Locally Delivered Polyclonal Antibodies Potentiate Intravenous Antibiotic Efficacy against Gram-Negative Infections

Nazir A. Barekzi,[1,2] Adrian G. Felts,[1,2] Kornelis A. Poelstra,[1,2] Jeffrey B. Slunt,[2] and David W. Grainger[3,4]

Received May 21, 2002; accepted August 29, 2002

Purpose. Comparison of the anti-microbial efficacy of locally delivered antibodies in tandem with conventional systemic administration of ceftazidime antibiotic therapy in two lethal gram-negative animal infection models.

Methods. Previously published lethal *E. coli*-induced closed peritonitis and *Klebsiella*-induced burn wound infections were generated in outbred female CF-1 mice cohorts. Pooled human polyclonal antibodies were injected locally into sites of infection in these mice simultaneously with intravenous infusions of the broad-spectrum antibiotic, ceftazidime. Mouse survival was compared in sham control cohorts vs. both ceftazidime-alone or antibody-alone systemically infused cohorts as well as local antibody-systemic ceftazidime combination therapy cohorts. Microbial burdens in blood and tissue samples (by agar plating), as well as interleukin-6 cytokine levels (using ELISA) correlated with sepsis, were monitored in sacrificed animals as a function of antimicrobial treatment regimen.

Results. Local delivery of human polyclonal antibodies to infection sites was shown to produce synergistic therapeutic efficacy in combination with systemic antibiotic administration in these lethal wound infection models in mice. Enhanced benefits of the unique combination therapy included host survival, bacterial burden both locally and systemically, and IL-6 levels in host serum.

Conclusions. Commercial pooled human antibodies contain a broad spectrum of antimicrobial activity against gram-negative pathogens. Prevention of systemization of infection correlates with host survival in these models. Local control of infection using doses of local, high-titer polyclonal antibodies can enhance traditional approaches to curb systemic spread of infection using intravenous antibiotics. Antibodies provide antimicrobial efficacy independent of known pathogen resistance mechanisms.

KEY WORDS: antibody delivery; infection; antibiotic; synergy; immunoglobulin.

INTRODUCTION

Current clinical standards of care often use, either prophylactically or therapeutically, systemic antibiotics to manage and control the threat of infection in many indications. Antibiotic resistant pathogens are an increasingly problematic cause of hospital-based infections (1). A wide variety of gram-negative pathogens now demonstrate clinical resistance to antibiotics of choice including *Klebsiella pneumoniae* and *Escherichia coli* isolates resistant to β-lactam antibiotics due to production of β-lactamases and *Pseudomonas aeruginosa* strains with multi-drug efflux pumps (2). The continuously increasing prevalence of antibiotic resistant bacteria has greatly elevated concern that front-line antibiotics will eventually become ineffective in managing clinical infections (2–5). Proof that selective pressure from increasing antibiotic use promotes even more intrinsic resistance (2–5) has prompted renewed attention directed both to understanding mechanisms of antibiotic resistance, as well as to developing alternative anti-microbial methods (6,7). New *de novo* antibiotic synthesis is a logical, compelling area of investigation. Yet few new, original synthetic antibiotics are in clinical phase trials (6), as many prospective antibiotic candidates represent iterations on long-standing drug structure-function paradigms susceptible to resistance mechanisms (6,7).

Commercial pooled polyclonal human immunoglobulins (IgG antibodies) represent a broad-spectrum antimicrobial approach currently FDA-approved and administered by intravenous infusion to millions of patients annually (IVIG) (8,9). Commercial IgG pools exhibit measurable titers against numerous pathogens, representing the collective immunity from thousands of human donors. Clinicially, polyclonal antibodies are delivered systemically to supplement host humoral immunity and, because antibodies facilitate antimicrobial mechanisms distinct from those of antibiotics, they do not engender resistance. Moreover, the appearance of pathogen antibiotic resistance does not alter bacterial susceptibility to antibody opsonization and phagocytic neutralization (10). However, therapeutic benefit of IVIG alone in various treatments has not been compelling (11,12). By contrast, combination therapy comprising both systemic antibiotics and systemic polyclonal IVIG has demonstrated benefit against numerous indications including sepsis in newborns (13) and to reduce post-operative infection rates leading to sepsis after surgery for colorectal cancer (14). Clinical problems with antibiotic-induced release of pathogen toxins in septic patients have been addressed using antibodies administered systemically against these toxins (15,16). Systemic monoclonal antibody infusion has also been used successfully in cystic fibrosis patients infected with therapy resistant *Pseudomonas aeruginosa* (17), and as the treatment of choice against respiratory syncytial virus (RSV) (18). Systemic antimicrobial antibodies, therefore, have shown to be complementary to antibiotics in preventing and facilitating clearance of infection. Combination therapies represent the clinical capability to exploit pathogen susceptibility to multiple antimicrobial agents that individually exhibit sub-optimal clinical efficacy.

In this study we report observation of enhanced efficacy for ceftazidime, a common clinically used systemic antibiotic, in combination with *locally* delivered polyclonal antibodies, as a new treatment strategy against infection. Ceftazidime is a

[1] First three authors have contributed equally to this work.

[2] Anthony G. Gristina Institute for Biomedical Research, (formerly Medical Sciences Research Institute), 520 Huntmar Park Drive, Herndon, Virginia 20170.

[3] Department of Chemistry, Colorado State University, Ft Collins, Colorado 80523.

[4] To whom correspondence should be addressed. (e-mail: grainger@lamar.colostate.edu)

ABBREVIATIONS: IgG, immunoglobulin G; IL-6, interleukin-6 cytokine; ELISA, enzyme-linked immunosorbent assay; IVIG, intravenous immunoglobulin; LD_{90}, lethal dose for 90% mortality; CFU, colony forming units; ANOVA, analysis of variance; MIC, minimum inhibitory concentration; *K. pneumoniae, Klebsiella pneumoniae; E. coli, Escherichia coli.*

third-generation cephalosporin and first-line antibiotic against *Klebsiella pneumoniae*, *Serratia marcescens, Escherichia coli* and *Proteus mirabilis* infections . However, recent emergence of ceftazidime-resistant *Klebsiella* (19), *Escherichia coli* (20), and *Pseudomonas* (21) strains in hospital-acquired infection scenarios warrants assessment of strategies that enhance clinical antimicrobial efficacy. Importantly, combination antibiotic/human antibody therapy recently exhibited efficacy in treatment of ceftazidime-resistant *Klebsiella* lethal burn wound infection in a mouse model (22), providing compelling initial evidence for new opportunities for this strategy.

In this study, two different murine lethal infection models (using outbred CF-1 mice) detailed previously (23,24) are used to study the efficacy of combination systemic antibiotic/local antibody therapy over each monotherapy, respectively. Each infection model—one involving *Klebsiella* burn wound infection, the other *E. coli*-induced closed peritonitis—is subject to combination therapies using prophylactic, intravenous ceftazidime (tail vein infusion) and/or pooled human polyclonal antibodies either systemically infused (IVIG) or locally delivered to each site of infection.

MATERIALS AND METHODS

Animals and Animal Care

Outbred female Crl-CF-1 mice (22–24 g) were obtained from Charles River Laboratories (Wilmington, MA) and housed five per cage in a biosafety level 2 facility with a 12-h light/dark cycle. Standard mouse chow and water were provided *ad libitum*. All animals were maintained according to the publication Guide for the Care and Use of Laboratory Animals (25) and all protocols were approved by the Gristina Institute Animal Care and Use Committee.

Bacteria

Klebsiella pneumoniae 2270 and *Escherichia coli* KI08ACH7 (donated by Dr. Ian Holder, Shriners Burn Institute, Cincinnati, OH and Dr. J. Curtis Nickel, Queens University, Kingston Ontario, Canada, respectively) were grown for 18 h in 20 ml trypticase soy broth at 37°C while agitated at 150 RPM using a benchtop incubator shaker. The 2270 strain and the KI08ACH7 strain both exhibit ceftazidime MIC values of 8 μg/ml in cultures, (I. A. Holder, pers. com.). Cultured bacteria were twice sedimented by centrifugation at 7649 *x* g for 10 min, washed and diluted in saline to obtain a concentrated bacteria suspension. Serial bacterial dilutions were plated on trypticase soy agar (TSA) and colonies were counted to determine initial colony forming units (CFU) per ml after 24 h incubation at 37°C. In parallel, optical density (λ = 650 nm) of these bacterial dilutions was measured (Beckman DB-GT grating spectrophotometer) and standard curves plotting optical density vs. CFU/ml concentrations were then constructed for each organism. Optical density values of 1.16 for *K. pneumoniae* and 1.05 for *E. coli* suspensions resulted in ~10^9 CFU/ml.

Local and Systemic Antimicrobial Therapies

Mice were treated locally (sub-eschar, *s.c.*, intravenously (tail vein), *i.v.*, or intraperitoneally, *i.p.*) with commercially pooled human intravenous immunoglobulin (antibody, Lot# 2620M039A, Gammagard® S/D, protein content >98% IgG subclasses, Baxter Healthcare Corporation, Glendale, CA) and/or i.v. with sub-optimal doses of ceftazidime (Lot# 8ZP0340, Fortaz®, Glaxo Wellcome Inc., Research Triangle Park, NC). Gammagard® S/D was supplied as a clinical grade freeze-dried preparation, and reconstituted with supplied diluent (Sterile Water for Injection, USP) to 10 wt% protein/ml at an approximate pH of 6.8. Manufacturer specifications indicate that this reconstitution provides an iso-osmolar antibody solution comprising dextrose and saline. Titers of this pooled human antibody product against each bacterial strain were determined by using a published ELISA method (23,26). Antibody binding titers against the *K. pneumoniae* and *E.coli* strains determined by this assay were 398 and 100, respectively, representing significant antibody binding activity against the pathogens as described previously (23).

Ceftazidime, supplied as a lyophilized powder, was reconstituted to the optimal human equivalent clinical dose (200 and 22.7 mg/kg for pediatric burn wounds and adult peritonitis, respectively) in sterile distilled water. Stock antibody solutions were diluted in 5% dextrose (recommended by the manufacturer) and ceftazidime was diluted in sterile water to obtain the desired working concentrations. Injections of sterile diluents (0.1 ml for s.c. and i.v. injections, 0.5 ml for i.p. injections) and human serum albumin (10 mg doses, (23)) served as local and systemic control treatments in all experiments.

Murine Lethal Infection Models

A previously described full thickness murine burn wound infection model was used (22,24). Briefly, mice were shaved and then anesthetized by methoxyflurane inhalation (Metofane®, Schering Plough, Union, NJ) and a heat-resistant plastic board with a 1.0 (25 mm) by 1.5 inch (38 mm) window was pressed firmly against the shaved dorsum. Ethanol (200 proof; 0.50 ml) was spread evenly over the window opening, ignited, and allowed to burn for 10 s. The procedure alone produced a non-lethal full thickness burn wound over 10%–15% of the body surface. Immediately after the burn, mice were given 0.5 ml of sterile saline intraperitoneally as fluid replacement therapy and acetaminophen (0.25 mg/ml; Children's Tylenol suspension liquid) in drinking water as a post-burn analgesic. A lethal dose of *K. pneumoniae* (LD_{100} = 10^2 CFU/0.1 ml for strain 2270) and local polyclonal antibody treatment (10 mg/0.1 ml) were independently injected s.c. under the burn site immediately following the burn. Intravenous sub-optimal single doses of ceftazidime (44 μg/0.1 ml; 2 mg/kg) were infused by tail vein injection either alone (monotherapy) or followed by antibody treatment (combination therapy).

In the closed peritonitis model (23), mice were injected i.p. with a lethal dose inoculum dose of *E. coli* (LD_{90} = 10^6 CFU/0.5 ml for strain KI08ACH7) followed immediately by antibody treatment (1, 5, or 10 mg/0.5 ml i.p. or 10mg/0.1ml i.v.) and/or sub-optimal i.v. ceftazidime tail vein infusion (25 or 50 μg/0.1 ml).

Animal survival in both infection models was assessed for ten days thereafter, beyond which all animals would survive without further treatment.

Quantitative Microbiology

Approximately 1 ml of blood was collected via cardiac puncture into heparinized tubes (40 U; Sigma Chemical Co., St. Louis, MO) from each anesthetized mouse either 24 h (burn wound model) or 12 h (peritonitis model) post-bacterial challenge. Immediately following blood collection each anesthetized mouse was euthanized by cervical dislocation. Blood was serially diluted in sterile saline and plated on trypticase soy agar (TSA) plates for enumeration of bacteria. In the peritonitis model, each peritoneal cavity was lavaged with 5 ml sterile saline. Lavage was serially diluted and plated on TSA plates for enumeration of bacteria. Resulting colony counts are expressed as log CFU/ml blood or lavage, respectively. Post-euthanasia in the burn wound model, the burned eschar was surgically removed and homogenized in 10 ml sterile saline. In both models, livers were excised postmortem and placed in 10 ml sterile saline. All tissue samples were weighed prior to homogenization (Omni-International GLH Homogenizer, Marietta, GA). Homogenate fluids were then serially diluted in sterile saline and plated on TSA plates for bacterial cultures and enumeration. Resulting colony counts are expressed as log CFU/g tissue.

Quantitation of Systemic Interleukin-6 (IL-6)

Immediately following serial dilution and plating of the blood samples, serum was separated by centrifugation at 3,000 rpm for 10 min at room temperature, collected and assayed with an ELISA (23,26) specifically designed to detect IL-6. Optical density was measured at 405 nm (SLT Spectra Reader, Tecan Company, Durham, NC). The detection range for the assay was 15–2000 pg/ml. The concentration of IL-6 present in each sample is reported as pg/ml serum.

Statistical Analysis

Data are expressed as the mean ± standard error of the mean (SEM). Student's *t* tests were used to compare the control and therapy groups of the bacterial burden enumeration and IL-6 studies while two-tailed pairwise ANOVA with Tukey's tests were used to compare rates of mortality. All probabilities less than 5% ($p < 0.05$) were considered significant. For the burn tissue bacterial assessments, data outliers, defined as any datum outside the range of the mean ± two times the standard deviation, were excluded. Commercial software (SigmaStat 2.01, Jandel Corp., San Rafael, CA) on desktop computers was used.

RESULTS

Antibiotic/Antibody Therapy Benefits in Burn Wound Infection

In untreated murine burn wounds or those treated with control treatments (saline i.v. or serum albumin locally), low levels of *K. pneumoniae* challenge (10^2 CFU s.c.) consistently killed 100% of burned and 80% of unburned mice (Fig. 1 n = 10). Significantly, previous work (22,24) has shown that other relevant burn wound pathogens required substantially higher inocula (e.g., *Pseudomonas aeruginosa*, LD_{90} ~10^4 CFU) to produce consistent lethal infections in this model, and that locally delivered polyclonal antibody monotherapy protected

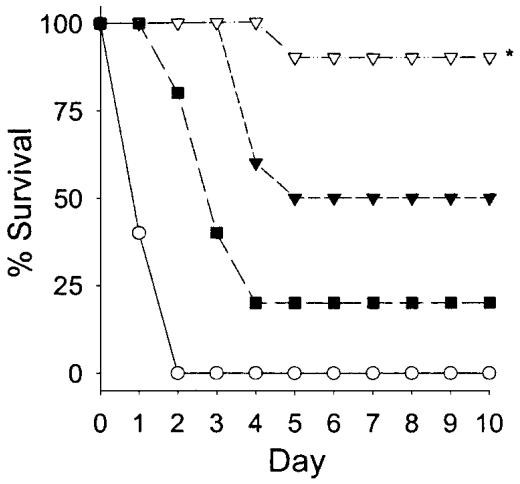

Fig. 1. Host survival over time for systemic sub-optimal antibiotic dose (44 μg ceftazidime via tail vein infusion) or locally delivered polyclonal antibody monotherapy (sub-eschar, s.c., injection) compared to various combination therapies in a murine burn wound infection model using a lethal dose (10^2 CFU) of *K. pneumoniae* as the pathogen inoculum. Mice were left unburned or burned and then lethally challenged following established protocols (22,24). Combination therapy of systemic ceftazidime and locally delivered polyclonal antibodies s.c. into the burn wound confers synergistic survival benefit over either systemic ceftazidime or locally applied antibody alone. (Significance: two-tailed pairwise ANOVA and Tukey's test comparing survival at each day over 10 total days, n = 10; *$p < 0.05$ compared to all other data). (▽, 44 μg ceftazidime i.v. + 10 mg Antibody s.c.; ▼, 44 μg ceftazidime i.v; ■, infected, unburned controls; O, placebo or 10 mg antibody s.c. treated, burned)

~90% of these animals from *P. aeruginosa* lethal infection. However, Fig. 1 shows that local polyclonal antibody monotherapy, or sub-optimal i.v. ceftazidime (44 μg/animal, 2.0 mg/kg) monotherapy with the more virulent *Klebsiella*-infected burn wounds each produced low survival (0% for local antibody, 50% for i.v. ceftazidime). Nevertheless, the combination of both local antibody deliveries with systemic ceftazidime produced 90% survival, a significant enhancement (40%) in synergistic benefit ($p < 0.05$).

Quantification of tissue bacterial burdens in the burn infection model at 24 h post-infection exhibits similar trends (Fig. 2 n = 10). In the harvested burned eschar, *K. pneumoniae* colony counts in dextrose-treated controls (placebo) and in cohorts treated with local s.c. antibody monotherapy increased dramatically (>6 log order increase over challenge inoculum dose) by 24 h after bacterial challenge. Pathogen colonization of eschar tissue after systemic infusion of a sub-optimal dose of ceftazidime alone also was significantly increased (>2 log order increase over challenge inoculum dose). In contrast, combining local antibody delivery and systemic sub-optimal antibiotic treatment at the time of bacterial challenge decreases bacterial levels in this tissue by 0.5 log CFU/g tissue compared to the initial inoculum (Fig 2). Although high virulence exhibited by this strain of *K. pneumoniae* is attributed to the presence of an extracellular polysaccharide capsule (27), a 24-h incubation period in the host is still too brief for the infection to become fully systemized. No colonies were detected in blood samples from all groups after 24 h (data not shown). Colonies were detected in liver homogenates, however, exhibiting similar, higher bacterial counts for placebo

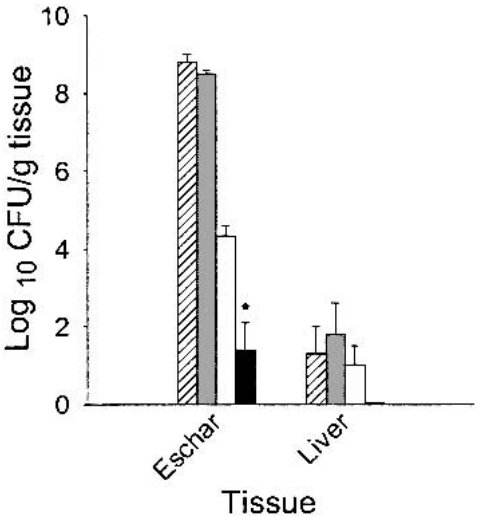

Fig. 2. Bacterial tissue burden assayed 24 h post-burn and post-challenge with 10^2 CFU of *K. pneumoniae* following mono- or combination antimicrobial therapy. Combination therapy of systemic ceftazidime and locally delivered polyclonal antibodies provides substantial reduction in burn eschar bacterial burden over either ceftazidime or locally applied antibody monotherapies (Student's *t* test, n = 10, *p < 0.01 vs. all harvested eschar groups, data expressed as mean ± SEM.). (hatched), placebo i.v. and s.c.; (shaded), 10 mg antibody, s.c.; (open), 44 μg ceftazidime i.v.; (solid), 44 μg ceftazidime i.v + 10 mg antibody s.c.

and both monotherapy-treated mice cohorts after 24 h. These counts will increase to lethal levels in the following 24 h period, resulting in the notable increase in mortality over time (Fig. 1). By contrast, livers of all mice treated with both locally delivered antibodies and systemic sub-optimal antibiotics exhibited average pathogen levels below the 10^2 CFU/g detection limit, conferring protection consistent with survival benefits observed for this therapy group.

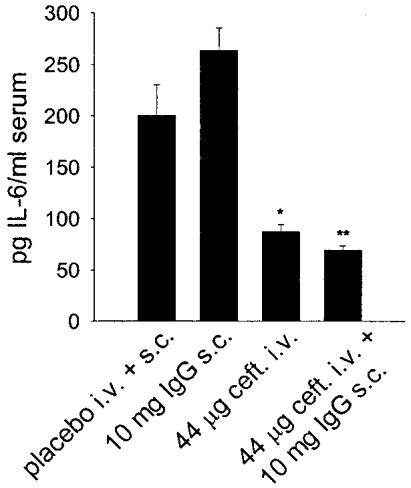

Fig. 3. Levels of interleukin-6 (IL-6) in serum assayed 24 h post-burn and post-challenge with 10^2 CFU of *K. pneumoniae* following mono- or combination antimicrobial therapy. Combination therapy of systemic ceftazidime and locally delivered polyclonal antibodies provides substantial reduction in circulating IL-6 over either ceftazidime or locally applied antibody alone (Student's *t* test, n = 5–10, *p < 0.003 vs. placebo and antibody s.c. treatment, **p < 0.05 vs. all treatments, data expressed as mean ± SEM)., placebo i.v. and s.c.; 10 mg IgG s.c.;, 44 μg ceftazidime i.v;, 44 μg ceftazidime i.v. + 10 mg IgG s.c.

Cytokine IL-6, a clinical indicator of systemic inflammatory acute phase reactions, was detected in serum of all groups post-infection. Both systemic antibiotic monotherapy and combination therapy groups had significantly reduced IL-6 levels over that observed for placebo or local antibody monotherapy groups (Fig. 3 n = 10). A significant difference in IL-6 reduction was observed for the combination treatment group compared to all other treatment groups. Reduced systemic IL-6 is consistent with the absence of detectable systemic bacteria in blood and low liver bacterial counts observed for both of these treatments (22–24).

Antibiotic/Antibody Combination Therapy Benefits in Closed Peritonitis.

In the second model, intra-abdominal injection of a clinically isolated *E. coli* (KI08ACH7, 10^6 CFU) produced consistent lethality in untreated mice (Fig. 4 n = 5–20). Analogous to the burn infection model survival synergy shown in Fig. 1, synergistic survival benefits were also observed in this peritonitis model for combinations of sub-optimal i.v. ceftazidime (50 μg/animal, 2.3 mg/kg) with intraperitoneally administered polyclonal antibodies (Fig. 4). In combination with sub-optimal 50 μg i.v. ceftazidime doses, two different, re-

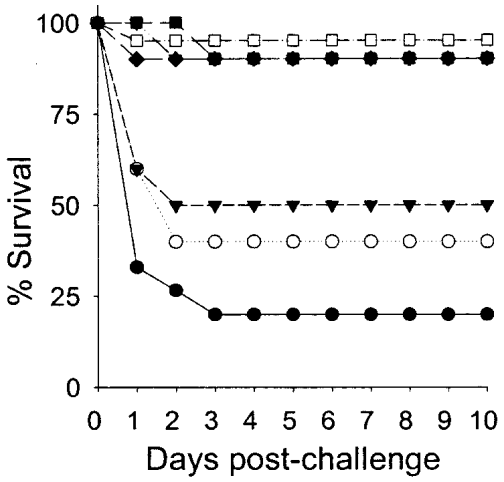

Fig. 4. Murine survival over time for systemic sub-optimal antibiotic dose (50 μg ceftazidime via tail vein infusion) or locally delivered polyclonal antibody monotherapy (intraperitoneal injection, i.p.) compared to various combination therapies in a murine peritonitis infection model challenged with a lethal dose (1×10^6 CFU) of *E. coli*, following established protocols (23). Combination therapy of systemic ceftazidime with 10 mg locally (i.p.) injected polyclonal antibodies confers synergistic survival benefit over either systemic ceftazidime, locally delivered antibody (i.p.) monotherapy, or the additive benefit of both monotherapies considered together. Additionally, survival benefits for combinations of systemic ceftazidime plus either 1 mg or 5 mg antibody i.p. were nearly identical to that for combination therapy of systemic ceftazidime and 10 mg antibody i.v. All of these survival benefits were comparable to the additive benefit provided by systemic ceftazidime and 10 mg antibody i.p. monotherapies considered together (significance: ANOVA and Tukey's test, n = 5–20; p < 0.001 for each combination antibiotic/antibody therapy vs. each monotherapy, and p < 0.001 for each monotherapy compared to placebo). □, 50 μg ceftazidime i.v. + 10 mg antibody i.p.; ♦ (A), 50 μg ceftazidime i.v. + 10 mg antibody i.v.; ▽(B), 50 μg ceftazidime i.v. + 1 mg antibody i.p.; ■ (C) 50 μg ceftazidime i.v. + 5 mg antibody i.p., ▼50 μg ceftazidime i.v.; ○, 10 mg antibody i.p.; ●, placebo i.v. and i.p.

duced doses of locally applied antibodies (1 and 5 mg antibody intraperitoneally (i.p.)) conferred survival equivalent to that observed for a substantially higher dose (10 mg i.v.) of systemically infused antibodies (conventional IVIG). Overall mouse survival with local antibody monotherapy is 40% against this pathogen, substantially less effective than previously observed for the same local antibody monotherapy in the same model against several different virulent strains of *P. aeruginosa* (23). Local antibody monotherapy proved slightly less effective than systemic antibiotic monotherapy that confers 50% survival with a single prophylactic sub-optimal dose of i.v. ceftazidime alone. Combination therapy comprising systemic antibiotic and locally delivered antibody produced a synergistic 95% survival (Fig. 4), compared to each monotherapy, a benefit significantly different than all other treatment groups (ANOVA, $p < 0.05$).

Enhanced efficacy of locally delivered antibody i.p. over systemic antibody is more apparent when both are compared in combination with a further reduced sub-optimal 25 μg ceftazidime systemic infusion and slightly reduced *E. coli* challenge (7×10^5 CFU, Fig. 5 n = 10) in this peritonitis model. Significantly, addition of any antibody therapy—systemic or local—to ceftazidime i.v. treatment improves survival over ceftazidime monotherapy. Combination therapy involving local antibody applications even at this low systemic antibiotic dose produced higher survival than systemic antibiotics combined with systemic antibody infusion at two different doses (1 and 10 mg IVIG). The lower local 1 mg antibody dose i.p. in combination therapy conferred equivalent survival to that observed for a log higher dose (10 mg) of systemic antibody with the same antibiotic infusion (Fig. 5).

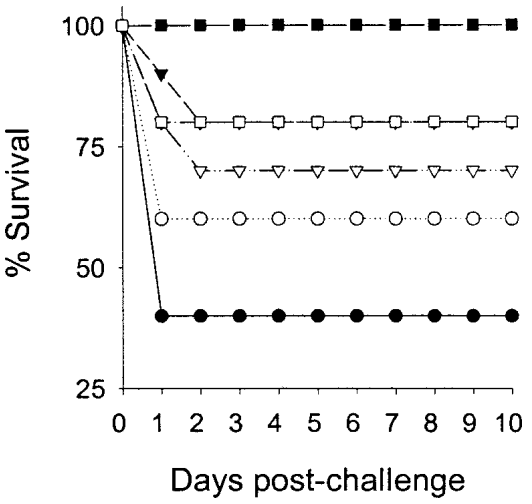

Fig. 5. Murine survival over time for a lower systemic sub-optimal antibiotic dose (25 μg i.v. ceftazidime) in combination with various locally or systemically applied polyclonal antibody doses (1 and 10 mg antibody i.p. or i.v.) compared to placebo and combination therapies in a murine peritonitis infection model using a lethal dose (7×10^5 CFU) of *E. coli* as the pathogen inoculum. Combination therapy of sub-optimal systemic ceftazidime and locally (i.p.) delivered polyclonal antibodies (1 or 10 mg antibody dose) confers improved survival benefit over either systemic ceftazidime monotherapy or combination of systemic ceftazidime with corresponding 1 mg or 10 mg systemic antibody dose i.v., respectively. Additionally, survival benefit for combination therapy comprising sub-optimal systemic ceftazidime plus 1 mg antibody i.p. was equivalent to that for combination therapy of systemic ceftazidime and 10 mg antibody i.v. (significance: ANOVA and Tukey's test, n = 10; $p < 0.001$ for each combination antibiotic/antibody therapy vs. each monotherapy, $p < 0.001$ for ceftazidime monotherapy compared to placebo, and $p < 0.001$ for all combination therapies using local antibody dosing (i.p.) vs. combination therapy using systemic antibody (i.v.)). ■, 25 μg ceftazidime i.v. + 10mg antibody i.p.;, ▼ 25 μg ceftazidime i.v. + 10 mg antibody i.p.; □, 25 μg ceftazidime i.v + 1 mg antibody i.p.; ▽, 25 μg ceftazidime i.v. + 1 mg antibody i.v.; ○, 25 μg ceftazidime i.v + placebo i.p.; ●, placebo i.v. and i.p.

Bacterial burdens found in tissues harvested from the peritonitis model at 12 h post-infection support these trends in survival (Fig. 6 n = 10). Enumeration of pathogens from peritoneal lavage, liver and blood post-treatment indicates that systemic antibiotic/local antibody therapy significantly and synergistically reduces viable bacteria in the host compared to either monotherapy. Local antibody monotherapy i.p. has little efficacy in reducing pathogen burden in all sites examined, while sub-optimal i.v. ceftazidime monotherapy reduced bacterial burdens substantially and consistently at all sites. Together, the combined therapy would be predicted to show a benefit similar to ceftazidime alone. However, data shown in Fig. 6 indicate that a bacterial reduction benefit substantially greater than simple additivity is produced by the combination of ceftazidime i.v. and local antibody i.p., consistent with the survival synergy noted in Fig. 4.

Analogous to treatment effects seen in Fig. 3 for the burn infection, levels of circulating IL-6 detected in serum of both antibody monotherapy and placebo treatment groups for peritonitis are dramatically increased over IL-6 quantified for antibiotic monotherapy or combination antibiotic/antibody treatment. (Fig. 7). Combination therapy reduces IL-6 to near-baseline detection limits and is significantly reduced

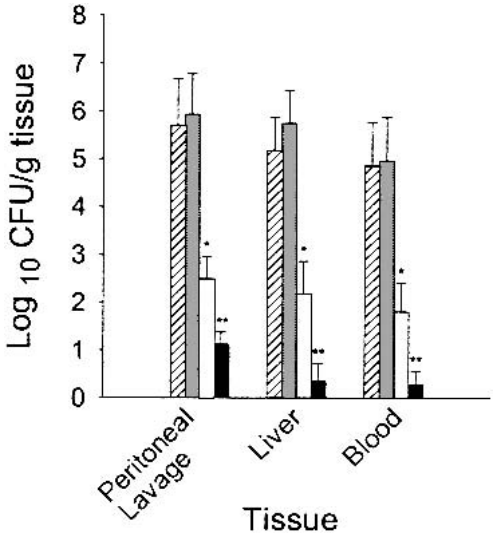

Fig. 6. Tissue and blood bacterial burden assayed 12 h post-challenge with 5×10^7 CFU of *E. coli* and mono- or combination antimicrobial therapy. Combination therapy comprising a sub-optimal systemic ceftazidime dose (25 μg i.v.) with locally injected polyclonal antibodies i.p. provides substantial reduction in bacterial burden in all sites assayed over either ceftazidime or locally applied antibody monotherapy (significance: Student's *t* test, n = 10, *$p < 0.05$ comparing ceftazidime vs. placebo and antibody monotherapy; **$p < 0.05$ comparing combination therapy vs. all treatments). (hatched), placebo i.v. and i.p.; (shaded) , 10 mg antibody i.p.; (open), 50 μg ceftazidime i.v; (solid), 50 μg ceftazidime i.v + 10 mg antibody i.p.

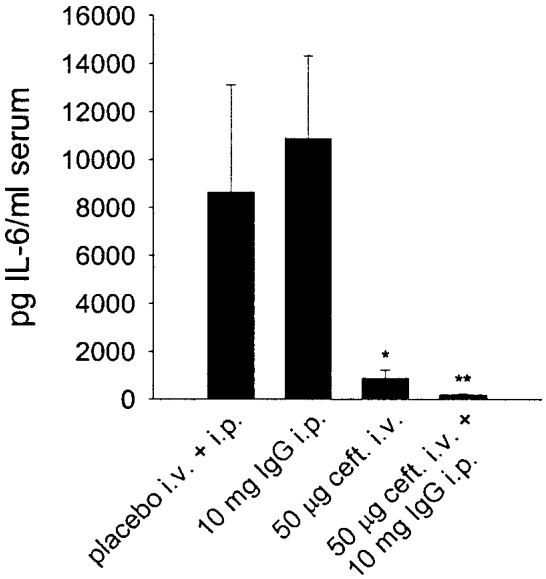

Fig. 7. Levels of interleukin-6 (IL-6) in serum assayed 12 h post-challenge with 1×10^6 CFU of *E. coli* following mono- or combination antimicrobial therapy. Combination therapy comprising systemic ceftazidime with locally delivered polyclonal antibodies provides substantial reduction in circulating IL-6 over placebo, ceftazidime i.v. or locally delivered antibody monotherapies (Student's *t* test, n = 10, *p < 0.05 comparing ceftazidime vs. placebo and antibody monotherapy, **p < 0.05 comparing combination therapy vs. all treatments)., placebo i.v. and i.p.;, 10 mg IgG i.p.;, 50 μg ceftazidime i.v;, 50 μg ceftazidime i.v. + 10 mg IgG i.p.

compared to i.v. ceftazidime monotherapy. Serum IL-6 levels after combination therapy show a significant and synergistic reduction compared to placebo or either monotherapy, consistent with low bacterial burden (Fig. 6) and survival synergy (Figs. 4 and 5).

DISCUSSION

Combining locally delivered polyclonal antibodies with sub-optimal systemic i.v. antibiotic prophylaxis produces synergistic survival benefits over that observed for each separate monotherapy in both lethal infection models. Significantly, mortality resulting from administration of sub-optimal i.v. antibiotic doses was consistently improved by low level, local co-administration of human antibodies. Survival benefits from combination therapy in both infection models using two different pathogens directly correlate with observed synergistic reductions in both tissue bacterial burdens at several sites, and systemic levels for a cytokine indicator of sepsis (IL-6). The data suggest that prevention of systemization of each infection generally enhances survival, and that such prevention is significantly enhanced using combination therapy over monotherapies or double systemic infusion therapy of both antibiotic and antibody. The interesting yet complicated question surrounds the mechanism for this effect because antibodies have no intrinsic microcidal or bacteriostatic properties. Systemic clearance of intraperitoneally administered human antibody in mice is known to be rapid (~3 h) (23,28,29). Similarly, human antibodies injected s.c. into murine full thickness burn wounds are first detectable by ELISA in mouse serum after 3 h (data not shown). Once present in murine systemic circulation, however, human antibodies are detectable in serum beyond seven days by ELISA (23,29). Hence, the synergy observed in reducing infection mortality and morbidity in these models could be attributable to the same, unelucidated yet therapeutically beneficial systemic interactions reported between systemic antibiotics and IVIG (30).

Improved, synergistic benefit observed for the combination of systemic antibiotic/local antibody therapy over the efficacy of combination systemic antibody therapy also supports a distinct, locally enhanced contribution from doses of exogenous polyclonal antibody at the sites of infection. Local pathogen opsonization by exogenous antibody in the peritoneal cavity can occur immediately by both specific opsonization and non-specific bacterial surface adsorption (31), reducing clearance of antibody to systemic circulation. Abundant, endogenous peritoneal macrophages could conceivably become rapidly activated by antibody binding (both specific opsonization and non-specific adsorption) of the bacteria as well as by pathogen proliferation, clearing bacteria in early stages of contamination locally prior to systemic spread of infection. Opsonization would not only serve to promote local phagocytic clearance but also hinder or delay rapid pathogen proliferation kinetics necessary to achieve systemization of the infection and mortality. Because the burn wound is severely immunocompromised, avascular and often necrotic, clearance of local infection would rely on local, extravascular transport of fresh antibodies, other immunocomponents (e.g., complement), and cellular elements including macrophages, monocytes and neutrophils from the unburned surrounding tissue. Direct antibody delivery into the immune-depleted site overcomes transport limitations for this important facilitating component. No protective benefit of topically applied antibodies externally onto burned eschar stratum corneum is observed (data not shown), supporting the contention that rapid s.c. local antibody delivery and transport is required. Lastly, increased capability for antibody binding and neutralization of toxins locally limits septicemia and systemic infection, as supported by IL-6 results.

Sub-optimal systemic antibiotic monotherapy has shown reduced efficacy against pathogens, but in conjunction or in combination with other antibiotics, synergistic effects have been previously observed (32). Sub-optimal antibiotic doses were chosen in this current study to facilitate discrimination of therapeutic benefits attributable to antibody in each infection model. Specifically, sub-optimal prophylactic antibiotic doses for each infection model were calculated from published human equivalent full clinical i.v. ceftazidime doses relevant to treating pediatric burn wounds or adult peritonitis. The full equivalent antibiotic monotherapy doses in mice (440 or 500 μg/animal i.v., respectively) produced 75% survival in the burn wound model and 100% survival in the peritonitis model, regardless of initial bacterial challenge between 10^2 and 10^6 CFU (data not shown). Ceftazidime reduction to 10% of the full dose constitutes a legitimate regimen to experimentally determine anti-microbial synergy, producing consistent infection mortality while permitting specific therapeutic benefit from local antibody delivery to be distinguished from antibiotic therapy.

Observed anti-infective benefits using combinations of antibiotics and antibodies might be general to many other infections. Such an approach could have important clinical implications for both reducing selective pressure involved with producing antibiotic resistance, improving the perfor-

mance and extending the clinical lifetime of current front-line antibiotics facing resistance, as well as in treating antibiotic resistant infection. Because IVIG is currently an expensive therapy with fluctuations in clinical availability, combination antibiotic/antibody therapies might lower treatment costs by reducing required antibody doses and simplifying delivery requirements for antibodies delivered locally and directly to the site of infection in tandem with routine systemic antibiotics. Importantly, reduction of systemic antibiotic levels by suboptimal dosing mimics a possible future clinical scenario in which selective pressures encouraging ceftazidime resistance (4) are decreased while optimizing therapeutic benefit against infection using exogenous, locally delivered antibodies. An additional study aimed at ascertaining this combination therapy efficacy against a ceftazidime-resistant *P. aeruginosa* strain in the burn wound infection model (22) recently validated the efficacy of this approach.

ACKNOWLEDGMENTS

This work was supported by NIH grant 2 R01 AR26957-11A1 provided to the Anthony G. Gristina Institute for Biomedical Research. We thank Henk Busscher, Ian A. Holder, Ignacio Rojas, J. Campbell Palmer, Greg Anderson, and Jennifer McClary for their technical assistance and suggestions.

REFERENCES

1. H. C. Neu. The crisis in antibiotic resistance. *Science* **257**:1064–1073 (1992).
2. H. S. Gold and R. C. Moellering. Antimicrobial-drug resistance. *N. Engl. J. Med.* **335**:1445–1453 (1996).
3. S. Monroe and R. Polk. Antimicrobial use and bacterial resistance. *Curr. Opin. Microbiol.* **3**:496–501 (2000).
4. W. Wong and D. L. Pompliano. In B. Rosen and S. Mobashery (eds.), *Resolving the antibiotic paradox: progress in drug design and resistance*, Plenum, New York, 1999, pp. 1–21.
5. D. T. Moir, K. J. Shaw, R. S. Hare, and G. F. Vovis. Genomics and antimicrobial drug discovery. *Antimicrob. Agents Chemother.* **43**:439–446 (1999).
6. A. Persidis. Antibacterial and antifungal drug discovery. *Nature Biotechnol.* **17**:1141–1142 (1999).
7. J. A. DeVito, J. A. Mills, V. G. Liu, A. Agarwal, C. F. Sizemore, Z. Yao, D. M. Stoughton, M. G. Cappiello, M. D. F. S. Barbosa, L. A. Foster, and D. L. Pompliano. An array of target-specific screening strains for antibacterial discovery. *Nature Biotechnol.* **20**:478–483 (2002).
8. A. Casadevall. Antibody-based therapies for emerging infectious diseases. *Emerg. Infect. Dis.* **2**:200–208 (1996).
9. R. H. Buckley and R. I. Schiff. The use of intravenous immune globulin in immunodeficiency diseases. *N. Engl. J. Med.* **325**:110–117 (1991).
10. C. G. Gemmell. Does the appearance of drug resistance during therapy alter bacterial susceptibility to opsonophagocytosis? *Drugs Exp. Clin. Res.* **22**:51–55 (1996).
11. A. Cometta, J. D. Baumgartner, and M. P. Glauser. Polyclonal intravenous immune globulin for prevention and treatment of infections in critically ill patients. *Clin. Exp. Immunol.* **97**:69–72 (1994).
12. G. R. Siber. Immune globulin to prevent nosocomial infections. *N. Engl. J. Med.* **327**:269–271 (1992).
13. K. N. Haque, C. Remo, and H. Bahakim. Comparison of two types of intravenous immunoglobulins in the treatment of neonatal sepsis. *Clin. Exp. Immunol.* **101**:328–333 (1995).
14. F. Cafiero, M. Gipponi, U. Bonalumi, A. Piccardo, C. Sguotti, and G. Corbetta. Prophylaxis of infection with intravenous immunoglobulins plus antibiotic for patients at risk for sepsis undergoing surgery for colorectal cancer: results of a randomized, multicenter clinical trial. *Surgery* **112**:24–31 (1992).
15. K. S. Lamp, M. J. Rybak, B. J. McGrath, and K. K. Summers. Influence of antibiotic and E5 monoclonal immunoglobulin M interactions on endotoxin release from *Escherichia coli* and *Pseudomonas aeruginosa*. *Antimicrob. Agents Chemother.* **40**:247–252 (1996).
16. H. S. El-Zaim, A. K. Chopra, J. W. Peterson, M. L. Vasil, and J. P. Heggers. Protection against exotoxin A (ETA) and *Pseudomonas aeruginosa* infection in mice with ETA-specific antipeptide antibodies. *Infect. Immun.* **66**:5551–5554 (1998).
17. J. E. Van Wye, M. S. Collins, M. Baylor, J. E. Pennington, Y. P. Hsu, V. Sampanvejsopa, and R. B. Moss. *Pseudomonas* hyperimmune globulin passive immunotherapy for pulmonary exacerbations in cystic fibrosis. *Pediatr. Pulmonol.* **9**:7–18 (1990).
18. R. Malley, J. DeVincenzo, O. Ramilo, P. H. Dennehy, H. C. Meissner, W. C. Gruber, P. J. Sanchez, H. Jafri, J. Balsley, D. Carlin, S. Buckingham, L. Vernacchio, and D. M. Ambrosino. Reduction of respiratory syncytial virus (RSV) in tracheal aspirates in intubated infants by use of humanized monoclonal antibody to RSV F protein. *J. Infect. Dis.* **178**:1555–1561 (1998).
19. L. B. Rice, E. C. Eckstein, J. DeVente, and D. M. Shlaes. Ceftazidime-resistant *Klebsiella pneumoniae* isolates recovered at the Cleveland Department of Veterans Affairs Medical Center. *Clin. Infect. Dis.* **23**:118–124 (1996).
20. J. Wiener, J. P. Quinn, P. A. Bradford, R. V. Goering, C. Nathan, K. Bush, and R. A. Weinstein. Multiple antibiotic-resistant *Klebsiella* and *Escherichia* coli in nursing homes. *JAMA* **281**:517–523 (1999).
21. S. C. Lee, C. P. Fung, P. Y. Liu, T. C. Wang, L. C. See, N. Lee, S. C. Chen, and W. B. Shieh. Nosocomial infections with ceftazidime-resistant *Pseudomonas aeruginosa*: risk factors and outcome. *Infect. Control Hosp. Epidemiol.* **20**:205–207 (1999).
22. A. Felts, D. W. Grainger, and J. B. Slunt. Locally delivered antibodies combined with systemic antibiotics confer synergistic protection against antibiotic resistant burn wound infections. *J. Trauma* **49**:873–878 (2000).
23. N. A. Barekzi, K. A. Poelstra, A. G. Felts, I. A. Rojas, J. B. Slunt, and D. W. Grainger. Efficacy of locally delivered polyclonal immunoglobulin against *Pseudomonas aeruginosa* peritonitis in a murine model. *Antimicrob. Agents Chemother.* **43**:1609–1615 (1999).
24. A. G. Felts, G. Giridhar, D. W. Grainger, and J. B. Slunt. Efficacy of locally delivered polyclonal immunoglobulin agains *Pseudomonas aeruginosa* infection in a murine burn wound model. *Burns* **25**:415–423 (1999).
25. National Research Council. In N.Grossblatt (ed.), *Guide for the Care and Use of Laboratory Animals*, National Academy Press, Washington DC, 1996.
26. P. S. Hiemstra, J. Brands–Tajouiti, and R. van Furth. Comparison of antibody activity against various microorganisms in intravenous immunoglobulin preparations determined by ELISA and opsonic assay. *J. Lab. Clin. Med.* **123**:241–246 (1994).
27. D. C. Straus. Production of an extracellular toxic complex by various strains of *Klebsiella pneumoniae*. *Infect. Immun.* **55**:44–48 (1987).
28. M. S. Collins, R. F. Hector, R. E. Roby, A. A. Edwards, D. K. Ladehoff, and J. H. Dorsey. Prevention of gram-negative and gram-positive infections in rodents with three intravenous immunoglobulins and therapy of experimental polymicrobial burn wound sepsis with *Pseudomonas* immunoglobulin and ciprofloxacin. *Infection* **15**:S51–S59 (1987).
29. K. A. Poelstra, N. A. Barekzi, A. M. Rediske, A. G. Felts, J. B. Slunt, and D. W. Grainger. Prophylactic treatment of gram-positive and gram-negative abdominal implant infections using locally delivered polyclonal antibodies. *J. Biomed. Mater. Res.* **60**:206–215 (2002).
30. A. Dalhoff. Synergy between acylureidopenicillins and immunoglobulin G in experimental animals. *Am. J. Med.* **76**:91–100 (1984).
31. D. R. Absolom, C. J. van Oss, W. Zingg, and A. W. Neumann. Phagocytosis as a surface phenomenon: Opsonization by aspecific adsorption of Antibody as a function of bacterial hydrophobicity. *J. Reticuloendothel. Soc.* **31**:59–70 (1982).
32. O. Mimoz, A. Jacolot, C. Padoin, J. Caillon, K. Louchahi, M. Tod, K. Samii, and O. Petitjean. Cefepime and amikacin synergy against a cefotaxime-susceptible strain of *Enterobacter cloacae* in vitro and in vivo. *J. Antimicrob. Chemother.* **39**:363–369 (1997).