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Experimental Gram-Negative Bacterial Sepsis: Optimal Methylprednisolone Requirements for Prevention of Mortality Not Preventable by Antibiotics Alone (41455)

SHELDON E. GREISMAN

Departments of Medicine and Physiology, University of Maryland School of Medicine, Baltimore, Maryland 21201

Abstract. Outbed ICR mice were inoculated ip with one LD_{90-100} *Escherichia coli* 0:18, *Proteus mirabilis*, or *Klebsiella pneumoniae*. After carefully timed intervals, aminoglycoside antibiotics were begun at dosages and intervals predetermined to constitute optimal therapy. With progressive delay of antibiotic therapy, mortality rates increased progressively from 0 to 90–100%. Standardized models of infection were obtained by selecting delay periods before initiating antibiotic therapy such that 50 to 70% mortality rates resulted. In these models, 30 mg/kg methylprednisolone (MP) given prior to or concomitantly with the delayed antibiotic therapy and repeated 4 hr later was previously shown capable of preventing gram-negative septic mortality not preventable by the optimal antibiotic therapy alone. The present studies quantitate the optimal quantities of MP required for such protection. It was found that (1) in the absence of aminoglycoside antibiotics, MP consistently failed to reduce mortality; (2) in the antibiotic-treated animals, a single im injection of 30 mg/kg MP provided definite protection. A second and a third injection of 30 mg/kg of MP at 4-hr intervals provided additional, but decremental, increments of protection; (3) decreasing the dose of MP to 10 mg/kg or less consistently reduced its protective activity; (4) increasing the dose of MP to 60 mg/kg or greater consistently reduced its protective activity; (5) short additional increments in delay in initiating antibiotic and MP therapy annulled the protective activity of MP. The findings indicate that the ability of MP to reduce murine mortality from gram-negative bacterial sepsis is not only critically restricted by the requirements for its early administration in conjunction with appropriate antibiotics, but also by its relatively narrow optimal dose–response range and the decremental increments in its effectiveness upon repetitive administration.

The ability of adrenal corticosteroids to reduce human mortality during antibiotic-treated gram-negative bacterial sepsis has remained controversial for almost two decades (1–5). Currently, only one critically controlled study supports the efficacy of adjunctive corticosteroid therapy in man (4). That adrenal corticosteroids can indeed prevent mortality not preventable by appropriate antibiotics alone has recently been demonstrated in several experimental models employing the rat, dog, and baboon (6–9). In these models, however, such large numbers of gram-negative bacteria were inoculated that it could be argued that the beneficial effects of the corticosteroids were based entirely upon their ability to protect against a lethal bolus of endotoxin contained preformed in the challenge inoculum. Another experimental model was therefore specifically designed to exclude this possibility (10). The present studies utilize this latter model to further explore the efficacy of adrenal corticosteroids for prevention of gram-negative septic mortality not preventable by antibiotics alone. In particular, two questions are addressed: (i) whether there is a wide or only a narrow optimal dose–response range for adrenal corticosteroid protection during sepsis; and (ii) whether repetitive injections of adrenal corticosteroids provide greater protection than a single optimal dose.

Materials and Methods. The models of antibiotic treated gram-negative bacterial sepsis employed were identical to those previously described (10) except that (a) the present outbred mice were ICR, not Swiss, and were obtained from a different source (Flow Laboratories, Dublin, Va.) and (b)

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animals were all males (rather than mixed sexes) weighing 25 to 30 g at time of receipt. The mice 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 ip with 1 ml of 16- to 18-hr cultures of *Escherichia coli* 0:18, *Proteus mirabilis,* or *Klebsiella pneumoniae* (Caroli strain). These organisms, characterized previously (10), were grown in Trypticase soy broth (BBL) at pH 7.4 and 37°, harvested by centrifugation in pyrogen-free sterile tubes, washed with pyrogen-free sterile 0.9% saline at room temperature, and suspended in the saline at concentrations determined turbidometrically at 580 nm. Control and test animals were always challenged alternately with the bacterial suspension to minimize possible effects of changing bacterial viability during the total injection period. Immediately after ip inoculation, each animal was examined for external leakage; animals with leakage of more than three drops of injectate (generally <10% of any experimental group) were rejected since greater losses would reduce mortality rates by more than 10% as judged from mortality dose–response curves previously obtained with these organisms (10). This precaution accounts for the small differences in numbers of control and test animals as indicated under Results. In each study, one LD_{90-100} of bacteria was inoculated. This required 1×10^8 and 5×10^8 of the *E. coli* and *P. mirabilis* cultures respectively. The *K. pneumoniae* proved uniformly lethal, the size of the inoculum determining only rate of death; 1×10^7 *K. pneumoniae* was administered in all present studies. With each bacterial species, no mortality occurred following injection of comparable quantities of heat-killed inocula.

After carefully timed intervals following bacterial challenge, aminoglycoside antibiotics were begun; these were diluted in pyrogen-free sterile physiologic saline and given im in 0.2-ml volumes. *In vitro* sensitivities of each of the bacterial strains to these aminoglycoside antibiotics, determined by minimal inhibitory concentrations of antibiotic in Trypticase soy broth (BBL) at pH 7.4 and 37°, were as follows: *E. coli* 0:18, 3.1 μg gentamicin/ml; *P. mirabilis,* 6.25 μg kanamycin/ml; *K. pneumoniae,* 1.0 μg gentamicin/ml. In each case, bactericidal levels of antibiotic were achieved with twofold higher concentrations. The antibiotics were administered at intervals and dosages determined from previous studies (10) to constitute optimal therapy, i.e., 0.1 mg gentamicin for *E. coli* and *K. pneumoniae* sepsis and 0.5 mg kanamycin for *P. mirabilis* sepsis repeated every 2 hr for 32 hr (omitting the 2 AM to 6 AM doses). The requirement for such closely spaced injections of aminoglycoside antibiotics to obtain optimal protection is consistent with the reported rapid decline in their blood levels following im or sc injections in mice. Indeed, bactericidal levels for each of the microbes presently employed would be achievable only within the initial hour after the aminoglycoside administration (11, 12). Unlike the *E. coli* and *P. mirabilis* infections, mortality from *K. pneumoniae* failed to plateau by 48 hr if the aminoglycoside antibiotics were discontinued after 32 hr; gentamicin was therefore continued beyond 32 hr in this latter model at intervals of 6 to 8 hr for 96 hr. All mortality rates were recorded at 96 hr because animals surviving for this period survived "permanently", i.e., >3 weeks. (With *K. pneumoniae,* such long-term survival required maintenance antibiotic therapy as described above.)

In each model of sepsis, when antibiotic therapy was begun immediately after bacterial challenge, 0% mortality resulted. With progressive delay in initiating antibiotic therapy, mortality rates increased progressively. Standardized models of antibiotic-treated infection were obtained by selecting delay periods before initiating antibiotic therapy such that 50 to 70% mortality resulted. The required delay periods were 2.5, 4, and 5.5 hr for the *E. coli, P. mirabilis,* and *K. pneumoniae* septic models, respectively. Utilizing these models, the effect of varying dose schedules of methylprednisolone sodium succinate (Solu-

Medrol, The Upjohn Co.) was evaluated. Solutions of this steroid were freshly prepared immediately before each study by gentle agitation of the sterile powdered steroid in pyrogen-free sterile 0.9% saline at 37° for 1 hr. Differing quantities were given by varying the concentration of the steroid, the volume being held constant at 0.25 ml; control animals received 0.25 ml sterile pyrogen-free saline. The corticosteroid was administered im and the dose is expressed in terms of equivalents of methylprednisolone (MP). For reasons considered in the Discussion, treatment with MP was begun between the time of bacterial challenge and the initiation of antibiotic therapy. A χ^2 test was employed for evaluation of differences in mortality.

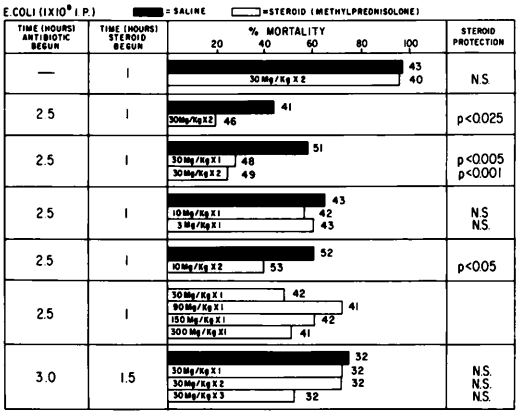

FIG. 1. Effect of methylprednisolone on survival of ICR mice following ip challenge with *E. coli* 0:18 (1×10^8) in the absence or presence of antibiotic therapy (gentamicin). See text for detailed schedule of antibiotic and methylprednisolone therapy. Numbers after bars in this and subsequent figures represent numbers of animals in each trial.

Results. *E. coli 0:18 sepsis.* Figure 1 summarizes the effect of differing dose schedules of MP when combined with optimal, but delayed, antibiotic (gentamicin) treatment. The antibiotic therapy was begun after a standard delay period of 2.5 hr following bacterial challenge (except for the last study where the effect of further delay to 3 hr was assessed). The effect of MP was tested 1 hr after bacterial challenge (except for a further delay to 1.5 hr in the last study); the effects of second and third doses of MP were tested at 4-hr intervals after the first. The findings, considered in the sequence shown in each descending panel of Fig. 1, indicate: (i) in the absence of antibiotic administration, two doses of 30 mg/kg MP (at 1 and 5 hr) following an LD_{90-100} *E. coli* 0:18 challenge failed to alter mortality; (ii) optimal gentamicin therapy, when begun after the standard delay period following *E. coli* 0:18 challenge, reduced mortality into the preselected range and now the administration of two doses of 30 mg/kg MP (at 1 and 5 hr) significantly further reduced this mortality ($P < 0.025$); (iii) the initial dose of MP (given at 1 hr) provided the most marked protection; the second dose of MP (given at 5 hr) provided only minimal additional protection; (iv) when the MP dose was reduced to 10 or 3 mg/kg, no significant protection was achieved by a single dose of MP given at 1 hr. (The slightly higher control mortality rate in this study compared to the previous one may have contributed to this lack of protection); (v) two doses of 10 mg/kg MP (at 1 and 5 hr) did provide significant protection ($P < 0.05$). Such protection, however, was not as marked as that following a single injection of 30 mg/kg MP; (vi) increasing the dose of MP from 30 mg/kg to levels 3-, 5-, or 10-fold greater failed to improve upon its protective activity; indeed, less protection resulted; (vii) increasing the delay in initiation of both the antibiotic and MP therapy by a relatively short interval (½ hr) increased the control mortality rate to levels higher than in any of the previous antibiotic treatment studies (as would be expected) and annulled the protective activity of both one and two doses of 30 mg/kg MP; under these conditions, three doses of 30 mg/kg MP (at 1.5, 5, and 9 hr) appeared to have some protective effect that did not, however, attain statistical significance.

P. mirabilis sepsis. Figure 2 summarizes the effect of differing dose schedules of MP when combined with optimal, but delayed, antibiotic (kanamycin) treatment. The antibiotic therapy was begun after a standard delay period of 4 hr following bacterial challenge. The effect of MP was tested 2 hr

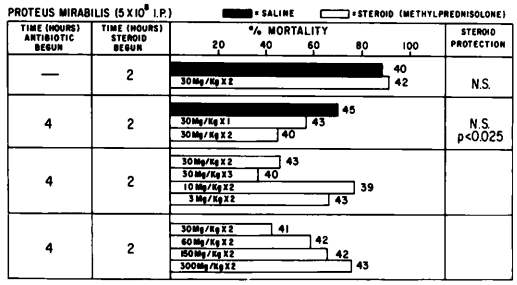

FIG. 2. Effect of methylprednisolone on survival of ICR mice following ip challenge with *P. mirabilis* (5×10^8) in the absence or presence of antibiotic therapy (kanamycin). See text for detailed schedule of antibiotic and methylprednisolone therapy.

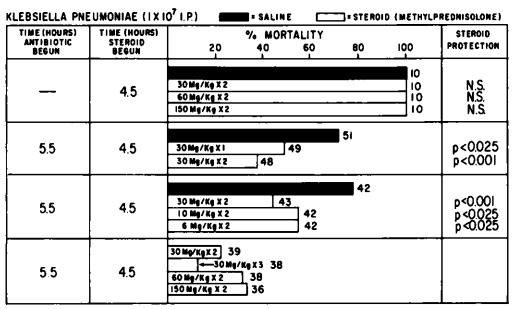

FIG. 3. Effect of methylprednisolone on survival of ICR mice following ip challenge with *K. pneumoniae* (1×10^7) in the absence or presence of antibiotic therapy (gentamicin). See text for detailed schedule of antibiotic and methylprednisolone therapy.

after bacterial challenge; the effects of second and third doses of MP were tested at 4-hr intervals after the first. The findings, considered in the sequence shown in each descending panel of Fig. 2, indicate: (i) in the absence of antibiotic administration, two doses of 30 mg/kg MP (at 2 and 6 hr) following an LD_{90} *P. mirabilis* challenge failed to alter mortality; (ii) optimal kanamycin therapy, when begun after the standard delay period following *P. mirabilis* challenge, reduced mortality into the preselected range and now the administration of a single dose of 30 mg/kg MP (at 2 hr) further reduced this mortality, although this was not statistically significant. However, two doses of 30 mg/kg MP (at 2 and 6 hr) significantly reduced mortality in the antibiotic treated animals ($P < 0.025$); (iii) compared with the effects of two doses of 30 mg/kg MP (at 2 and 6 hr), three doses (at 2, 6, and 10 hr) further reduced mortality, although this augmentation of protection was not statistically significant. Reduction of the MP dose from 30 to 10 or to 3 mg/kg significantly reduced its protective activity ($P < 0.05$ for each of these lower doses); (iv) increasing the dose of MP from 30 mg/kg to levels 2-, 5-, or 10-fold greater progressively impaired its protective activity.

K. pneumoniae sepsis. Figure 3 summarizes the effect of differing dose schedules of MP when combined with optimal, but delayed, antibiotic (gentamicin) therapy. The antibiotic therapy was begun after a standard delay period of 5.5 hr following bacterial challenge. The effect of MP was tested at 4.5 hr after bacterial challenge; the effects of second and third doses of MP were tested at 4-hr intervals after the first. The findings, considered in the sequence shown in each descending panel of Fig. 3, indicate: (i) in the absence of antibiotic administration, two doses of either 30, 60, or 150 mg/kg MP (at 4.5 and 8.5 hr) following an LD_{100} challenge with *K. pneumoniae* failed to affect mortality; (ii) optimal gentamicin therapy, when begun after the standard delay period following the *K. pneumoniae* challenge, reduced mortality into the preselected range and now the administration of a single dose of 30 mg/kg MP (at 4.5 hr) significantly further reduced this mortality ($P < 0.025$). A second dose of 30 mg/kg MP at 8.5 as well as 4.5 hr provided some additional protection and increased the significance to $P < 0.001$; (iii) repetition of the previous study confirmed the significant protection ($P < 0.001$) provided by two doses of 30 mg/kg MP. Reduction of the MP dose from 30 to 10 or 6 mg/kg reduced its protective activity, although both these smaller doses still provided significant protection ($P < 0.025$ for each trial); (iv) three doses of 30 mg/kg MP (at 4.5, 8.5, and 12.5 hr) provided more protection than did the initial two, although this increment in protection was not statistically significant. Increasing the dose of MP from 30 mg/kg to levels two- or fivefold greater did not enhance its protective activity; indeed some reduction in protection was seen.

Effect of saline loading. The use of optimal schedules of aminoglycoside antibiot-

ics, entailing their repetitive administration im in 0.2 ml saline at closely spaced (2 hr) intervals, led to appreciable volume loading (≥2.4 ml saline per 25- to 30-g animal per 32 hr). To test the possibility that such fluid loading might contribute to edematous changes in the septic animals and that MP might be protective simply by inhibiting this edema formation, groups of mice were challenged with the *E. coli, P. mirabilis,* or *K. pneumoniae* as in the previous studies but were given 10-fold higher concentration of the aminoglycoside antibiotics in 0.2 ml saline every 8 rather than every 2 hr during the initial 32 hr. (Since this schedule provided less protection (10), the antibiotic treatment was begun ½ to 1 hr earlier than previously.) In half of each of these groups, additional injections of 0.2 ml sterile nonpyrogenic physiologic saline were given im every 2 hr to mimic the volume loading associated with the standard optimal antibiotic therapy. The results, shown in Table I, indicate that the volume loading did not produce detrimental effects; in fact, significant reductions in mortality occurred in the *P. mirabilis* and *K. pneumoniae* models of sepsis. Thus, the beneficial effects of MP observed in the antibiotic-treated models of gram-negative sepsis do not appear relatable simply to protection against adverse effects of saline loading with antibiotic administration.

Discussion. A major question concerning the therapy of gram-negative bacterial sepsis is whether adrenal corticosteroids can prevent mortality not preventable by appropriate antibiotics alone. The few controlled trials in man have led to divergent conclusions (1, 2, 4, 5). Differences in corticosteroid dosage, number of repetitive injections, and time of initiation of steroid and antibiotic therapy after onset of sepsis, as well as variations in underlying disease states and host physiologic status, are some critical variables that may account for these divergent clinical conclusions.

TABLE I. EFFECT OF SALINE LOADING ON MURINE MORTALITY DURING ANTIBIOTIC TREATED SEPSIS[a]

Inoculum	Percentage mortality (no. animals)	
	Controls	Saline loaded[b]
E. coli	65 (28)	75 (29)
P. mirabilis	70 (30)	43 (30)*
K. pneumoniae	38 (72)	12 (72)*

[a] Aminoglycoside antibiotics given im every 8 hr in 0.2 ml saline.

[b] Physiologic saline (sterile and nonpyrogenic), 0.2 ml, given im every 2 hr for 32 hr (omitting 2 to 6 AM doses).

* $P < 0.005$ compared to respective controls.

Recently, a number of experimental studies on antibiotic treated gram-negative bacterial sepsis have been conducted in different laboratories, all employing *Escherichia coli* challenge and pharmacologic doses of adrenal corticosteroids, i.e., ≥30 mg/kg methylprednisolone or ≥8 mg/kg dexamethasone; all agree that such steroid treatment can prevent mortality not preventable by antibiotics alone (6–9). However, one major problem is inherent in these studies. As emphasized previously (10), it is crucial to demonstrate that the challenge inoculum of gram-negative bacteria does not contain a lethal quantity of preformed endotoxin. Adrenal corticosteroids, if given early and in pharmacologic doses, protect against endotoxemic mortality (13–18). Therefore, if gram-negative bacteria are inoculated in numbers sufficient to cause death from both preformed endotoxin as well as from infection, then adrenal corticosteroids alone or antibiotics alone, even if given promptly, could not be expected to prevent mortality whereas their combination, each protective against the respective lethal factors, would be protective. That preformed lethal quantities of endotoxin per se might indeed account for the synergistic protection of corticosteroids and antibiotics observed in the experimental studies cited above employing rats, dogs, and baboons (6–9) is suggested by the rapid and high mortality rates after *E. coli* challenge in each of these species despite the prompt administration of appropriate antibiotics. This possibility is further supported by a repetition of the study in the rat model of antibiotic-treated sepsis which demonstrated the lethal effects of comparable inocula of heat-killed *E. coli* and the ability of adrenal corticosteroids to prevent such lethality (10). It is less likely that preformed lethal doses of endotoxin were adminis-

tered in the baboon model of antibiotic-treated *E. coli* sepsis since such primates are so markedly resistant to endotoxin (19), but this possibility has not yet been evaluated. Thus, while the above experimental studies attest to the value of corticosteroids given promptly and concomitantly with antibiotics when the host is challenged with an overwhelming inoculum of *E. coli,* they do not address the crucial issue of whether corticosteroids can be protective when given during gram-negative bacterial sepsis in which a lethal bolus of preformed endotoxin does not comprise part of the initial challenge. The present and previous (10) studies from our laboratory deal with this key issue, since only those models of gram-negative bacterial infection were employed in which a lethal quantity of endotoxin was not part of the initial challenge, as proven by complete absence of mortality after both (a) prompt administration of appropriate antibiotics, and (b) heat killing the challenge inoculum.

Utilizing these latter models, the present studies demonstrate that in the absence of antibiotic therapy, 30 mg/kg methylprednisolone (MP) consistently failed to decrease murine mortality following ip infection with an LD_{90-100} *E. coli* 0:18, *P. mirabilis,* or *K. pneumoniae*. However, in each of these models, when mortality was reduced to between 50 and 70% by optimal aminoglycoside antibiotic therapy begun after an appropriate delay period, then the addition of 30 mg/kg MP further reduced mortality significantly. Since optimal antibiotic therapy was used, this protective activity of MP during sepsis could not be duplicated simply by increasing the antibiotic dosage. These results fully confirm earlier findings in which Swiss, rather than ICR, strains of mice were used (10). In addition, the present findings demonstrate that the effectiveness of MP is not an "artefact" based upon protection against adverse effects of saline loading consequent to the multiple antibiotic injections since such saline loading was found nonharmful (*E. coli* sepsis) or significantly beneficial (*P. mirabilis* and *K. pneumoniae* sepsis).

The major objectives of the present studies were to determine whether the optimal protection achievable with MP during antibiotic treated gram-negative bacterial sepsis occurs within a broad or narrow dose range, and whether repetitive injections of MP are more effective than a single optimal dose. For this purpose, MP was given at a time that its protective action would be readily apparent. From our previous studies in the antibiotic-treated murine models of sepsis, it was found that the earlier the steroid was given after bacterial challenge, the greater was its protective activity (10); moreover, in the present studies, a relatively short additional increment in delay in initiating antibiotic and MP therapy after *E. coli* challenge annulled the protective effect of the MP. Based upon these considerations, a delay period for MP administration was chosen intermediate between that expected to yield the highest levels of protection (immediately post-bacterial inoculation) and the lowest levels (time of initiation of antibiotic therapy). Whether the optimal quantities of MP required for protection are significantly affected by varying the timing of its administration remains to be determined. This seems unlikely, however, since a similar pattern of MP dose–protection relationships emerged using three different challenge organisms, each with different timing of treatment. Thus 30 mg/kg MP proved optimal; decreases to 10 mg/kg or lower and increases to 60 mg/kg or greater consistently reduced the protective activity of the corticosteroid. The mechanism underlying the reduced protection with the 60 mg/kg or greater doses of MP is unknown. However, a similar reversal of corticosteroid protection at these high dose levels has been noted in other shock models, i.e., as the amount of various adrenal corticosteroids given iv or ip was increased, increasing protection was observed against endotoxin-induced mortality in mice and against hemorrhagic shock lethality in purebred beagles, but at very high doses (240 to 512 mg/kg prednisolone phosphate in mice and 60 mg/kg MP in beagles) protection decreased (16, 20, 21). This was attributed to a "toxic" effect of these large quantities of corticosteroids and such toxicity appeared to be exaggerated by the shock state, since comparably "toxic" doses of the steroids did not evoke mortality in healthy animals (20, 21).

Further increments in corticosteroid dose, however, proved lethal even to healthy animals (20). The possibility that slower rates of administration of corticosteroids might reduce their acute toxicity and thereby increase the upper range of their protective activity is an important question that remains to be explored.

In the present studies, when optimal protective doses of MP (30 mg/kg) were given, the initial injection of the corticosteroid consistently evoked the greatest increments in protection. Further increments in protection were achieved by additional injections of 30 mg/kg MP given at 4-hr intervals, but each increment was progressively less marked. Thus the present findings, together with our earlier data (10), indicate that the ability of MP to reduce murine mortality from gram-negative bacterial sepsis is not only critically restricted by the requirement for its early administration in conjunction with appropriate antibiotic therapy, but also by its relatively narrow optimal dose–response range and the decremental increments in its effectiveness upon repetitive administration.

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