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Supplemental Complement Component C9 Enhances the Capacity of Neonatal Serum to Kill Multiple Isolates of Pathogenic *Escherichia coli*[1]

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ABSTRACT. Previous studies demonstrated that, compared with adult serum, neonatal serum contained a diminished concentration of complement component C9 and that supplemental C9 enhanced the capacity of neonatal serum to kill an isolate of *Escherichia coli*. Therefore, experiments were designed to determine the mechanisms by which supplemental C9 enhances the bactericidal capacity of neonatal serum and to determine whether supplemental C9 enhances the capacity of neonatal serum to kill several different pathogenic strains of *E. coli*. A radiobinding assay and immunogold electron microscopy using a monoclonal anti-C9 antibody revealed that, compared with 40% adult serum, neonatal serum deposited a diminished quantity of C9 onto *E. coli* O7w:K1:NM. Supplemental C9 (75 mg/L) significantly enhanced the quantity of C9 deposited by the neonatal serum. Treatment with 10 mM MgEGTA (a mixture of 100 mM $MgCl_2$ and 100 mM EGTA that blocks activation of the classic complement pathway but leaves the alternative pathway intact) abolished the capacity of neonatal serum to deposit C9 and to kill the bacteria. Supplemental C9 enhanced the capacity of neonatal serum to kill eight different blood isolates of *E. coli*. Therefore, supplemental C9 enhanced the capacity of neonatal serum to kill *E. coli* by increasing the total quantity of C9 deposited via activation of the classic complement pathway. Neonatal serum contained sufficient quantities of classic pathway components, other than C9, to deposit the supplemental C9 onto *E. coli* and to enhance bacterial killing. The bactericidal activity of neonatal serum against multiple isolates of pathogenic *E. coli* was increased after C9 supplementation. We speculate that C9 deficiency may be one of the defects in antibacterial host defense that predisposes neonates to the acquisition of *E. coli* sepsis. (*Pediatr Res* 35: 389–396, 1994)

Abbreviations

MgEGTA, 100 mM $MgCl_2$ and 100 mM EGTA
LC_{90}, minimum concentration of adult serum required to kill 90% of an isolate of *Escherichia coli* during 90 min of incubation
$BPBS^+$, PBS with 1 mM $MgCl_2$ and 0.5% BSA
$BPBS^{++}$, PBS with 1 mM $MgCl_2$, 0.5% BSA, and 0.15 mM $CaCl_2$

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Escherichia coli is the second most common bacterium isolated from the blood of septic neonates (1, 2). Newborn infants acquire *E. coli* sepsis for reasons that are not completely understood. In the adult host, complement-mediated cytolysis (bacteriolysis) has been proposed as a mechanism of defense against invasion by gram-negative bacteria (3–5). However, compared with serum from adults, serum from human neonates kills *E. coli* inefficiently (6, 7). The ninth component of complement (C9) is a circulating glycoprotein that is required for efficient complement-mediated cytolysis of *E. coli* (8–10). The concentration of C9 is diminished in the sera of human neonates (11, 12). Moreover, supplemental C9 enhanced the capacity of sera from newborn infants to kill a pathogenic isolate of *E. coli* (12).

The mechanism by which supplemental C9 increases the bactericidal capacity of neonatal serum is not known. Also, whether supplemental C9 affects the bactericidal activity of neonatal serum against other isolates of *E. coli* is not known. Therefore, experiments were designed to determine the effect of supplemental C9 on the kinetics of bacterial killing by neonatal serum, the deposition of C9 onto the surface of *E. coli*, and the capacity of neonatal serum to kill several different isolates of pathogenic *E. coli*.

MATERIALS AND METHODS

Buffers and reagents. The following buffers and reagents were used: Dulbecco PBS (Media Tech, Washington, DC), pH 7.4; $BPBS^+$; $BPBS^{++}$; 100 mM MgEGTA (Sigma, St. Louis, MO), pH 7.4; and tryptic soy broth (Difco Laboratories, Detroit, MI). Buffers used in the radiobinding assays and the immunogold assays contained 0.02% sodium azide (Sigma). Buffers used in the bactericidal assays contained no bacteriostatic agents.

Bacteria. Eight isolates of *E. coli* were obtained from the blood of eight septic neonates who were patients at the Neonatal Intensive Care Unit of Kosair Children's Hospital, Louisville, KY. The serotype of each organism was determined at the *E. coli* Reference Center of Pennsylvania State University, University Park, PA (Table 1). The bacteria were grown overnight and stored in 5-mL aliquots of tryptic soy broth at −70°C. Before an experiment, 35 mL of fresh broth were added, and the bacteria were incubated at 37°C for 3 to 4 h to bring the organisms to log phase. The bacteria were then washed three times in $BPBS^{++}$ or $BPBS^+$ and adjusted by absorbance at 600 nm to a concentration

Table 1. *Characteristics of E. coli isolates**

Isolate	LC_{90} (%)	Serotype
1	20	O7w:K1:NM
2	30	O(−):H(−)
3	40	O7w:K1:8w
4	60	O(−):K1:H(−)
5	30	O1:K1:NM
6	30	O(−):K1:NM
7	40	O(−):H(−)
8	50	O7:K1:NM

* Each isolate was obtained from the blood of a septic neonate.

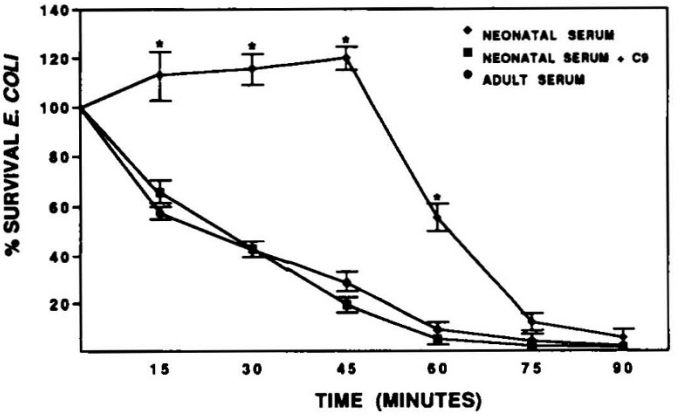

Fig. 1. Effect of supplemental C9 on the bactericidal activity of neonatal serum. *E. coli* O7w:K1:NM were incubated in pooled neonatal serum (*diamonds*), pooled neonatal serum supplemented with C9 (*squares*), and pooled adult serum (*circles*). As determined by bactericidal assay, the values are depicted as the mean bacterial survival ± SE. *, $p <$ 0.05 *vs* adult serum and $p < 0.05$ *vs* C9-supplemented neonatal serum. The onset of bacterial killing was delayed in neonatal serum compared with adult serum. However, in neonatal serum, supplemental C9 accelerated the onset of bacterial killing.

Fig. 2. Deposition of C9 onto *E. coli* by neonatal sera. *E. coli* O7w:K1:NM were incubated with pooled adult serum (*AdS*) and with sera from 10 healthy neonates (numbers 1–10). As determined by radiobinding assay, the results are depicted as the mean (+ SE) quantity of ^{125}I-labeled anti-C9 antibodies specifically bound to the bacteria ($n = 4$). *, $p < 0.01$ *vs* adult serum. Compared with adult serum, the neonatal sera deposited C9 onto *E. coli* inefficiently.

of 8×10^5 or 4×10^9 colony forming units/mL. The concentration of organisms was confirmed by quantitative culture.

Serum. Neonatal sera were obtained from the cord blood of 20 healthy full-term neonates who were delivered vaginally or by elective Cesarean section between July 2 and July 7, 1991, at Norton Hospital, Louisville, KY. The cord blood was obtained by sterile needle puncture of the umbilical vein of the placenta.

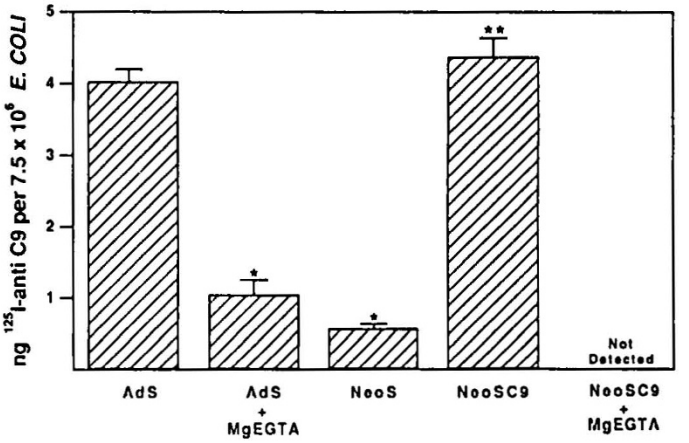

Fig. 3. Effect of supplemental C9 on the deposition of C9 onto *E. coli* by neonatal serum. *E. coli* O7w:K1:NM were incubated with pooled adult serum (*AdS*), AdS treated with MgEGTA, pooled neonatal serum (*NeoS*), NeoS supplemented with C9 (*NeoSC9*), and C9-supplemented NeoS treated with MgEGTA. As determined by radiobinding assay, the results are depicted as the mean (+ SE) quantity of ^{125}I-labeled anti-C9 antibodies specifically bound to the bacteria ($n = 3$). *, $p < 0.01$ *vs* adult serum; **, $p < 0.01$ *vs* pooled neonatal serum. C9 deposition onto *E. coli*, mediated by the classic complement pathway, was inefficient in the pooled neonatal serum but was enhanced by supplemental C9.

The serum from each neonate contained less than 0.1 g/L of IgA and less than 0.2 g/L of IgM, as determined by single radial immunodiffusion (Endplate IgA and Endplate IgM, Kallestad, Austin, TX) (13). Most experiments used serum that was pooled from the blood of the 20 neonates. Sera were also obtained and pooled from five healthy adult volunteers. The serum was separated from the clotted blood by centrifugation and passed through a 0.2-μm filter to ensure the removal of bacterial contaminants. Individual sera and the pooled serum were stored at −70°C in sterile vials.

Complement component C9. Purified C9 (Lot no. A40TT23901) was purchased from Quidel Corporation (San Diego, CA) as a solution containing 1000 μg of C9/mL of PBS without bacteriostatic agents. This C9 preparation was isolated by the method of Biesecker and Müller-Eberhard (14) and was subjected by the manufacturer to an antiimpurities solid-phase immunoaffinity column containing the IgG fraction isolated from monospecific goat antihuman C3, -C5, -C6, -C7, and -C8 to insure the complete removal of these complement proteins. SDS-PAGE and hemolytic assays by the manufacturer demonstrated that more than 95% of the protein in the solution was pure C9 and that the solution was completely free of C3, C5, C6, C7, and C8 functional activities.

To confirm the purity of the C9 preparation used in these studies, various assays were also performed in our laboratory. By nephelometry (Beckman Array Protein System, Beckman Instruments, Brea, CA), the solution did not contain detectable IgA, IgM, or IgG. Because neonatal serum normally contains diminished concentrations of various complement components, such as C3 and C5, assays were performed to determine the amount of these two proteins in the C9 preparation used to supplement the neonatal serum. By radial immunodiffusion using Human C3 NL-RID and Human C5 NL-RID Kits obtained from The Binding Site (San Diego, CA), the C3 and C5 concentrations in the pooled neonatal serum were 977 mg/L and 124.3 mg/L, respectively, compared with 1149 mg/L and 149.3 mg/L in the adult serum. The C9 preparation was subjected to Western-blot analysis (15) using *1*) rabbit antisera specific for C3, C5, and C9 (Harlan Bioproducts, Indianapolis, IN) and *2*) affinity purified antirabbit IgG that had been absorbed with human serum proteins and coupled to alkaline phosphatase (Sigma). Trace

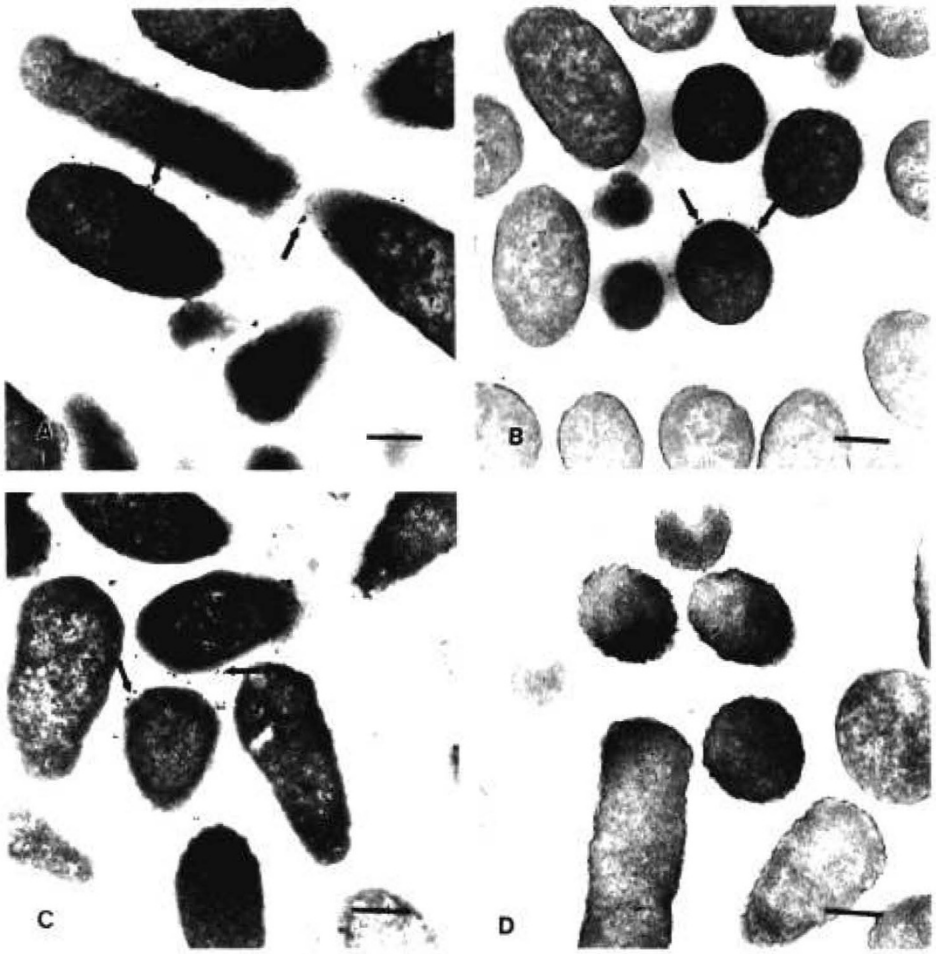

Fig. 4. Effect of supplemental C9 on the capacity of neonatal serum to deposit C9 onto *E. coli*. *E. coli* O7w:K1:NM were incubated with pooled adult serum (*A*), pooled neonatal serum (*B*), neonatal serum supplemented with C9 (*C*), and heat-treated neonatal serum supplemented with C9 (*D*). *Arrows* indicate the gold particles in the photomicrographs obtained after immunogold electron microscopy. Bar = 0.5 μm. Supplemental C9 enhanced the capacity of neonatal serum to deposit C9 onto *E. coli*.

amounts of C3 were detected on the basis of a series of standards but represented less than 0.1% of the protein in the C9 preparation. C5 was not detected and represented less than 0.2% of the protein in the C9 preparation. SDS-PAGE analysis revealed that more than 90% of the protein in the solution was represented by a single band with an approximate molecular mass of 71 000 D. Each of the minor bands detected by SDS-PAGE, including a band with a molecular mass of more than 180 000 D, reacted with the anti-C9 antiserum in the Western-blot assay. Therefore, the proteins detected by SDS-PAGE, other than monomeric C9, may represent aggregated C9 or C9 degradation products that developed during storage. The C9 was stored in 10-μL aliquots at −70°C.

Quantitation of C9 in serum by radial immunodiffusion. A radial immunodiffusion kit (Human C9 NL RID Kit) was purchased from The Binding Site. In duplicate, 5-μL of serum was pipetted into the agar well, and the diameter of the immunoprecipitation ring was measured after 72 h of incubation at room temperature. The concentration of C9 was calculated from the regression equation derived from concurrent assays of control sera, provided by the vendor, which contained known concentrations of C9.

Antibodies. Affinity-purified, mouse monoclonal antihuman C9 antibody (Product No. 0947) was produced and supplied as a lyophilized powder by Accurate Chemical and Scientific Corporation (Westbury, NY). Morgan *et al.* (16, 17) demonstrated that this antibody binds to C9 that has been deposited onto biologic membranes and also to monomeric C9 in solution. The concentration of the anti-C9 antibody was adjusted to 1 mg/mL in PBS. In some experiments, the anti-C9 antibody was labeled with ^{125}I (Amersham, Arlington Heights, IL) using 1,3,4,6-tetrachloro-3α,6α-diphenylglycouril (Sigma) by the method of Parker *et al.* (18). The radiolabeled antibodies (sp act, 8.8×10^5 cpm/μg) were dialyzed against three changes of PBS and stored at 4°C. Protein concentrations were determined spectrophotometrically (19, 20).

Affinity-purified, colloidal gold-labeled goat antimouse IgG antibody was purchased from Energy Beam Sciences (Agalon, MA). The mean diameter of the gold particles was 15 nm, and the antibody was supplied as a liquid formulation with an optical density of 4.0 at 520 nm.

Bactericidal assay. E. coli isolate 1 (Table 1) was used to assess the effect of supplemental C9 on the kinetics of bacterial killing by neonatal serum. *E. coli* O7w:K1:NM (1.6×10^4 organisms suspended in 20 μL of $BPBS^+$) were incubated at 37°C for 90 min with 80 μL of serum in reaction mixtures that contained a final volume of 200 μL. In some experiments, the mixtures were supplemented with 15 μg C9. Also, some reaction mixtures were treated with 20 μL of 100 mM MgEGTA and were incubated 10 min at 37°C before the addition of the bacteria. In each case, the volume of the mixture was adjusted to 180 μL with $BPBS^+$ before the addition of the bacteria.

Immediately after adding the bacteria and at 15-min intervals during the incubation, 10 μL of the mixture was removed and

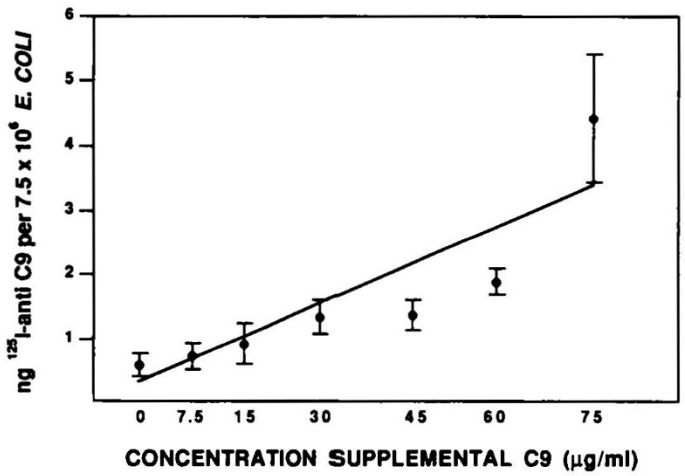

Fig. 5. Correlation between the concentration of supplemental C9 in neonatal serum and the quantity of C9 deposited onto *E. coli*. *E. coli* O7w:K1:NM were incubated with pooled neonatal serum containing various concentrations of supplemental C9. As determined by radiobinding assay, the results are depicted as the mean (± SE) quantity of ^{125}I-labeled anti-C9 antibodies specifically bound to the bacteria ($n = 3$). $r = 0.88$; $p < 0.01$. The quantity of C9 deposited onto the bacteria by the neonatal serum was positively correlated with the concentration of supplemental C9 in the serum.

diluted in BPBS$^+$ for quantitative culture in triplicate on tryptic soy agar by the pour-plate technique. The percentage of bacteria that survived was determined by dividing the number of colony forming units present at each 15-min interval by the number originally present in the mixture. The assays, including concurrent control mixtures, were performed three times on separate days.

The bactericidal assay was modified to assess the effect of supplemental C9 on the capacity of neonatal serum to kill the eight different isolates of *E. coli*. Each isolate of bacteria (8×10^3 organisms suspended in 10 μL of BPBS^{++}) was incubated with serum at 37°C for 120 min. The quantity of serum used varied according to the experimental requirements (see below). Some mixtures containing neonatal serum were supplemented with 1 μg of C9 per 5 μL of serum. In each case, the volume of the mixture was adjusted to 90 μL with BPBS^{++} before the addition of the bacteria. Immediately after adding the bacteria and at 20-min intervals during the incubation, 10 μL of the mixture was removed and diluted in BPBS^{++} for quantitative culture in triplicate on tryptic soy agar.

Preliminary assays were conducted to determine the minimal concentration of adult serum required to kill each of the eight isolates of *E. coli*. Each isolate was incubated with various concentrations of adult serum for 90 min, and the number of surviving bacteria were determined by quantitative culture. The LC_{90} was determined. The LC_{90} of the eight isolates ranged from 20 to 60% (Table 1).

To determine the effect of C9 on the survival of each isolate of *E. coli*, the concentration of serum used in the assay was equal to the LC_{90} of that particular isolate. For instance, the LC_{90} of *E. coli* isolate 1 was 20%. Therefore, the concentration of adult and neonatal serum used in the reaction mixtures was 20%.

Radiobinding assay. A radiobinding assay was developed to measure the quantity of C9 deposited onto the surface of *E. coli* during incubation with serum.

E. coli O7w:K1:NM (isolate 1; 8×10^7 organisms suspended in 20 μL of BPBS$^+$) were incubated at 37°C for 30 min with 80 μL of serum in reaction mixtures containing a final volume of 200 μL. In some experiments, the mixtures contained various quantities of supplemental C9. In each case, the final volume of the mixture was adjusted to 180 μL with BPBS$^+$ before the addition of the organisms. Quantitative cultures in pilot experiments revealed that the concentration of bacteria did not change significantly during 30 min of incubation with adult serum or heat-treated adult serum under these experimental conditions. In contrast to the bactericidal assay, the radiobinding assay used a higher concentration of bacteria—4×10^8 rather than 8×10^5 organisms/mL of reaction mixture.

In some experiments, the reaction mixtures contained 20 μL of 100 mM MgEGTA and were incubated 10 min at 37°C before the addition of the bacteria. A hemolytic assay using rabbit (21) and antibody-sensitized sheep red blood cells was used to verify that in pooled adult serum diluted to 40% with BPBS$^+$, 10 mM MgEGTA blocked activation of the classic, but not the alternative, complement pathway (22). During incubation (37°C, 30 min) with the adult serum but not heat-treated (56°C, 30 min) adult serum, both rabbit and sheep red blood cells were completely lysed. However, during incubation with MgEGTA-treated adult serum, the rabbit cells but not the sensitized sheep cells were lysed.

After the incubation with serum, the bacteria were washed three times and resuspended in 400 μL of BPBS$^+$. Next, in quintuplicate, 50 μL (10^7 organisms) were mixed with 50 μL of PBS containing 1.5 μg of radiolabeled anti-C9. Preliminary equilibrium binding experiments had previously demonstrated that this concentration of radiolabeled antibody was saturating. After incubation at 37°C for 30 min, 75 μL of the mixture was layered over 200 μL of phthalate oils (18) and centrifuged at $12\,000 \times g$ for 15 min at room temperature. Pilot studies using organisms labeled with ^{125}I had shown that more than 95% of the radioactivity associated with the bacteria was present in the cell pellet after centrifugation under the conditions described above. The cell-associated radioactivity was quantified with a gamma counter (RIASTAR model #B5005, Packard Instruments, Downers Grove, IL).

The amount of anti-C9 antibody bound to the bacteria was calculated by using the specific activity of the radiolabeled ligand. The quantity of anti-C9 antibody specifically bound during activation of the complement system was calculated by determining the radioactivity of the bacteria incubated with serum and subtracting the radioactivity of the bacteria incubated with heat-treated (56°C, 30 min) serum. If the serum was supplemented with C9, the quantity of specifically bound anti-C9 antibody was calculated by determining the radioactivity of the bacteria incubated with C9-supplemented serum and subtracting the radioactivity of the bacteria incubated with C9-supplemented, heat-treated serum.

Immunogold electron microscopy. E. coli O7w:K1:NM (*E. coli* isolate 1) were incubated with serum at 37°C for 30 min. Each 500-μL mixture contained 200 μL of serum and 50 μL of BPBS$^+$ containing 2×10^8 bacteria. Some mixtures contained 37.5 μg of C9. The volume of the mixture was adjusted to 450 μL with BPBS$^+$ before the addition of the bacteria. After the incubation, the organisms were washed three times and suspended in 100 μL of BPBS$^+$. Fifty microliters of the bacterial suspension (1×10^8 organisms) were incubated and gently rotated for 18 h at 4°C with 50 μL of PBS containing 20 μg of the anti-C9 MAb. Next, the bacteria were washed three times, suspended in 50 μL of BPBS$^+$, and incubated for 6 h at 4°C with 50 μL of PBS containing 20 μg of the colloidal gold-labeled antimouse IgG antibody. The organisms were then washed three times in BPBS$^+$ and suspended in 2.5% glutaraldehyde. The bacteria were fixed in 1% osmium tetroxide, rapidly dehydrated with graded concentrations of ethanol, and embedded in LX-112 (Ladd, Burlington, VT). Thin sections were cut with an ultramicrotome, collected on copper grids, and doubly stained with uranyl acetate and Reynolds lead citrate. The cross-sectioned organisms were then visualized and photographed on a Philips CM-10 electron microscope.

The gold particles that bound to 100 bacteria were counted using the photomicrographs. The length of the bacterial surface

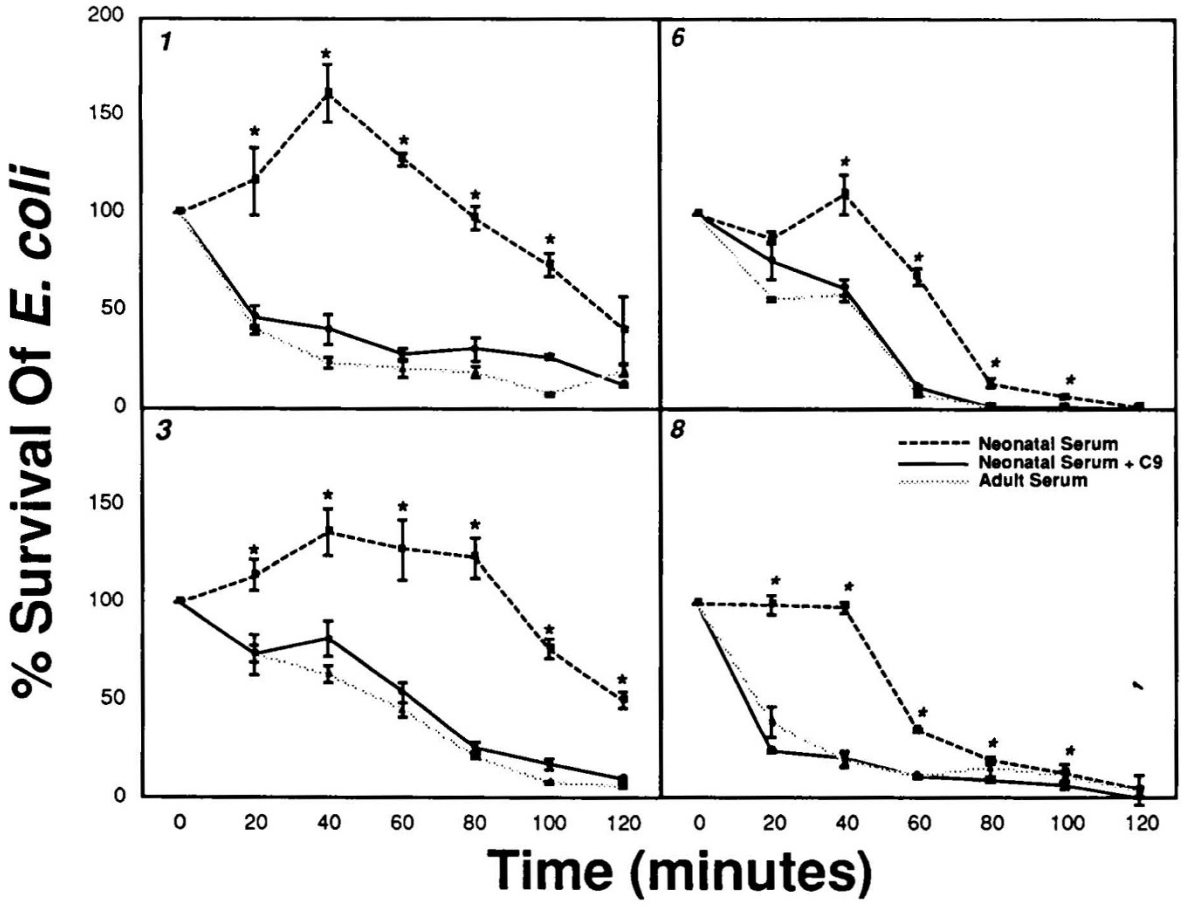

Fig. 6. Effect of supplemental C9 on the capacity of neonatal serum to kill four isolates of *E. coli*. Shown are representative experiments that analyzed isolates 1, 3, 6, and 8. Each isolate was incubated with pooled neonatal serum (*broken lines*), neonatal serum supplemented with C9 (*solid lines*), and pooled adult serum (*stippled lines*). As determined by bactericidal assay, the results are depicted as the mean bacterial survival ± SE. *, $p < 0.03$ *vs* neonatal serum supplemented with C9. The C9 supplemented serum, similar to adult serum, killed *E. coli* isolates 1 and 3. Supplemental C9 accelerated killing of isolates 6 and 8 by the neonatal serum.

visualized in cross section was measured by computer-assisted morphometric analysis with the Bioquant System 4 (R & M Biometrics, Nashville, TN). The results were expressed as the number of gold particles bound per linear micrometer of bacterial surface. The number of gold particles specifically bound to the bacteria during activation of the complement system was calculated by subtracting the number of particles bound to the bacteria incubated in heat-treated control serum from the number of particles that bound to organisms incubated in serum not treated with heat. The assays for electron microscopy, including concurrent control mixtures, were performed three times on separate days.

Statistics. Correlations between continuous variables were determined by multiple linear regression analysis. Comparison of the means of continuous variables was performed with *t* test for unpaired data.

For kinetic data derived from the bactericidal assays (Fig. 1), differences between groups were determined by repeated measures analysis and Bonferroni simultaneous *t* tests (23). Nonlinear differences were compared with full model—reduced model test using indicator variables (24, 25).

RESULTS

Serum concentrations of C9. The concentration of C9 in serum pooled from the adults was 211.5 mg/L. In contrast, the C9 concentration in sera from 10 neonates was 23.6 ± 4.0 mg/L (mean ± SEM; range, 8.4–34.7 mg/L). In the serum pooled from 20 neonates, the C9 concentration was 25.5 mg/L.

Effect of supplemental C9 on the kinetics of bacterial killing by neonatal serum. Both pooled adult serum and pooled neonatal serum killed *E. coli* O7w:K1:NM. In pooled adult serum, a reduction in the number of surviving bacteria was detectable at 15 min of incubation. In contrast, in the pooled neonatal serum, a reduction in the number of surviving bacteria was not detectable until the organisms had incubated for 60 min. During incubation of *E. coli* in pooled neonatal serum that had been supplemented with C9 (75 mg/L), the time required to detect a reduction in the bacterial count and the rate of decline in the number of surviving bacteria was indistinguishable from that observed in the pooled adult serum (Fig. 1). The number of surviving bacteria did not change significantly during incubation with $BPBS^+$ or with $BPBS^+$ supplemented with C9 in the absence of serum. The bacteria proliferated during incubation with adult serum, neonatal serum, or C9-supplemented neonatal serum treated with heat (56°C, 30 min) or MgEGTA.

Deposition of C9 onto E. coli by neonatal serum. During incubation of *E. coli* O7w:K1:NM with pooled adult serum, C9 was specifically deposited onto the bacteria. However, during incubation with each of the neonatal sera (Fig. 2) or with the pooled neonatal serum (Fig. 3), the quantity of C9 that was deposited onto the bacteria was diminished compared with that observed in adult serum. The quantities of C9 deposited onto the organisms by the 10 individual neonatal sera were not

correlated with the concentrations of C9 in these sera (data not shown; $r = 0.20$, $p = 0.58$).

Effect of supplemental C9 on the deposition of C9 onto E. coli by neonatal serum. C9 deposition onto the surface of *E. coli* O7w:K1:NM during incubation with 40% serum was quantified by the radiobinding assay and by immunogold electron microscopy. During incubation of *E. coli* with pooled neonatal serum that had been supplemented with C9 (75 μg/mL), the quantity of C9 deposited onto the organisms was increased to an amount equivalent to that observed in adult serum (Fig. 3). MgEGTA impaired the deposition of C9 during incubation of the bacteria with pooled adult serum or with C9-supplemented, pooled neonatal serum.

Electron microscopy revealed that the number of gold particles that specifically bound to each linear micrometer of bacterial surface was as follows: *1*) after incubation with adult serum, 0.93 ± 0.2; *2*) after incubation with neonatal serum, 0.30 ± 0.1 ($p < 0.01$ *versus* adult serum); and *3*) after incubation with neonatal serum supplemented with C9, 1.39 ± 0.3 ($p < 0.01$ *versus* unsupplemented neonatal serum). Only 0.055 gold particles/μm were detected on the surface of bacteria incubated in heat-treated adult serum. Gold particles were not detected on the surface of bacteria incubated in heat-treated neonatal serum, in heat-treated neonatal serum supplemented with C9, in C9 and buffer in the absence of serum, or in buffer without serum or C9 (Fig. 4).

In separate experiments, C9 deposition was quantified on *E. coli* that were incubated with pooled neonatal serum supplemented with various quantities of C9 (the concentration of supplemental C9 ranged from 0 to 75 μg/mL of reaction mixture). The quantity of C9 deposited onto the bacteria by the pooled neonatal serum was positively correlated with the concentration of supplemental C9 in the reaction mixture (Fig. 5).

Effect of supplemental C9 on the capacity of neonatal serum to kill eight isolates of E. coli. Adult serum killed all eight isolates of *E. coli*. Bacterial killing was defined as a reduction in bacterial survival to less than 20% of the original inoculum during 120 min of incubation. However, after 120 min of incubation of neonatal serum with *E. coli* isolates 1 through 4, more than 20% of the original inoculum survived (Fig. 6). In contrast, the C9-supplemented neonatal serum, like the adult serum, killed each of these four isolates. In each case, the number of surviving bacteria was reduced more rapidly during incubation with the C9-supplemented serum than with neonatal serum that was not supplemented with C9.

Neonatal serum killed isolates 5 through 8. However, the rate of bacterial killing in the neonatal serum was accelerated by supplemental C9 (Fig. 6). In the neonatal serum, isolates 5 through 8 were killed within 85 ± 5 min (mean ± SEM). In contrast, in the C9-supplemented neonatal serum, these four isolates were killed within 50 ± 13 min ($p < 0.05$ *versus* unsupplemented neonatal serum).

Each of the eight isolates of *E. coli* proliferated during incubation with heat-treated (56°C for 30 min) adult serum, heat-treated neonatal serum, heat-treated neonatal serum supplemented with C9, C9 and buffer in the absence of serum, and in buffer without serum or C9.

DISCUSSION

Previous studies determined that, compared with adults, neonatal serum killed an isolate of *E. coli* inefficiently. This observation was due at least in part to diminished concentrations of C9 in the sera of neonates (7, 12). The studies reported herein revealed that the onset of a detectable decline in the number of surviving bacteria was delayed during incubation with pooled neonatal serum (Figs. 1 and 6). After supplementation of the neonatal serum with C9, the time of onset and the rate of bacterial killing were indistinguishable from that observed in adult serum. These findings suggest that *1*) the diminished C9 concentration retarded the onset and rate of bacterial killing and *2*) the pooled neonatal serum contained sufficient quantities of the complement components, other than C9, required to kill *E. coli* at a rate similar to that observed in adult serum.

Although some isolates of *E. coli* are slowly killed in C9-deficient serum, C9 is required for efficient bactericidal activity of adult serum against *E. coli* (9, 10, 26–31). During complement-mediated cytolysis, the proteins C5, C6, C7, and C8 are sequentially assembled on the cell surface to form C5b-8 complexes that insert into the cell membrane (9, 27, 28). Complete membrane attack complexes are formed when the C5b-8 complexes bind several molecules of C9, which polymerize to form large membrane channels that may result in cell injury, death, and lysis (31–36).

Supplemental C9 has been shown to increase the rate of killing of a laboratory strain of *E. coli* by serum from a C9-deficient adult (10). With a pathogenic isolate of *E. coli* (serotype O7w:K1:NM), this phenomenon is now reported in the serum of healthy neonates (Fig. 1). However, unsupplemented 40% neonatal serum did kill the bacteria, albeit slowly compared with serum from healthy adults. Possibly, the isolate of *E. coli* used was an isolate that is killed slowly by serum in the absence of C9 by the assembly and deposition of C5b-8 complexes on the bacterial surface. Alternatively, C9 may have been required to kill the bacteria, but the diminished concentration of C9 in the neonatal serum may have limited the number of C9 molecules incorporated into each membrane attack complex or retarded the assembly of the complexes. After supplementation with C9, pooled neonatal serum killed *E. coli* as rapidly as the adult serum. These results suggest that the bactericidal activity of the neonatal serum was restricted by the concentration, rather than activation, of C9.

To determine the mechanisms by which supplemental C9 accelerates bacterial killing in neonatal serum, we used a radiobinding assay and immunogold electron microscopy. Previous studies revealed that radiolabeled supplemental C9 was deposited onto *E. coli* by neonatal serum (12). However, these studies did not determine whether the supplemental C9 was displacing intrinsic C9 from the bacterial surface or whether the total amount of C9 deposited was enhanced. The studies reported herein revealed that, compared with adult serum, neonatal serum deposited a diminished quantity of C9 onto *E. coli*. Supplemental C9 increased the total quantity of C9 deposited onto the organisms by neonatal serum to a level that equaled or exceeded that observed in adult serum. The quantity of C9 deposited by the neonatal serum was dependent on the quantity of supplemental C9 provided. C9 deposition was abolished by treatment of serum by heat or MgEGTA. MgEGTA blocks activation of the classic pathway of complement but leaves the alternative pathway intact (22). Hence, complement-mediated killing of *E. coli* O7w:K1:NM by serum required activation of the classic pathway. Therefore, supplemental C9 enhanced the bactericidal activity of neonatal serum by increasing the deposition of C9 onto *E. coli* mediated by activation of the classic pathway of complement. Furthermore, the results suggest that the pooled neonatal serum contained sufficient quantities of the other components of the classic pathway to kill the bacteria rapidly if sufficient exogenous C9 was provided. However, the results do not exclude the possibility that other factors, such as antibody-deficiency or diminished complement components in addition to C9, may also contribute to inefficient serum bactericidal activity in the sera of individual neonates.

In the sera of individual neonates, the quantity of C9 deposited onto *E. coli* was variable and was unrelated to the concentration of intrinsic C9. This observation is consistent with previous experiments that revealed that the survival of *E. coli* in the sera of individual neonates was variable (12). These prior studies also revealed that the effect of supplemental C9 on the bactericidal activity of neonatal sera was variable and was potentiated by supplemental IgG that contained anti-*E. coli* antibodies. In most cases, the C9 supplementation reduced the survival of *E. coli*

incubated with sera from individual neonates, but the bacteria were not killed (12). Taken together, the prior and present studies suggest that the sera of some individual neonates may contain not only diminished concentrations of C9 but also diminished activity of the other components of serum required to deposit C9 onto *E. coli* and to kill the bacteria, such as anti–*E. coli* antibodies or components of the classic pathway of complement.

Eight pathogenic isolates of *E. coli* were incubated with serum pooled from 20 neonates. The serum contained IgG antibodies transferred from the blood of their mothers. This experimental design diminished the probability that a deficiency of *E. coli* antibodies would confound the assessment of the effect of supplemental C9 in neonatal serum. Supplemental C9 enhanced the capacity of pooled neonatal serum to kill each of the bacterial isolates. The results were consistent with the theory that a diminished concentration of C9 contributes to the bacteriolytic defect observed in neonatal serum. The results also indicated that several different isolates of *E. coli* that cause sepsis in neonates were killed inefficiently by serum from neonates compared with adults. Furthermore, the resistance to rapid bacteriolysis manifested by the various isolates during incubation with pooled neonatal serum was due, at least in part, to a diminished serum concentration of C9.

The clinical consequences of diminished C9 serum concentrations in newborn infants are not clear. Previous studies revealed that the isolates of *E. coli* obtained from the blood of septic neonates were predominantly serum sensitive (killed during incubation with serum from healthy adults) (7). The studies reported herein revealed that supplemental C9 enhanced the bactericidal capacity of neonatal serum to kill several isolates of pathogenic bacteria. Taken together, the studies suggest that a diminished concentration of C9 may possibly be one of the defects in host immunity that predispose neonates to invasion by serum sensitive isolates of *E. coli*.

Unlike neonates, C9-deficient adults have not been shown to be predisposed to invasion by *E. coli* (37). However, the immune system of newborn infants, unlike C9-deficient adults, is characterized by multiple deficiencies, such as reduced serum concentrations of various complement components, variable serum concentrations of anti–*E. coli* antibodies, and diminished function and quantity of neutrophils (11, 38–41). Therefore, it is possible that C9 deficiency is one of several immune defects that, in combination, predispose neonates to the acquisition of *E. coli* sepsis.

In summary, compared with adult serum, neonatal serum deposited a diminished quantity of C9 onto *E. coli* and killed the bacteria inefficiently. Supplementation of neonatal serum with C9 accelerated the onset and rate of bacterial killing, increased the total quantity of C9 deposited onto the surface of *E. coli*, and enhanced the bactericidal activity against eight different pathogenic isolates of *E. coli*. Therefore, C9 deficiency restricted the capacity of neonatal serum to deposit C9 and to kill bacteria. C9 deficiency may possibly be one of the defects in antibacterial host defense that predisposes neonates to the acquisition of *E. coli* sepsis. However, the role of C9 deficiency in the pathogenesis of *E. coli* sepsis in human neonates remains speculative and will require assessment with continued investigation, such as the development of an animal model.

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