The Effect of Administration of Immunoglobulin to Newborn Rats with *Escherichia coli* Sepsis and Meningitis

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ABSTRACT. Newborn rats 24–36 h old were injected transthoracically with various doses of *Escherichia coli* K1. Eighty-six of 92 rats which received 10^4 colony-forming units/g body weight were dead within 48 h. Two h after injection, *E. coli* were recovered from the blood of six of six rats and from the cerebrospinal fluid of two of six. Sixteen h after injection, *E. coli* were recovered from all blood (7/7) and spinal fluid (11/11) specimens cultured. Animals inoculated with 10^4 *E. coli*/g exhibited neutropenia and depletion of the neutrophil storage pool. In other studies, newborn rats inoculated with *E. coli* were injected intraperitoneally with various doses of human serum immunoglobulin, modified for intravenous use (MISG). One hundred percent (25/25) injected with 1500 mg/kg lived. In contrast to infected animals injected with albumin (controls), MISG recipients did not develop neutropenia nor did they deplete their neutrophil reserves. The effects of treating infected animals with MISG, antibiotics, or a combination of antibiotics plus MISG were compared. When administered within 2 h after the *E. coli*, all treatments resulted in survival rates of over 75%. However, when delayed for 6 h, 63% (17/27) of antibiotic recipients, 50% (12/24) of MISG recipients, and 91% (30/33) of those receiving both treatments lived ($p < 0.01$ versus antibiotics or MISG). (*Pediatr Res* 22: 455–460, 1987)

Abbreviations

GBS, group B streptococcus
MISG, human immune serum globulin, modified for intravenous use
PBS, phosphate-buffered saline
KRP, Krebs-Ringer phosphate solution
CSF, cerebrospinal fluid
CFU, colony-forming units

septic, neutrophil-depleted patients, the prognosis is better for those who do not develop neutropenia and who sustain only a moderate reduction in the marrow neutrophil reserve (6).

Although GBS accounts for most instances of neonatal sepsis in the United States, *Escherichia coli* is responsible for 30–40% of cases (7), and for approximately 30–40% of cases of neonatal bacterial meningitis (8). We previously observed neutropenia and depletion of the marrow neutrophil reserve in four human neonates with *E. coli* infection (2). However, it is not known whether *E. coli* regularly produces these abnormalities in the manner described during neonatal GBS infection (5). The suckling rat has been used as a model of *E. coli* sepsis and meningitis by several investigators (10, 11), who have demonstrated the severity of infection to be related to the larger doses of organisms, the younger aged animals, and the presence of the K1 antigen. In experimental neonatal infection with GBS, specific antibacterial antibody has been shown to improve the outcome (12–16) and to prevent the development of neutropenia and depletion of the neutrophil reserves (13). Recent studies have demonstrated similar beneficial effects on outcome in suckling rat models of *E. coli* infection, whether the antibody is administered naturally through the colostrum of immunized dams (17) or orally as hyperimmune horse serum (17) or intraperitoneally as specific monoclonal IgM antibody (18) or MISG containing opsonic antibody (19). However, no studies have examined the effect of antibody to *E. coli* on neutrophil kinetics.

In order to further study the effects of antibody in *E. coli* infection, we developed a model for *E. coli* sepsis and meningitis, inoculating newborn rats with a strain of *E. coli* expressing the K1 antigen. In these studies we (1) quantified circulating and storage neutrophil populations serially, (2) compared the neutrophil kinetics of infected rats treated with MISG to the neutrophil response of others treated with albumin as a control, and (3) compared the survival of infected rats treated with MISG, antibiotics, and a combination of MISG plus antibiotics.

METHODS

Organism. *E. coli*, identified as 085w:K1:NM (*E. coli* Reference Center, Philadelphia, PA), were stored at -70°C until use. Then aliquots were thawed and allowed to grow overnight in fresh Todd-Hewitt or tryptic-soy broth. Organisms were sedimented by centrifugation and washed three times in PBS or KRP. The concentration of bacteria was standardized by its optical density at 600 nm, and suspensions of $0.9\text{--}2.9 \times 10^9$, 10^8 , 10^7 , or 10^6 organisms/ml were prepared. Concentrations were confirmed by colony counts on McConkey or tryptic-soy agar. The *E. coli* were sensitive to both ampicillin and gentamicin (Autobac, General Diagnostics, Morristown, NJ).

Animals. Pregnant Sprague-Dawley albino rats, obtained from Simonsen Laboratories (Gilroy, CA), were housed in the Uni-

The distinctive susceptibility of neonates to bacterial infection has been observed in many species, including rats (1). Using newborn rats as a model of neonatal GBS sepsis, investigators have observed that neutropenia and depletion of the marrow neutrophil reserves precede septic death (2, 3). These neutrophil abnormalities have also been observed in human neonates with GBS sepsis (4, 5). In contrast to the poor outcome observed in

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versity of Utah Vivarium until litters delivered. When the pups were 24–36 h old, litters were mixed; and, using a microliter syringe (Hamilton Co., Reno, NV), 6.5 μ l of *E. coli* suspension/g body wt was injected into the right lateral thorax after the skin was washed with 70% ethyl alcohol.

Cultures. Blood was obtained sterily from the jugular vein, after washing the area with 70% ethyl alcohol, or from the inferior vena cava. CSF was obtained by exposing the dura over the cisterna magna by dissection and sterily penetrating the dura with a needle or scalpel blade. Twenty-five μ l of CSF was collected in a micropipet. All blood-tinged specimens were discarded. To assess the degree of blood contamination, erythrocyte counts were performed on specimens from 10 uninfected animals. Specimens were incubated overnight in Todd-Hewitt or tryptic-soy broth and subcultured in 100 μ l aliquots on Todd-Hewitt or tryptic-soy agar plates. Colonies were identified as *E. coli* by morphology.

Quantitative cultures for *E. coli* were performed using 50 μ l of jugular venous blood or 25 μ l of CSF. Specimens were plated directly or in various dilutions of sterile water in a volume of 100 μ l on tryptic-soy plates and spread with a glass rod. After overnight growth, colonies were counted with the aid of a touch-sensitive automatic colony counter (Quebec Colony Counter, American Optical Corp., Buffalo, NY).

MISG. MISG (Gamimune-N, lot #PR 2964, Cutter Biological, Berkeley, CA) was prepared at an acid pH (4.25) as a 5% solution in 10% maltose. It was administered intraperitoneally to groups of 17–25 newborn rats in doses of 125, 250, 500, 1000, 1500, and 2000 mg/kg (corresponding to 5, 10, 15, 20, 30, and 40 ml/kg). Other animals (controls) were given 0.1% albumin in 10% maltose, administered in the same volume as the MISG.

In other studies, groups of 24–48 newborn rats inoculated with 10^4 *E. coli*/g were given one of three forms of therapy: 1) MISG (1500 mg/kg intraperitoneally), 2) antibiotics (ampicillin, 200–300 mg/kg/day, and gentamicin, 5–7 mg/kg/day, intramuscularly twice daily for 2 days), or 3) MISG and antibiotics. Other animals (controls) were given injections of sterile normal saline, rather than antibiotics, and albumin in 10% maltose, rather than MISG. The treatments were administered at the same time as and at various intervals after the *E. coli*: 2, 6, 16, or 22 h.

Neutrophil kinetics. Two, 6, and 22 h after the simultaneous administration of *E. coli* and either MISG or albumin, venous blood was obtained from groups of five to nine rats and counted electronically (Coulter Electronics, Hialeah, FL). Cover slip smears were prepared for a 100-cell differential count. The number of neutrophils within the storage pool was determined by methods we have previously described (2, 13), in which the components of the reserve within the skeletal marrow, liver, and spleen were determined by electronic and differential counts of cells from these organs.

Radiolabeled MISG. MISG was radioiodinated by employing 125 I and 1,3,4,6-tetrachloro-3 α ,6 α -diphenylglycoluril (Iodogen) (Pierce Chemical Co., Rockford, IL) (20). Iodogen was dissolved in chloroform to a concentration of 416 μ g/ml and 60 μ l were placed in a pyrex tube (10 $\times$ 75 mm). The tube was rapidly vacuum-dried and placed on ice. Thirty μ l of 0.2 M sodium phosphate dibasic buffer or PBS plus 10 μ l of MISG were added, followed by 10 μ l (1 mCi) of carrier-free Na^{125}I in 0.1 N NaOH (Amersham Corp., Arlington Heights, IL), resulting in a total reaction volume of 50 μ l. The mixture was kept on ice for 20 min with gentle agitation. Bound ^{125}I was collected by passage over a G-10 Sephadex column and concentrated by membrane cone ultrafiltration (Type CF25, Amicon Corp., Danvers, MA). The protein content was determined by the Lowry method (21). Radiolabeled MISG was then diluted with unlabeled MISG to give $3.0\text{--}3.2 \times 10^7$ cpm/ml or $7.4\text{--}7.6 \times 10^5$ cpm/ μ g protein. The final diluted produce was administered to neonatal rats intraperitoneally in a volume of 225–250 μ l (1500 mg/kg of MISG).

Eleven newborn rats were inoculated with 10^4 *E. coli*/g and at

the same time or 6 h later, injected intraperitoneally with radio-labeled MISG. Three control animals were given KRP instead of *E. coli*, followed by radiolabeled MISG. Twenty-two h after the MISG administration, blood and CSF were obtained and counted on an automated gamma counter (Gamma 8000, Beckman Instruments, Inc., Fullerton, CA). The concentrations of radiolabeled MISG, expressed in mg/100 ml, in blood and CSF were then calculated.

Statistics. Differences in neutrophil kinetic studies were analyzed using the Student's *t* test. Survival was analyzed using the Fisher exact test.

RESULTS

In mortality studies, 21% of newborn rats inoculated with 10^3 *E. coli* CFU/g body weight were dead within 7 days (Fig. 1). However, 93% which received 10^4 /g were dead by 48 h, and 99% had died by 6 days. All animals which received larger inocula of 10^5 /g or 10^6 /g were dead within 40 h of injection. Since 10^4 CFU/g resulted in greater than 90%, but less than 100% mortality, this inoculum was selected for all subsequent experiments.

The results of blood and CSF cultures following inoculation with 10^4 *E. coli* CFU/g are displayed in Figure 2. *E. coli* were

Fig. 1. Survival of groups of 16–92 newborn rats following transthoracic inoculation with four different doses of *E. coli* K1.

Fig. 2. Cultures of the blood ($n = 32$) and CSF ($n = 68$) in groups of newborn rats following transthoracic inoculation of 10^4 *E. coli* K1/g body weight.

recovered from the blood of five of six animals $\frac{1}{2}$ after the inoculation and from all blood cultures (26/26) at each time thereafter. *E. coli* were recovered from the CSF of one of six rats $\frac{1}{2}$ after the bacterial inoculation, from two of six after 2 h, from five of six after 4 h, from 24 of 32 (75%) after 6 h, and from 18 of 18 (100%) thereafter.

Quantitative blood and CSF cultures were also performed at various times following the inoculation. Sixteen h after injection, blood cultures were obtained on 12 animals and found to contain 1.4 ± 1.4 (mean $\pm$ SD) $\times 10^6$ *E. coli*/ml. Seven CSF samples obtained at this time contained $6.6 \pm 3.1 \times 10^2$ *E. coli*/ml. Within 22 h blood cultures ($n = 10$) contained $9.8 \pm 14.8 \times 10^6$ *E. coli*/ml and CSF specimens ($n = 2$) $1.1 \pm 1.1 \times 10^5$ /ml. The red blood cell concentration in the CSF was 459 ± 371 cells/mm³ (mean $\pm$ SD). Because the red blood cell concentration in peripheral blood of newborn rats is about 2×10^6 /mm³ (22), at most the CSF specimens were contaminated by one part blood for every 4000 parts CSF. Therefore, contamination of CSF by blood containing $<2.5 \times 10^6$ *E. coli* CFU/ml could not explain the quantity of organisms recovered from the CSF of these animals. These data support the diagnosis of meningitis.

Fig. 3. Survival of groups of 17-25 newborn rats after a transthoracic inoculation of 10^4 *E. coli* K1/g body weight, followed immediately thereafter by the intraperitoneal administration of various doses of MISG.

Fig. 4. The blood neutrophil count in groups of newborn rats 2, 6, and 22 h following inoculation with 10^4 *E. coli* body weight. Five to six infected animals received an intraperitoneal injection of 1500 mg/kg of MISG, and their neutrophil counts are shown by solid circles. Eight to nine infected animals received an intraperitoneal injection of the same volume of albumin (control), and their neutrophil counts are shown by open squares.

The survival of groups on newborn rats, transthoracically injected with 10^4 *E. coli*/g followed immediately by an intraperitoneal injection of various doses of MISG, is shown in Figure 3. No deaths occurred among 25 infected animals receiving 1500 mg/kg and in only one of 18 animals which received 1000 mg/kg MISG. When less MISG was administered, survival diminished (Fig. 3). In all subsequent experiments 1500 mg/kg of MISG was given.

The blood neutrophil concentration in groups of five to nine newborn rats following inoculation of 10^4 *E. coli*/g is shown in Figure 4. One group of infected rats received an intraperitoneal injection of MISG, 1500 mg/kg, while the other group received albumin. Six h after the bacterial inoculation, blood neutrophils had diminished to 1.19 ± 0.21 (mean $\pm$ SEM) $\times 10^3$ /mm³ in the albumin recipients but remained normal ($2.18 \pm 0.43 \times 10^3$ /mm³) in those which received MISG ($p = 0.06$). By 22 h after inoculation, the infected animals which had received MISG had increased concentration of blood neutrophils ($3.08 \pm 0.53 \times 10^3$ /mm³), while infected animals which received albumin exhibited marked neutropenia ($0.16 \pm 0.08 \times 10^3$ /mm³, $p < 0.001$).

The ratio of immature neutrophils (band neutrophils + metamyelocytes) to total neutrophils (segmented neutrophils + band neutrophils + metamyelocytes) in the blood of infected animals is shown in Figure 5. Two h after the bacterial inoculation, animals which had received MISG displayed a more elevated ratio (0.71 ± 0.05 , mean $\pm$ SEM) than did albumin recipients (0.48 ± 0.07 , $p < 0.03$). However, by 22 h the ratio was greater in animals treated with albumin (0.86 ± 0.08) than in those which received MISG (0.47 ± 0.05 , $p < 0.005$).

The neutrophil storage pool (the total of all segmented neutrophils, band neutrophils, and metamyelocytes within the skeletal marrow, liver, and spleen) in groups of five to nine newborn rats 2, 6, and 22 h after inoculation of 10^4 *E. coli*/g body weight is shown in Figure 6. MISG promoted early mobilization of neutrophils from the marrow. Two h after inoculation, neutrophil reserves were more reduced in the MISG recipients [containing 4.44 ± 0.64 (mean $\pm$ SEM) $\times 10^6$ cells/animal] than in the controls, which received albumin ($7.93 \pm 0.86 \times 10^6$ cells, $p < 0.01$). Later, MISG prevented exhaustion of the neutrophil reserves. After 22 h control animals displayed exhaustion of their neutrophil supply (containing only $1.38 \pm 0.30 \times 10^6$ cells)

Fig. 5. The ratio of immature neutrophils (band neutrophils + metamyelocytes) to total neutrophils (segmented neutrophils + band neutrophils + metamyelocytes) in groups of newborn rats 2, 6, and 22 h following inoculation with 10^4 *E. coli*/g. Five to six infected animals received an intraperitoneal injection of 1500 mg/kg of MISG and their immature/total blood neutrophil ratios are shown by solid circles. Eight to nine infected animals received an intraperitoneal injection of the same volume of albumin (control), and their ratios are shown by open squares.

Fig. 6. The neutrophil storage pool (all segmented neutrophils + band neutrophils + metamyelocytes within the skeletal marrow + liver + spleen) in groups of newborn rats 2, 6, and 22 h following inoculation with 10^4 *E. coli*/g body weight. Five to six infected animals received an intraperitoneal injection of 1500 mg/kg of MISG, and their neutrophil storage pool values are shown by solid circles. Eight to nine infected animals received an intraperitoneal injection of the same volume of albumin (control), and their neutrophil reserves are shown by open squares.

Fig. 7. The survival of groups of 24–33 newborn rats inoculated with 10^4 *E. coli*/g body weight and after 6 h treated with one of three therapies. Survival of infected rats treated with MISG (1500 mg/kg) is shown by solid circles. Survival of those treated with antibiotics is shown by open circles and of those treated with MISG and antibiotics by solid squares. Infected animals treated with albumin (intraperitoneal) and saline (intramuscular) injections are shown by open squares.

whereas MISG recipients had a larger population of stored neutrophils ($3.30 \pm 0.70 \times 10^6$ cells, $p < 0.05$). Thus, newborn rats infected with *E. coli* and treated with MISG released neutrophils from their reserves more promptly than did controls. In addition, MISG recipients did not completely exhaust their neutrophil reserves as did controls.

Other groups of animals were inoculated with *E. coli* and following 2 h treated with either MISG, antibiotics, or a combination of the two. Substantial survival was observed in all groups: 75% (18/24) with MISG, 86% (24/28) with antibiotics, and 79% (38/48) with the combination. However, when treatments were

Fig. 8. Survival of groups of 25–43 newborn rats inoculated with 10^4 *E. coli*/g body weight who were alive after 22 h and treated with one of three therapies. Survival of infected rats treated with MISG (1500 mg/kg) is shown by solid circles. Survival of those treated with antibiotics is shown by open circles and of those treated with MISG and antibiotics by solid squares. Infected animals treated with albumin (intraperitoneal) and saline (intramuscular) injections are shown by open squares.

Table 1. Accumulation of administered IgG in blood and CSF*

<i>E. coli</i> inoculation	<i>n</i>	Blood concentration (mg/100 ml)	CSF concentration (mg/100 ml)
Yes	11	372 ± 59	24 ± 5
		NS	NS
No	3	309 ± 25	26 ± 2

* All animals received 1500 mg/kg 125 I-MISG intraperitoneally. Assays were performed on blood and CSF obtained 16 or 22 h after MISG administration to rats inoculated with either 10^4 *E. coli*/g or KRP (mean ± SD).

delayed until 6 h after the bacterial inoculation (when neutrophil stores had already been significantly reduced), MISG plus antibiotics was significantly better than either MISG or antibiotics alone ($p < 0.01$, Fig. 7). Even when delayed until 22 h after the *E. coli* inoculation, the therapeutic advantage of the combined treatment persisted (Fig. 8).

Eleven animals with meningitis and three control animals received radiolabeled MISG intraperitoneally (Table 1). After 22 h the blood and CSF levels of IgG were similar in the two groups, reaching blood concentrations of labeled IgG of more than 300 mg/100 ml. These increments probably represent a substantial increase in blood IgG levels since newborn rats have a blood level of IgG_{2a} (the primary rat IgG) of only 100 mg/100 ml in contrast to the adult level of 691 mg/100 ml (21).

DISCUSSION

Specific antibacterial antibody has an important role in neonatal defense against bacterial infection (24). In clinical studies of neonatal GBS disease, a lack of opsonic antibody toward GBS correlates with a poor outcome (25). Furthermore, in experimental models of neonatal GBS sepsis, infected animals treated with anti-GBS antibody exhibit markedly improved survival (26–28). Preparations of MISG have been successfully used as a source of anti-GBS antibody, improving the outcome of infected neonatal rats (13, 15, 16). Many *in vitro* studies have also demonstrated the requirement of antibody for effective resistance to encapsulated *E. coli* (29–31). Furthermore, animal studies have shown effectiveness of treating *E. coli* infections with antibody directed against specific *E. coli* antigens, especially the K1 polysaccharide

(17, 18, 32–34). In humans, however, the K1 antigen is only weakly immunogenic (34–36) and in fact elicits predominantly an IgM antibody response (37). Preparations of MISG, therefore, often have very low anticapsular-*E. coli* titers (37). Cross *et al.* (34) did not demonstrate any opsonic antibody against *E. coli* K1 in five preparations of MISG. However, Hill and Bathras (38) detected modest opsonic antibody in six different lots of the preparation used in the present study. Bortolussi and Fischer (19) has also recently reported the use of MISG as a source of opsonic antibody which conferred improved survival on newborn rats infected with *E. coli* K1. Nonetheless, specific or cross-reactive antibody to the O-antigen may also play a role in humoral defense against *E. coli* K1 (39).

The beneficial effect of antibody appears, at least in part, to be mediated through its effect on the neutrophil response to bacterial infection. Neutropenia and depletion of the neutrophil reserves have been well documented in fatal neonatal GBS infection (2–6), and antibody has been shown to cause a marked diminution in this adverse response. However, little information has been available previously on either neutrophil kinetics during gram-negative infection in neonates or on the effect of antibody on the neutrophil response.

In the present studies we determined that *E. coli* K1, administered transthoracically into rats 24–36 h old, resulted in mortality in a dose-dependent fashion. Animals inoculated with 10^4 CFU/g body weight developed neutropenia, depletion of neutrophil reserves, and death within 48 h. However, neutropenia and exhaustion of neutrophil reserves did not occur in animals which, immediately after the bacterial inoculation, received an intraperitoneal injection of MISG, 1500 mg/kg. Moreover, the increase in immature neutrophil forms in the peripheral blood of treated animals 2 h after *E. coli* inoculation along with the simultaneous decrease in storage neutrophils suggests a more rapid mobilization of neutrophils into the blood. MISG as a treatment for newborn rats with *E. coli* infection was also compared with antibiotics and with the combination of antibiotics plus MISG. If administered within 2 h following the bacterial inoculation, all treatments were equally effective in preventing mortality. However, if delayed for 6 h, a time at which 75% of animals had meningitis and all had diminished neutrophil reserves, the combined treatment was clearly superior. When delayed for 22 h, a time at which all animals had meningitis, neutropenia, and severe depletion of neutrophil reserves, none of the treatments was very effective. However, even under these circumstances, the combination of MISG and antibiotics resulted in significantly greater survival than did either treatment alone.

Givner *et al.* (40) demonstrated that MISG alone does not kill bacteria *in vitro* but rather acts as an opsonin. Similarly, we (13) reported that a major effect of MISG administration to infected neonatal rats was an improved efficiency in migration of neutrophils to inflammatory sites. Therefore, the fact that in the present studies MISG improved survival of infected rats when administration was delayed until after marked neutropenia and neutrophil storage pool depletion had occurred appears incongruous with its expected mechanism of action. These observations might be explained by the hypotheses that in neutropenic subjects MISG improves the efficiency of bacterial killing by nonneutrophilic phagocytes (41), enhances the function of the reticuloendothelial system (42), supplies noncellular mechanisms such as plasma factors (43), or improves the function of the relatively few remaining neutrophils (44).

Admittedly the present studies cannot distinguish whether the effects of MISG on neutrophil kinetics and survival were the result of specific anti-*E. coli* antibody or some other mechanism. However, the cooperative effect of MISG and antibiotics in improving survival of animals with sepsis and meningitis is striking.

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