversed" anaphylaxis to antigens liberated during *K. pneumoniae* sepsis. Other studies indicated that with the given i.p. inoculum of *K. pneumoniae* (10^7), such anaphylaxis became elicitable only after 4 h of sepsis, occurred whether or not antibiotics were used, and developed with both immunoglobulin G and immunoglobulin M (but not albumin) fractions of the antiserum.

TABLE 4. *Effect of dosage schedule of kanamycin on mortality of Swiss mice from P. mirabilis (5 × 10^8 i.p.)*

Expt	Kanamycin				
	Dose (mg/mouse)	Route	Schedule[a]	*n*	%
1	0	i.m.	A	17	100
	0.05	i.m.	A	30	100
	0.50	i.m.	A	49	92
	5.0	i.m.	A	47	62
2	0	i.m.	A	20	100
	0.5	i.m.	A	28	86
	0.5	i.m.	B	30	87
	5.0	i.m.	B	30	67
3	5.0	i.m.	B	30	70
	0.1	i.m.	C	32	41
	0.5	i.m.	C	30	23
	5.0	i.m.	C	34	44
4	0.5	i.m.	C	60	33
	0.5	i.v.	C	28	36

[a] A = kanamycin, single dose, 3 h after *P. mirabilis* inoculation; B = same as A, but then repeated every 8 h for 72 h; C = same as A, but then repeated every 2 h for 32 h, omitting 2 a.m. to 6 a.m. doses.

Since the antisera against the Re (595) and Rc (J5) mutants of *S. minnesota* and *E. coli* O:111, respectively, failed in each of the above models to prevent mortality not preventable by aminoglycoside antibiotics alone, these studies were repeated using new pools of antisera to the rough mutants. The additional pool of Re (595) mutant *S. minnesota* antiserum was prepared against a culture of this rough mutant generously furnished a second time by Siegfried Schlecht, and the additional pool of Rc (J5) mutant *E. coli* antiserum was prepared using a boiled suspension of these organisms kindly supplied by Abraham Braude. The antisera against these additional rough mutants were prepared as described above for the initial studies and had been stored at −20°C for several years. Antibody titers in these pooled antisera were as follows: anti-Re (595) *S. minnesota*—bacterial agglutinating titer = 1:2,560, HA titer = 1:640, and bentonite flocculation titer = 1:640; anti-Rc (J5) *E. coli*—bacterial agglutinating titer = 1:2,560, HA titer = 1:640, and bentonite flocculation titer = 1:320. Neither pool of rough mutant antisera possessed bentonite flocculation antibody titers greater than 1:10 against lipopolysaccharides from their respective smooth wild-type parent organisms, in contrast to titers of 1:640 in concomitantly prepared pooled antisera against the smooth wild-type parent strains. The results obtained with these second preparations of anti-rough mutant sera in the antibiotic-treated models of *E. coli* O:18, *P. mirabilis*, and *K. pneumoniae* were entirely comparable to the initial trials reported above; i.e., in groups of 35 to 40 animals each, no protection was observed when compared to similar numbers of controls treated with the corresponding preimmune sera lacking detectable antibodies against the rough mutant and challenge bacterial strains.

(ii) Adrenal corticosteroids. In the following studies, each test animal received two i.m. injections each of either 30 mg of methylprednisolone sodium succinate per kg in sterile pyrogen-free physiological saline or 8 mg of dexamethasone sodium phosphate per kg. (Since the dexamethasone was supplied in a solution containing parabens as a preservative, control animals were given sterile pyrogen-free physiological saline containing comparable concentrations of the parabens.) The use of two steroid injections, and their relatively high dosages, was based upon the report of the effectiveness of such regimens during antibiotic-treated, gram-

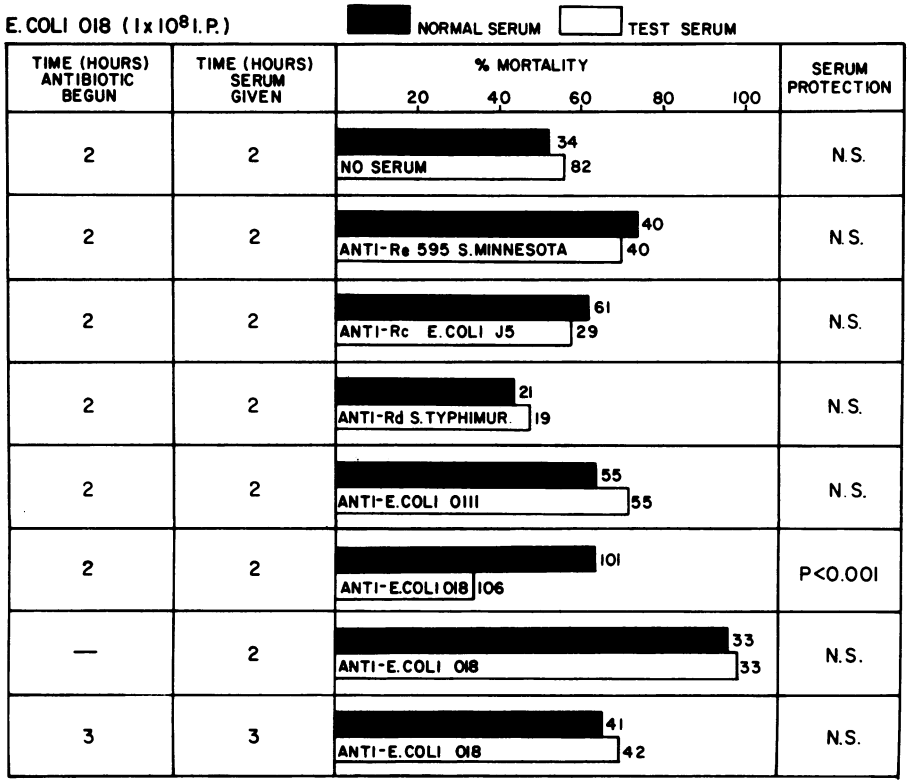

FIG. 3. *Ability of rabbit antisera (0.25 ml) administered i.v. to Swiss mice to prevent mortality from E. coli O:18 (10^8 i.p.) not preventable by optimal aminoglycoside antibiotic therapy (gentamicin) alone. Numbers after bars in this and subsequent figures represent the number of animals in each trial. (See text for dosage schedule of antibiotic.) (Solid bar) Normal serum; (open bar) test serum. N.S., Not significant.*

negative bacterial sepsis in humans (41). The effect of both adrenal corticosteroids on mortality was virtually identical in all studies, and the data are therefore considered collectively under the term "steroid" therapy.

The effect of steroid administration after the standard delay period during *E. coli* O:18 sepsis is shown in Fig. 5. Steroids prevented mortality not preventable by optimal doses of gentamicin alone. Such protection, however, was not dramatic, and relatively large numbers of animals were required to demonstrate statistical significance. The administration of steroids before the standard delay period (i.e., beginning at 0 h) provided more effective protective activity. No significant protection by steroids was evident when antibiotic dosage was suboptimal (each dose one-fifth optimal) or when both antibiotic and steroid therapy were postponed 1 h past the standard delay period (total of 3 h after challenge).

The effect of steroid therapy after the standard delay period during *P. mirabilis* sepsis is shown in Fig. 6. Steroids prevented mortality not preventable by optimal doses of kanamycin alone. As in the *E. coli* O:18 studies, administration of steroids before the standard delay period (i.e., beginning at 0 h) provided even more effective protective activity, and similarly, no significant protection by steroids was evident when antibiotic dosage was suboptimal (each dose one-fifth optimal) or when steroid therapy was postponed for short intervals beyond the standard delay period. It is apparent from Fig. 5 and 6 that the decline of protective activity of steroid with delay in initiating therapy, or with suboptimal antibiotic therapy, cannot be attributed simply to inordinately high mortality rates under these conditions. (The last two series of *P. mirabilis* studies shown in Fig. 6 were carried out 2.5 years after the other studies shown in Fig. 6. Changes in microbial virulence or host resistance, or both, over this period presumably account for the overall reductions in mortality rates.)

The effects of steroid therapy after *K. pneu-*

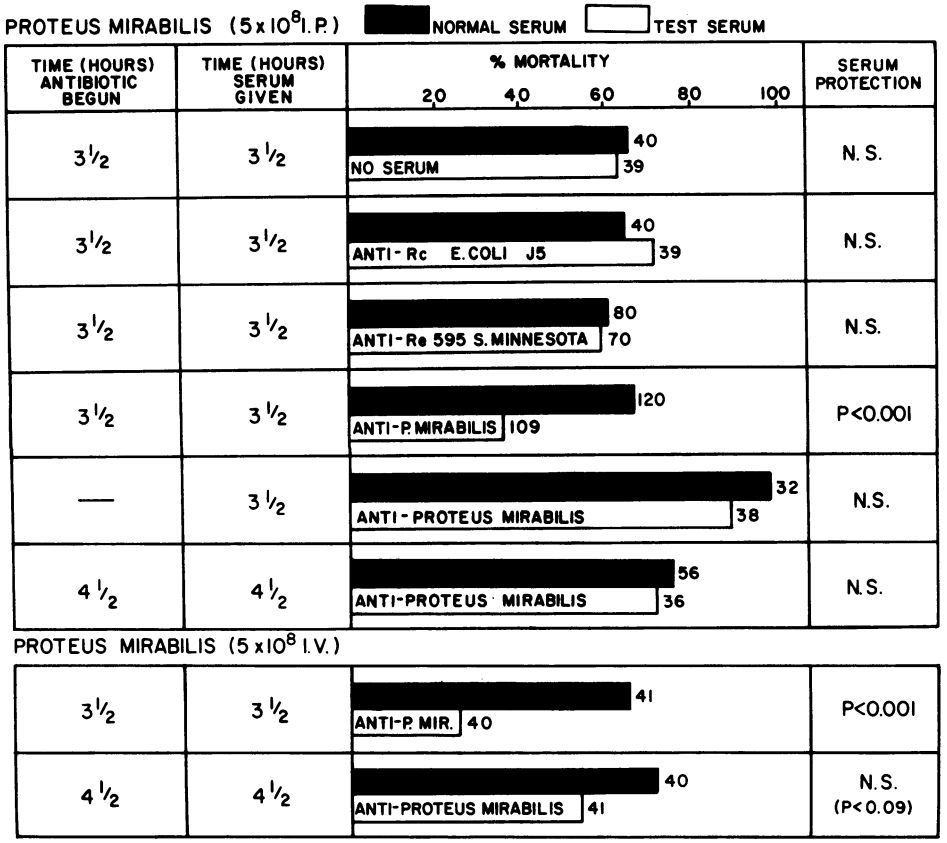

FIG. 4. *Ability of rabbit antisera (0.25 ml) administered i.v. to Swiss mice to prevent mortality from P. mirabilis (5 × 10⁸ i.p.) not preventable by "optimal" aminoglycoside antibiotic therapy (kanamycin) alone. (See text for dosage schedule of antibiotic.) (Solid bar) Normal serum; (open bar) test serum. N.S., Not significant.*

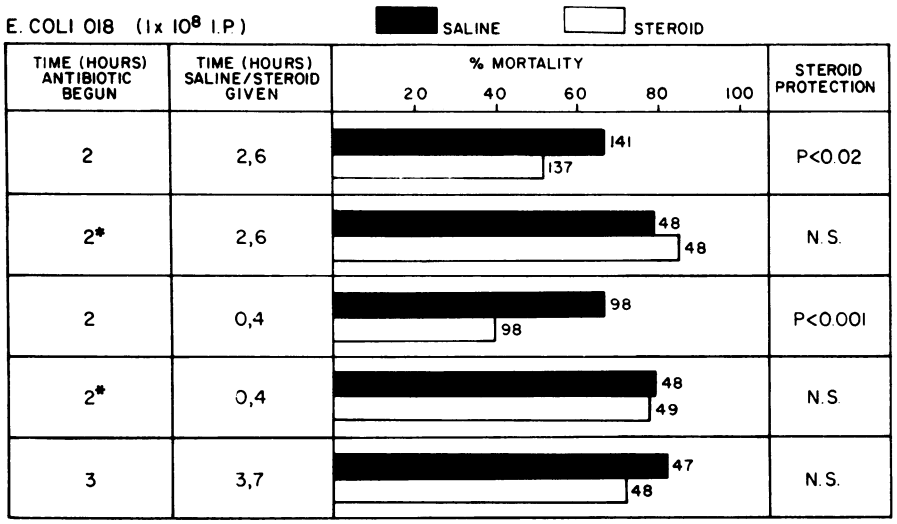

FIG. 5. *Ability of adrenal corticosteroids (30 mg of methylprednisolone or 8 mg of dexamethasone per kg) administered i.m. to Swiss mice to prevent mortality from E. coli O:18 (10⁸ i.p.) not preventable by optimal aminoglycoside antibiotic therapy (gentamicin) alone. (See text for dosage schedule of antibiotic.) (Solid bar) Saline; (open bar) steroid. N.S., Not significant. * Suboptimal antibiotic therapy.*

moniae challenge were similar to those described for *E. coli* O:18 and *P. mirabilis* sepsis (Fig. 7). Steroid given after the standard delay period prevented mortality not preventable by optimal doses of gentamicin alone, and this protective effect waned progressively as therapy with the antibiotic or steroid was delayed further. It is emphasized that these results pertained at 96 h, when mortality rates had reached a plateau and while the animals were continuing to receive aminoglycoside antibiotic therapy.

As with specific antiserum, the ability of ad-

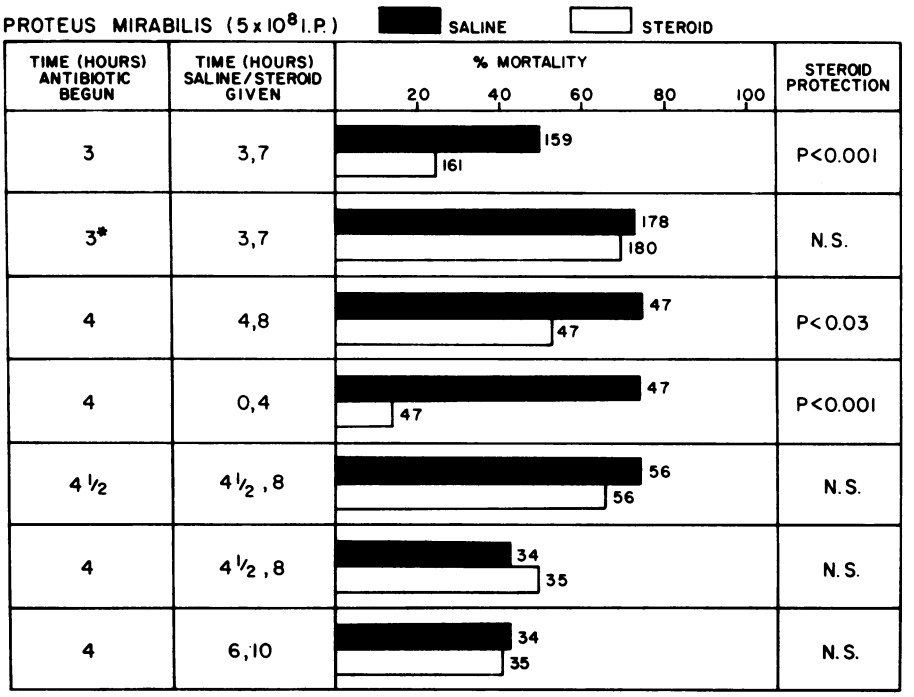

FIG. 6. *Ability of adrenal corticosteroids (30 mg of methylprednisolone or 8 mg of dexamethasone per kg) administered i.m. to Swiss mice to prevent mortality from P. mirabilis (5 × 10⁸ i.p.) not preventable by optimal aminoglycoside antibiotic therapy (kanamycin) alone. (See text for dosage schedule of antibiotic.) (Solid bar) Saline; (open bar) steroid. N.S., Not significant. * Suboptimal antibiotic therapy.*

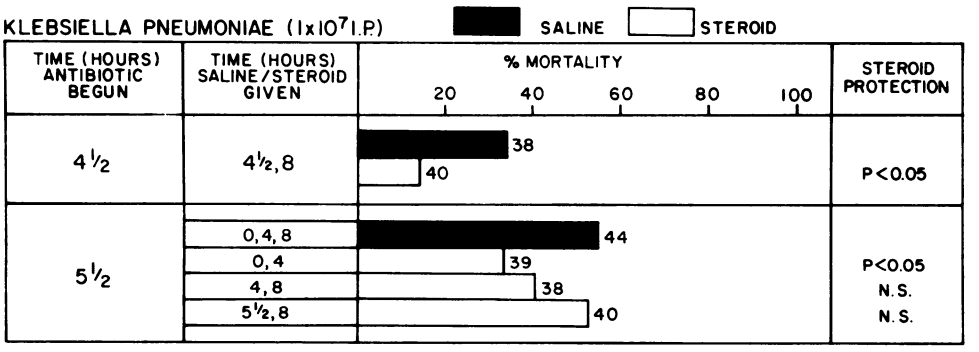

FIG. 7. *Ability of adrenal corticosteroids (30 mg of methylprednisolone or 8 mg of dexamethasone per kg) administered i.m. to Swiss mice to prevent mortality from K. pneumoniae (10⁷ i.p.) not preventable by optimal aminoglycoside antibiotic therapy (gentamicin) alone. If Scheffe's multiple comparisons test is applied to the study where antibiotic was begun at 5.5 h (since one control group is used for several comparisons), the P < 0.05 for protection by steroid given at 0 and 4 h decreases to not significant (N.S.). (See text for dosage schedule of antibiotic.) (Solid bar) Saline; (open bar) steroid.*

renal corticosteroids to prevent mortality no longer preventable by aminoglycoside antibiotics alone required the concomitant activity of the antibiotic. Thus, in the absence of antibiotic therapy, using inocula of *E. coli* O:18 or *P. mirabilis* that produced mortality rates approximating the 50 to 70% levels achieved in the antibiotic treatment studies, no significant protective (or detrimental) effect on mortality was evident with steroid administration (Fig. 8). Similarly, in the absence of antibiotics, steroid failed to retard the rate at which death occurred during *K. pneumoniae* sepsis (Fig. 9). The importance of antibiotics for the protective activity of steroids during sepsis was further evidenced by the late mortality results during *K. pneumoniae* infection. In contrast to the *E. coli* O:18 and *P. mirabilis* models of sepsis, in which discontinuance of antibiotic therapy after 32 h no longer significantly affected late mortality whether or not steroids had been used, mortality from *K. pneumoniae* increased progressively upon discontinuing antibiotic therapy at 96 h, and this increase occurred at an accelerated rate in the steroid-treated group. Within 3 to 6 days after cessation of antibiotic therapy (7 to 10 days after challenge), mortality rates in the steroid-treated group equalled those of the controls.

(iii) Effect of combined antiserum and adrenal corticosteroids. Since specific antiserum and adrenal corticosteroids each proved capable of preventing mortality not preventable by aminoglycoside antibiotics alone during *E. coli* O:18 and *P. mirabilis* sepsis, the effect of combining these agents was evaluated (Fig. 10). In conformity with the previous studies, after the standard delay period during *E. coli* O:18 sepsis, specific antiserum or steroids given concomitantly with gentamicin each prevented mortality not preventable by the administration of the aminoglycoside antibiotic alone. However, no further significant reduction in mortality occurred when the specific antiserum and steroid therapy were combined. Also in conformity with the previous studies, after the standard delay period during *P. mirabilis* sepsis, specific antiserum or adrenal corticosteroids given concomitantly with kanamycin each prevented mortality not preventable by the administration of the aminoglycoside antibiotic alone. In this model, however, specific antiserum and steroid when used together lowered mortality significantly further than either agent alone.

(iv) Effect of specific antiserum and adrenal corticosteroids in cyclophosphamide-treated animals. Since gram-negative bacteremia often occurs in patients with bone marrow suppression or on immunosuppressive therapy, the protective activity of specific antiserum and adrenal corticosteroids in the antibiotic-treated models described above was evaluated in animals treated with cyclophosphamide (Cytoxan, Mead Johnson Laboratories). In preliminary studies, varying amounts of this marrow-suppressive agent were administered i.m. A single dose of 300 mg/kg given 48 h before bacterial challenge increased susceptibility of the Swiss mice to i.p. *P. mirabilis* such that the LD_{80} decreased from 2×10^8 to 1.5×10^7. Such cyclophosphamide-pretreated animals were then tested in a manner similar to that described above to determine if under these conditions specific antiserum and adrenal corticosteroids would continue to prevent mortality not preventable by optimal aminoglycoside antibiotic (kanamycin) administration alone. Cyclophos-

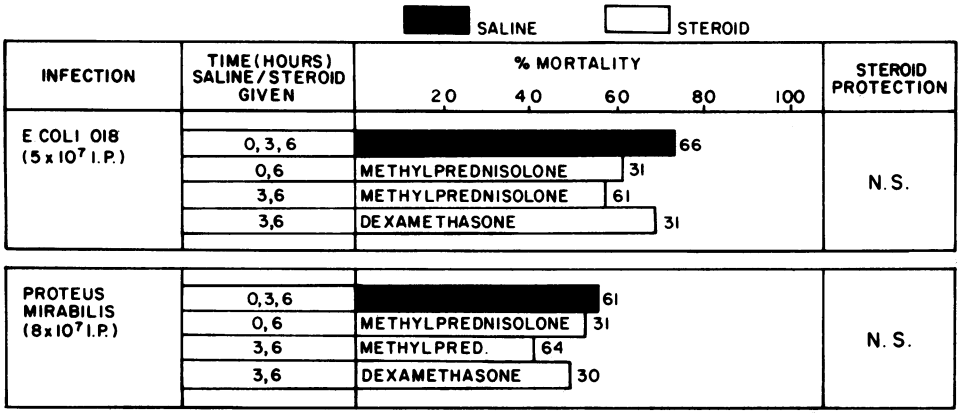

FIG. 8. *Effect of i.m. adrenal corticosteroids (30 mg of methylprednisolone or 8 mg of dexamethasone per kg) on mortality from E. coli O:18 and P. mirabilis in Swiss mice in the absence of antibiotic therapy. (Solid bar) Saline; (open bar) steroid. N.S., Not significant.*

phamide pretreatment profoundly modified the mortality responses to the delayed administration of the aminoglycoside, as well as to the protective activity of concomitantly delayed specific antiserum and adrenal corticosteroids (Fig. 11). Thus, in control animals given saline only ("no Cytoxan" group) and begun on kanamycin 4 h after i.p. *P. mirabilis* inoculation, mortality reached a plateau within 36 h. In contrast, mortality continued to climb steadily in the Cytoxan-treated group. Adrenal corticosteroids or specific antiserum, or both, given concomitantly with kanamycin after the standard delay period remained capable of preventing mortality not preventable by the antibiotic alone in the Cytoxan-pretreated animals, but such protection was not sustained, and by 48 h reductions in mortality were no longer evident. These negative results could not be attributed to a direct toxic effect of kanamycin in the Cytoxan-treated animals. As depicted in Fig. 11 (right panel), kanamycin caused no mortality in Cytoxan-pretreated animals not challenged with *P. mirabilis* and, indeed, completely prevented mortality when begun immediately after *P. mirabilis* inoculation (not shown in Fig. 11).

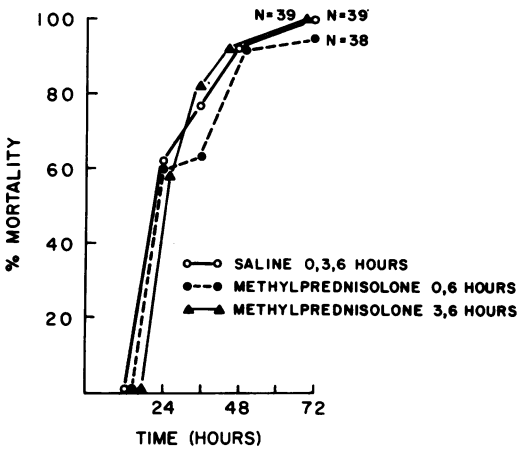

FIG. 9. *Effect of i.m. adrenal corticosteroids (30 mg of methylprednisolone per kg) on mortality from K. pneumoniae (2 × 10⁴ i.p.) in Swiss mice in the absence of antibiotic therapy.*

(v) Effect of anti-endotoxin agents other than antiserum and adrenal corticosteroids. A variety of agents reputed to possess anti-endotoxin activity were studied to determine if any could act similarly to specific antiserum or adrenal corticosteroids to prevent mortality not preventable by aminoglycoside antibiotics alone when begun after the standard delay period (Fig. 12). These agents included heparin, ascorbic acid, aprotinin, phenoxybenzamine, acetylsalicylic acid, sodium salicylate, indomethacin, nicotinamide, glucose, and glucose-insulin-potassium mixtures. Two other agents not reported to possess anti-endotoxin activity, glucagon and nicotinic acid, were also tested. Each agent was administered concomitantly with the delayed initiation of optimal aminoglycoside antibiotic therapy, in the same manner that permitted the

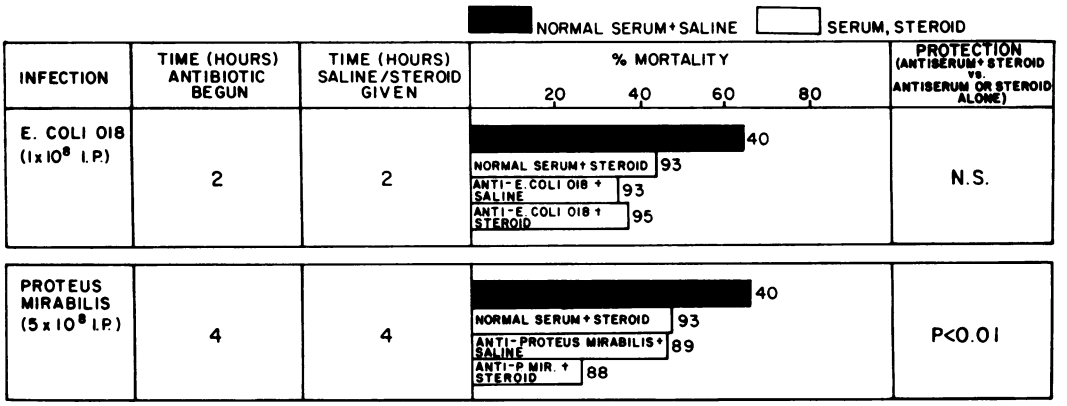

FIG. 10. *Ability of the combination of i.v. specific antiserum (0.25 ml) and i.m. adrenal corticosteroids (30 mg of methylprednisolone per kg) to prevent mortality from E. coli O:18 and P. mirabilis in Swiss mice not preventable by optimal aminoglycoside antibiotic therapy alone. Whereas specific antiserum and adrenal corticosteroids each reduced mortality, the combination of these agents resulted in significant additional protection only during P. mirabilis sepsis. If Scheffe's multiple comparisons test is applied, the difference between anti-P. mirabilis serum + steroid versus either anti-P. mirabilis serum + saline or normal serum + steroid each decreases from $P < 0.01$ to $P < 0.05$. (See text for dosage schedules of antibiotics.) (Solid bar) Normal serum + saline; (open bar) serum, steroid. N.S., Not significant.*

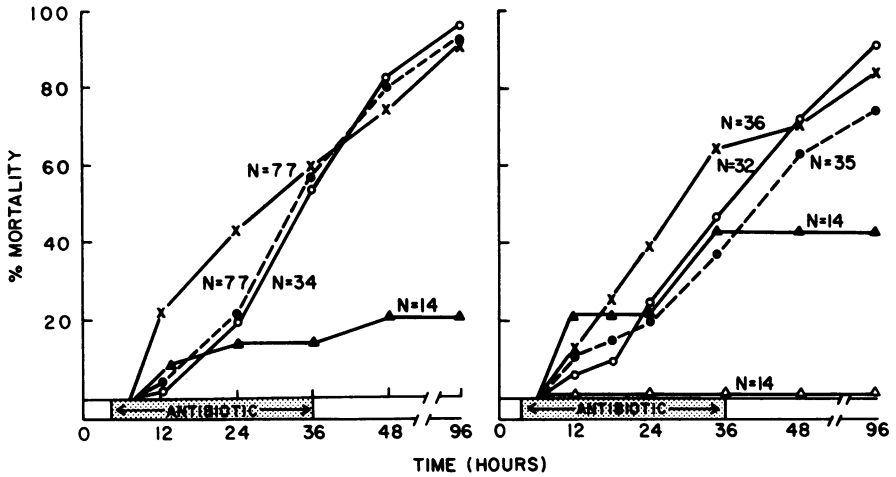

FIG. 11. *Effect of cyclophosphamide (Cytoxan) pretreatment (300 mg/kg) on the ability of i.v. specific antiserum (0.25 ml) and/or adrenal corticosteroids (30 mg of methylprednisolone per kg) to prevent mortality from P. mirabilis (5 × 10^8 i.p.) in Swiss mice not preventable by optimal aminoglycoside antibiotic therapy (kanamycin) alone. (See text for dosage schedule of antibiotic.) Symbols for left panel: Cytoxan pretreatment—(×) saline; (●) methylprednisolone (3, 8 h); (○) methylprednisolone (0, 4 h); no Cytoxan—(▲) saline. Symbols for right panel: Cytoxan pretreatment—(×) saline; (○) homologous antiserum (3 h); (●) homologous antiserum (3 h) + methylprednisolone (3, 8 h); (△) saline, noninfected; no Cytoxan—(▲) saline.*

demonstration of protection by specific antiserum and by adrenal corticosteroids (i.e., 2-h delay after i.p. *E. coli* O:18 inoculation and 3.5- to 4-h delay after i.p. *P. mirabilis*). None of the agents, given in dosages shown in Fig. 12, proved capable of preventing mortality not preventable by the aminoglycoside antibiotics alone. The possibility that the negative results obtained with niacinamide may have resulted from the chlorobutanol used as a preservative in the Eli Lilly preparation was tested by repeating the studies with niacinamide obtained from the U.S. Biochemical Corp. The latter was freshly dissolved in pyrogen-free sterile saline and membrane filtered (Millipore Corp.) to ensure sterility. The results remained unaltered.

DISCUSSION

Numerous studies have been conducted to identify agents which can protect against the endotoxin component of gram-negative bacteria despite the absence of proof of its importance in mediating mortality during infection. A number of agents with anti-endotoxin activity have now been identified. These include hyperimmune serum (6, 12, 16, 20, 39, 46, 47), adrenal corticosteroids (5, 9, 29, 33, 43), anticoagulants (14, 18, 21, 31), ascorbic acid (17), antiproteolytic drugs (13), alpha adrenergic blockers (36), prostaglandin synthetase inhibitors (15, 25), nicotinamide (4, 42), glucose (3, 24), and insulin (1, 23, 30). In some cases (anti-proteolytic drugs) confirmation of anti-endotoxin activity is not yet available, and in others (anticoagulants, ascorbic acid, alpha adrenergic blockers, glucose) negative results also have been obtained (5, 11, 28, 42). Although such studies involving protection against isolated endotoxin preparations are of great academic interest, the key issue, the effect of anti-endotoxin agents on mortality during gram-negative bacterial sepsis, remains largely unresolved. Moreover, since antibiotics are now universally used for treatment of gram-negative bacteremias, this key issue can be further narrowed to the question of whether agents possessing anti-endotoxin activity are capable of preventing mortality not preventable by antibiotic therapy alone.

In developing an experimental model to explore this problem, one of two general approaches may be followed. In one model, a bacterial inoculum can be injected which is of such magnitude that lethality ultimately results despite prompt institution of appropriate antibiotic therapy. Agents with anti-endotoxin activity can then be administered together with the antibiotics to determine if mortality can be reduced. In the second model, a bacterial inoculum can be injected in a dosage such that lethality is nil after prompt administration of appropriate antibiotics; the administration of antibiotics can then be delayed until lethality reaches apprecia-

ble levels, and anti-endotoxin agents can be tested at this point.

In the first model, wherein prompt and appropriate antibiotic administration does not prevent mortality, lethal doses of preformed endotoxin are likely to be inoculated. If this were so, this model would not be simply one of gram-negative sepsis, but one of exogenous lethal endotoxemia plus gram-negative sepsis. This important point is illustrated by two earlier studies purporting to demonstrate that adrenal corticosteroids (dexamethasone and methylprednisolone) signifi-

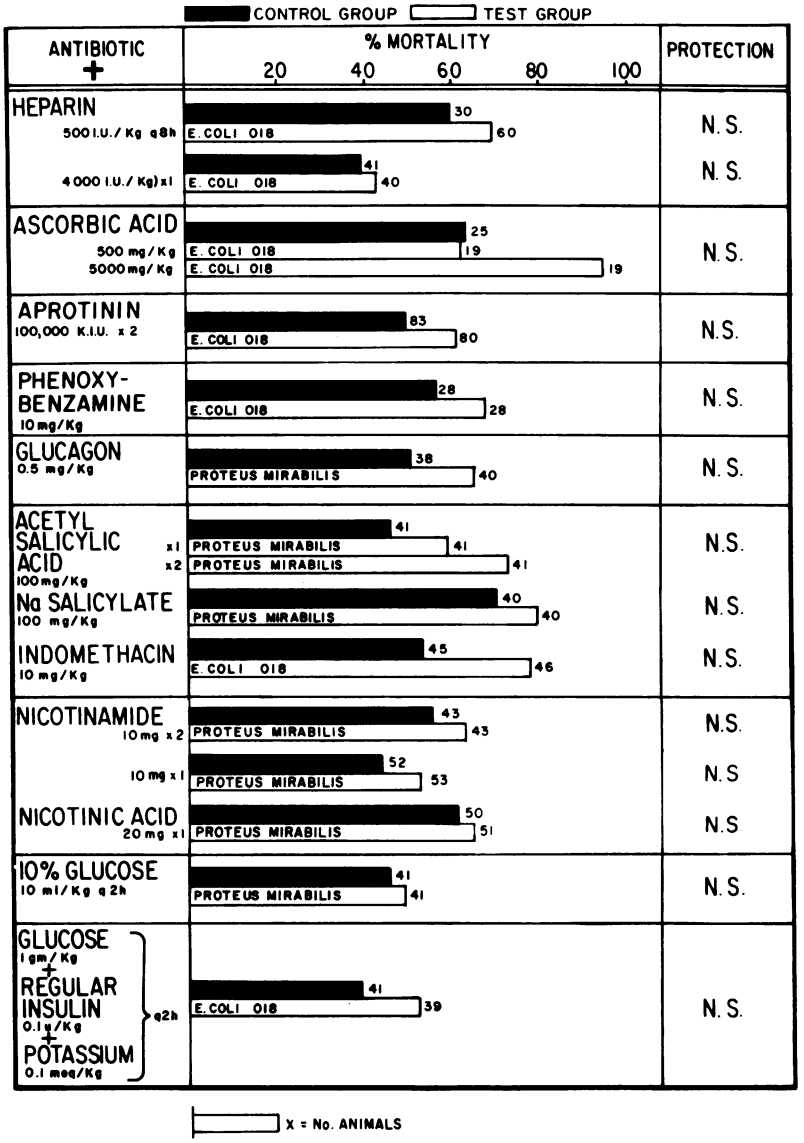

FIG. 12. *Inability of various reputed anti-endotoxin agents or of glucagon or nicotinic acid to prevent mortality from E. coli O:18 or P. mirabilis sepsis in Swiss mice not preventable by optimal aminoglycoside antibiotic therapy (gentamicin or kanamycin) alone. All drugs were given i.m. except indomethacin, which was given i.v. Therapy with the antibiotic and anti-endotoxin agent or diluent was begun after a standard delay of 2 h with E. coli O:18 and 3.5 to 4 h with P. mirabilis. (See text for dosage schedules of antibiotics.) (Solid bar) Control group; (open bar) test group. Number at end of bar = number of animals. N.S., Not significant.*

cantly potentiated the protective activity of antibiotics during gram-negative sepsis. In one study (37), 2×10^{10} *E. coli* were injected i.v. into 100- to 150-g Sprague-Dawley rats, and in the other study (2), 2.6×10^{10} *E. coli* were given i.v. to 280- to 320-g Sprague-Dawley rats; immediately thereafter, antibiotics with and without adrenal corticosteroids were administered. In both studies, significant reductions in mortality were achieved in the steroid-treated group. It is important to note, however, that in the control animals not given adrenal corticosteroids, despite immediate administration of antibiotics to which the *E. coli* were sensitive in vitro, high mortality rates rapidly ensued. (In one of these studies [2], the sensitivity of the *E. coli* [ATCC 25922] to gentamicin sulfate, expressed as minimal bactericidal and inhibitory concentrations, was stated to be 3 and 1.5 mg/ml, respectively. Presumably the authors mean 3 and 1.5 μg/ml, since in repeating their studies in our laboratory using *E. coli* ATCC 25922, we found the minimal bactericidal and inhibitory concentrations of gentamicin sulfate to be 3.2 and 1.6 μg/ml, respectively.) It seems entirely likely, based upon the present studies, that such immediate institution of appropriate antibiotic therapy in the dosages used would have prevented death from the *E. coli* infection, but that the massive inocula of bacteria contained lethal amounts of preformed endotoxin, thus accounting for both the high and rapidly occurring mortality despite prompt antibiotic administration and the beneficial effect of the prompt corticosteroid therapy. To verify this supposition, both of the above studies with live *E. coli* were repeated in our laboratory and the findings were entirely confirmed. It was also found, however, that the given i.v. inocula of *E. coli*, 2×10^{10} and 2.6×10^{10}, when heat killed and given to 100- to 150- or 300- to 325-g Sprague-Dawley rats, respectively, evoked mortality rates identical to those in the antibiotic (gentamicin)-treated live *E. coli* models. Moreover, dexamethasone or methylprednisolone reduced this mortality from the heat-killed *E. coli* just as effectively as in the antibiotic-treated live *E. coli* models. Since prompt adrenal corticosteroid administration reduces mortality from preformed endotoxin (5, 9, 29, 33, 43), it would be expected that in any study of gram-negative sepsis that commences with a lethal bolus of endotoxin, corticosteroids given promptly would prove beneficial, and thus no information is gained as to the effect of corticosteroids on the septic process per se.

In the present studies, infection was induced in outbred Swiss mice by one of three gram-negative bacterial species, *E. coli*, *P. mirabilis*, or *K. pneumoniae*, and the antibiotics used were the aminoglycosides gentamicin and kanamycin, to which these organisms were found to be sensitive in vitro. An inoculum of gram-negative bacteria was selected in each case that resulted in 90 to 100% lethality without antibiotic therapy, and in no mortality with prompt antibiotic administration. In addition to the above evidence that a lethal dose of endotoxin was not administered preformed in the bacterial inoculum, no mortality could be demonstrated when comparable inocula of heat-killed organisms were used. By progressively delaying the time before initiating therapy with the aminoglycoside antibiotics, mortality rates increased progressively. With each species of bacteria studied, a standard delay period was selected before starting antibiotic therapy such that despite optimal dosage schedules of antibiotics (i.e., schedules resulting in the lowest mortality rates), a 50 to 70% mortality ensued. It is possible that other schedules or combinations of antibiotics may have further reduced mortality. Nevertheless, sufficient studies were carried out to indicate that the antibiotic schedules used were probably close to optimal. In addition, the i.m. route of antibiotic administration was found to be as protective as the i.v. route, excluding the possibility that delayed absorption of antibiotic from muscle depots might interfere with antibiotic effectiveness. Using such a model of optimal but delayed aminoglycoside antibiotic therapy, the effect of addition of various reputed anti-endotoxin agents was evaluated. It should be noted that the use of optimal antibiotic therapy is stressed, since any protective activity conferred by addition of anti-endotoxin agents could thus not be duplicated simply by increasing antibiotic dosage. It should also be noted that the anti-endotoxin agents tested were selected solely on the basis of their having been cited in the literature as possessing antitoxin activity. As already indicated, confirmation of this activity is lacking in some cases and is controversial in others. It was not the purpose of the present studies to re-evaluate the anti-endotoxin activity of each of the numerous agents cited, but rather to determine if any could be identified that would be protective during antibiotic-treated gram-negative bacterial sepsis.

Of all the reputed anti-endotoxin agents tested, only specific antiserum and adrenal corticosteroids were found capable, when administered concomitantly with optimal aminoglycoside antibiotics after the standard delay period, of preventing mortality not preventable by the antibiotic alone. Some of the characteristics of such adjunctive protection were of especial in-

terest. When the antibiotic was omitted or given in suboptimal quantities, specific antiserum given after the standard delay period no longer exerted any protective activity, suggesting that the serum acted synergistically with the aminoglycoside antibiotics. In addition, potentiation of antibiotic protection by antiserum was achieved only during *E. coli* and *P. mirabilis*, not during *K. pneumoniae*, sepsis and only with strain-specific antiserum. For example, during *E. coli* O:18 sepsis, antisera to *E. coli* O:111 failed to reduce mortality, whereas anti-*E. coli* O:18 serum was highly protective. It is emphasized that when the administration of antibiotic and specific antiserum therapy were postponed beyond the standard delay period during *E. coli* and *P. mirabilis* sepsis by even relatively short increments, the adjunctive protective activity of the antiserum rapidly waned. Thus, there exists a short-lived period which occurs early in the course of *E. coli* and *P. mirabilis* sepsis wherein specific antisera, no longer protective by itself, can significantly potentiate the effectiveness of aminoglycoside antibiotic therapy. In contrast to the protection achieved by delayed administration of specific antiserum during *E. coli* and *P. mirabilis* sepsis, the delayed administration of specific antiserum during *K. pneumoniae* sepsis (with or without concomitant antibiotics) evoked an immediate high mortality. This appeared to be based upon reversed anaphylaxis since the same anti-*K. pneumoniae* serum was entirely without effect on mortality during *E. coli* and *P. mirabilis* sepsis, was highly protective when given as pretreatment for *K. pneumoniae* sepsis, and was produced by both the immunoglobulin G and immunoglobulin M fractions of the antiserum. Apparently, at some point during *K. pneumoniae* sepsis (i.e., 4 or more h after 10^7 organisms i.p.) sufficient amounts of antigen are released to predispose to fatal anaphylaxis when specific antiserum is administered i.v. The nature of these antigens was not identified, but the potential injurious effect of specific antiserum during delayed treatment of gram-negative sepsis is underscored, and this complication should be considered in any future therapeutic clinical trials.

It is of special interest that in none of the three models of gram-negative sepsis presently studied did antisera to rough mutant *Enterobacteriaceae* of chemotype Rc, Rd, or Re prevent mortality not preventable by aminoglycoside antibiotics alone, even though antisera to certain of these rough mutants have been reported to provide significant protection when administered prophylactically to non-antibiotic-treated mice (10, 32, 46). To further study this important point, the ability of antisera to these rough mutants to protect mice against gram-negative sepsis when administered as pretreatment to non-antibiotic-treated animals was re-evaluated. In confirmation of the findings by Ng and co-workers (35), pretreatment of mice with the rabbit antisera to the Re (595) *S. minnesota* mutant, as well as to the Rd (SL 1032) *S. typhimurium* and Rc (J5) *E. coli* O:111 mutants, was not found capable of reducing mortality from challenge with the currently used smooth *Enterobacteriaceae* (*E. coli* O:18, *P. mirabilis*, *K. pneumoniae*) any more effectively than preimmune sera from the corresponding donors possessing no detectable antibodies to these rough mutants (19). Therefore, the presently observed inability of these rough mutant antisera to prevent mortality no longer preventable by aminoglycoside antibiotics alone is not surprising. The possibility must be considered, however, that these negative results might be due to the use of rough mutant strains different from those used by previous investigators, or to reversion to their wild-type smooth parent form before use as a vaccine. This appears unlikely since (i) the Re 595 mutant of *S. minnesota* was obtained from the same source (Max Planck Institut für Immunbiologie) as that used by previous investigators (32, 46). Moreover, additional antisera prepared against a new culture of the Re 595 *S. minnesota* mutant received at a later date from the Max Planck Institut was also tested and again yielded negative results; (ii) the negative results with antisera to the Rc (J5) mutant of *E. coli* O:111 were observed not only with this mutant obtained directly from Edward Heath's laboratory, but also with this mutant obtained in the form of a boiled suspension from Abraham Braude; (iii) the rough mutant vaccines were prepared by selection of typical rough colonies from blood agar plates, and these were proven to have retained their rough colonial morphology; (iv) the rough mutant antisera possessed impressive antibody titers (passive HA and bentonite flocculation) against their respective purified lipopolysaccharides obtained from the laboratory of other investigators; indeed, the HA titers were as high as those recorded by previous investigators (7, 32). In addition, the rough mutant antisera exhibited markedly lower antibody titers against lipopolysaccharides from their respective smooth parent strains when compared with similarly prepared antisera against the smooth parent organisms.

Since differences in rough mutant strains from those used by previous investigators or reversion to wild type did not appear to account for the present inability of antisera against the Re (595)

S. minnesota or Rc (J5) *E. coli* O:111 rough mutants to protect mice against gram-negative bacterial sepsis, other possibilities should be considered. In both the present and pretreatment studies cited (19, 35), even though the gram-negative challenge inocula were selected to yield mortality within the sensitive dose-response range, relatively large inocula were used; this might have masked the protective activity of the antiserum. However, it should be noted that strain-specific smooth antisera were generally highly protective and that in two studies in mice challenged with relatively small inocula of *K. pneumoniae* (10^4 and 1.5×10^3), no significant protection was observed with rough mutant antisera, even when given as pretreatment (19, 35). Moreover, in a model of *Pseudomonas* sepsis in burned mice where relatively small inocula (10^4) administered intradermally produced lethal sepsis, pretreatment with 0.5 ml of rabbit sera against the J5 mutant of *E. coli* O:111 afforded no significant protection compared with animals receiving preimmunization sera (L. T. Callahan III and A. F. Woodhour, Merck Sharp, & Dohme, personal communication). One additional possibility for the inability to demonstrate protection with rough mutant antisera merits consideration. The present and previously cited negative results were obtained in mice that were not immunosuppressed or agranulocytic and that were inoculated by the i.p., i.v., or intradermal route. In contrast, protective activity of rough mutant antisera has been most convincingly demonstrated in agranulocytic rabbits infected via the gastrointestinal tract or conjunctival sac (7, 48). Differences in protection by rough mutant antisera may stem, therefore, from differences in host species, antimicrobial defenses, and/or route and tempo of bacterial invasion. This is supported by the observation that the same antisera to the J5 mutant of *E. coli* O:111 that failed to protect burned mice against intradermal challenge with *Pseudomonas* clearly protected about 50% of agranulocytic rabbits against *Pseudomonas* inoculated into their conjunctival sac (Callahan and Woodhour, personal communication). Whether rough mutant antisera can prevent mortality not preventable by appropriate antibiotic therapy alone in this latter setting, as could be accomplished in the present noncompromised murine model only with strain-specific smooth antisera, is a key issue that remains to be determined. The effect of compromising antimicrobial resistance on the protective activity of antiserum will be considered further after describing the activity of adrenal corticosteroids.

In each of the models of gram-negative sepsis presently studied, *E. coli* O:18, *P. mirabilis*, and *K. pneumoniae*, administration of adrenal corticosteroids (methylprednisolone or dexamethasone) concomitantly with optimal doses of aminoglycoside antibiotics after the standard delay period prevented mortality not preventable by the antibiotic alone. Increasing protection resulted when the adrenal corticosteroids were begun earlier, and maximum protection was achieved when the adrenal corticosteroids were given simultaneously with the bacterial challenge, even though the aminoglycoside antibiotic therapy continued to be delayed for the standard period. Conversely, the protective effect of the corticosteroids, as with specific antiserum, was no longer apparent if the steroid therapy was postponed beyond the standard delay period by even relatively short increments. The protective effect of corticosteroids, as with specific antiserum, was also no longer discernible if the antibiotic dosage was suboptimal, and in the case of *K. pneumoniae* sepsis, unlike *E. coli* O:18 or *P. mirabilis* sepsis, the protection by steroid was sustained only as long as the antibiotic therapy was continued. Moreover, in each of the three models of gram-negative bacterial sepsis, adrenal corticosteroids given simultaneously with, or several hours after, bacterial inoculation in the absence of antibiotic therapy failed to reduce (or enhance) mortality. Thus, as with specific antiserum, there exists a short-lived period early in the course of gram-negative sepsis wherein adrenal corticosteroids, not protective by themselves, can significantly potentiate the effectiveness of aminoglycoside antibiotics. The above findings are consistent with the recently reported controlled clinical trial indicating a significant protective effect of early adrenal corticosteroid administration on mortality during antibiotic-treated, gram-negative sepsis in humans (41).

Additional studies were carried out to determine if the effectiveness of specific antiserum in reducing mortality in the delayed aminoglycoside antibiotic-treated sepsis models could be additive to that of adrenal corticosteroids. The results were not uniform. During *E. coli* O:18 sepsis, no additive protection occurred, whereas during *P. mirabilis* sepsis, significant additional reduction in mortality occurred when specific antiserum and adrenal corticosteroids were combined. Since specific antiserum evoked anaphylaxis when given after the standard delay period during *K. pneumoniae* sepsis, the combination of specific antiserum and corticosteroids was not tested in this model. Further studies will be required to determine if the additive protection achieved by combining specific anti-

serum and adrenal corticosteroids during aminoglycoside-treated *P. mirabilis* sepsis is unique.

The mechanisms whereby specific antiserum and adrenal corticosteroids prevent mortality from gram-negative sepsis no longer preventable by optimal aminoglycoside antibiotics alone are unknown. Early administration of specific antiserum and of adrenal corticosteroids are each known to be capable of preventing death from endotoxemia (5, 6, 9, 12, 16, 33, 39, 43, 47), and it would be tempting to speculate that these agents act by virtue of their anti-endotoxemic effects. However, both specific antiserum and adrenal corticosteroids have numerous additional activities. Specific antiserum might protect primarily by its opsonic activity or by enhancing serum bactericidal activity (or both). Adrenal corticosteroids have been shown to prevent mortality not preventable by antibiotic (penicillin) alone in rabbits during pneumococcal sepsis, and, as in the present study, timing was crucial (40). Here, endotoxemia presumably is not a contributory factor to lethality. Thus, further studies will be required to determine whether the prevention of gram-negative septic mortality not preventable by aminoglycoside antibiotics alone bears any relationship to the anti-endotoxemic effects of the specific antiserum and adrenal corticosteroids. (Since aminoglycosides possess intrinsic toxicity, the possibility must be considered that agents that prevent mortality not preventable by these antibiotics alone might do so simply by neutralizing this intrinsic toxicity. This possibility, however, appears unlikely since it would be unreasonable to presume that specific bacterial antiserum would mitigate aminoglycoside toxicity and since adrenal corticosteroids, in dosages that potentiate aminoglycoside antibiotic protection during sepsis, failed to reduce mortality in noninfected animals given LD_{50} doses of either gentamicin or kanamycin.)

The above studies evaluating the effect of specific antiserum or adrenal corticosteroids, or both, in combination with aminoglycoside antibiotics during *E. coli*, *Proteus*, and *Klebsiella* sepsis were conducted in mice with no systemic impairment of their antimicrobial defense mechanisms. When resistance was compromised by pretreatment with the bone marrow-suppressive agent cyclophosphamide, the ability of specific antiserum and adrenal corticosteroids to prevent mortality not preventable by the delayed administration of aminoglycoside antibiotics alone became difficult to demonstrate. In this setting, initiation of antibiotic therapy for *P. mirabilis* sepsis after the standard delay period led to a continuously increasing mortality rate, rather than to the plateau seen after 36 h in control animals. Moreover, mortality was now merely delayed by addition of specific antiserum or adrenal corticosteroids, or both, rather than prevented. Whether more intensive therapy with specific antiserum, adrenal corticosteroids, or antibiotics or whether reductions in the challenge inoculum would permit the antiserum or corticosteroids to remain protective requires further study. It is apparent from the present studies, however, that decreasing host resistance with cyclophosphamide can impair the ability of antisera and adrenal corticosteroids to prevent mortality not preventable by antibiotics alone. These findings parallel the recently documented effect of cyclophosphamide in dogs challenged with *Pseudomonas aeruginosa*. In this setting, specific antiserum, even when used as pretreatment, loses its ability to prevent mortality and now merely delays the rate of death. Apparently, at least in part as a result of the granulocytopenia evoked by cyclophosphamide, specific antibody can no longer prevent septic mortality (22). Similar results have been reported in dogs rendered granulocytopenic by irradiation (27).

Of the reputed anti-endotoxin agents tested other than specific antiserum and adrenal corticosteroids, none prevented mortality from gram-negative sepsis not preventable by aminoglycoside antibiotics alone. Thus, anticoagulants (heparin), ascorbic acid, antiproteolytic agents (aprotinin), alpha adrenergic blockers (phenoxybenzamine), prostaglandin synthetase inhibitors (acetylsalicylic acid, sodium salicylate, indomethacin), nicotinamide, glucose, and glucose-potassium-insulin mixtures all failed to reduce mortality when administered after the standard delay period concomitantly with optimal aminoglycoside antibiotics. It remains to be determined whether such failure reflects the effects of the delay in therapy, the limited effectiveness of these compounds as anti-endotoxin agents, or a limited role of endotoxin in producing gram-negative septic mortality.

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