Targeting of *Pseudomonas aeruginosa* in the Bloodstream with Bispecific Monoclonal Antibodies[1]

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We examined the ability of a bispecific mAb reagent, consisting of a mAb specific for the primate erythrocyte complement receptor cross-linked with an anti-bacterial mAb, to target bacteria in the bloodstream in an acute infusion model in monkeys. In vitro studies demonstrated a variable level of complement-mediated binding (immune adherence) of *Pseudomonas aeruginosa* (strain PAO1) to primate E in serum. In vivo experiments in animals depleted of complement revealed that binding of bacteria to E was <1% before administration of the bispecific reagent, but within 5 min of its infusion, >99% of the bacteria bound to E. In complement-replete monkeys, a variable fraction of infused bacteria bound to E. This finding may have significant implications in the interpretation of animal models and in the understanding of bacteremias in humans. Treatment of these complement-replete monkeys with the bispecific reagent led to >99% binding of bacteria to E. Twenty-four-hour survival studies were conducted; several clinical parameters, including the degree of lung damage, cytokine levels, and liver enzymes in the circulation, indicate that the bispecific mAb reagent provides a degree of protection against the bacterial challenge. ***The Journal of Immunology*****, 2001, 167: 2240–2249.**

Humans and other mammals have several lines of defense against bacteria and viruses which may invade the bloodstream (1, 2). Due to protection afforded by innate immunity, immunologically naive animals challenged i.v. with small to moderate doses of most bacteria can clear and destroy the bacteria by a variety of mechanisms which make use of pattern recognition receptors, natural IgM Abs, and the complement system (3–5). Binding of Abs to Ags in immunized animals results in formation of immune complexes (4) which can fix complement, capture the complement activation product C3b, and bind to immune adherence receptors on circulating cells (6–9). More than 90% of these receptors (complement receptor 1 (CR1)[5]) in primates are found in the circulation on E. Nelson (6) first demonstrated, both in vitro and ex vivo, that opsonization of bacteria with specific Abs leads to complement-mediated binding of bacteria to primate E. His work and that of Robineaux and Pinet (10) suggest that immune adherence and immobilization of bacteria on primate E enhances their ingestion and destruction by phagocytic cells. This reaction may therefore be important in host defense against pathogens (10).

We have previously reported on the use of cross-linked mAb complexes (heteropolymers (HP)) to promote the binding of bacteria and viruses to primate E in vitro (11, 12). The HP consists of a mAb specific for the target pathogen cross-linked with a mAb specific for primate E CR1 (13); therefore, substrates are bound to E without a requirement for complement activation. Studies in monkey models indicate that HP can be used to bind prototype pathogens to E, thereby confining the pathogens within the vasculature until they are removed from the circulation (8, 14–16). The E are not destroyed and thus the HP-mediated clearance reaction appears to follow the natural primate E-mediated immune complex clearance mechanism (7, 16).

The goal of the present work was to evaluate the potential of the HP-E system to target bacteria in the bloodstream. We used an acute bacterial infection model based upon challenge with large doses of bacteria infused i.v. continuously over several hours (17–20). Under these conditions live bacteria can be demonstrated in the circulation and a variety of effector mechanisms can be analyzed, although the role of immune adherence in this model has not, to our knowledge, been previously evaluated. We studied handling of *Pseudomonas aeruginosa* (strain PAO1) in the bloodstream of complement-depleted and complement-replete animals, with and without HP treatment. We found that although a variable fraction of bacteria infused into the circulation of complement-replete monkeys binds to E in the absence of HP, infusion of HP leads to >99% binding of bacteria to E. In addition, experiments conducted with paired monkeys challenged with or without HP demonstrate that several parameters associated with resistance to the bacterial challenge are enhanced by HP treatment.

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[5] Abbreviations used in this paper: CR1, primate E complement receptor; HCT, hematocrit; NHS, normal human serum; CH50, hemolytic complement activity; HP, heteropolymer; CVF, cobra venom factor; CCS, cell culture supernatant; GFP, green fluorescent protein; RT, room temperature; SATA, *N*-succinimidyl *S*-acetylthioacetate; sSMCC, sulfosuccinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate; SN supernatant.

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Materials and Methods

Monoclonal Abs

Anti-CR1 mAbs 7G9 and 9H3, specific for human and monkey E CR1, have been described previously (21, 22). mAbs specific for *P. aeruginosa* PAO1 (23) and *Escherichia coli* (strain O type 2, cytotoxic necrotizing factor type 1 isolated from the clinical microbiology laboratory, University of Virginia Hospital) were generated from hybridomas after immunization of A/J mice with heat-killed bacteria. Cell culture supernatants (CCS) produced by hybridomas were screened for specific mAbs by measuring binding to microtiter plates coated with bacteria. Selection for high-avidity mAbs (15) employed flow cytometry, RIA, and magnetic separation. Bacteria were incubated with CCS, washed, and probed with FITC-labeled anti-mouse IgG or ^{125}I-labeled anti-mouse IgG. Washed samples were analyzed by flow cytometry or monitored for bound ^{125}I, respectively. Alternatively, BioMag anti-mouse IgG-coated iron particles (Polysciences, Warrington, PA) were added to bacteria incubated in CCS. Free bacteria were separated from particle-bound bacteria in a Polysciences Magnetic Separation unit and counted in a Coulter MultiSizer II (Coulter, Luton, U.K.). mAbs which bound the bacteria in these assays were selected as high-avidity mAbs. The anti-PAO1 mAb 2H4, isotype IgG2a, recognizes the LPS of PAO1 on Western blots (data not shown) and the anti-*E. coli* mAb 3E1 is isotype IgG1.

Preparation of HP

mAbs were purified from ascites fluid or CCS by affinity chromatography (24) and dialyzed exhaustively against borate saline (0.15 M NaCl and 0.03 M boric acid, pH 7.8). The cross-linking procedure was based on the method of Segal and Bast (25). The anti-CR1 mAb was reacted with *N*-succinimidyl *S*-acetylthioacetate (SATA, Pierce, Rockford, IL) at a ratio of 14 μg SATA/mg mAb for 2 h at room temperature (RT). The mixture was dialyzed with one change against HP buffer (50 mM sodium phosphate and 5 mM EDTA, pH 7.5) and then the SATA-mAb was deprotected to produce SH-mAb by treatment with 0.5 M hydroxylamine, 50 mM sodium phosphate, and 25 mM EDTA (pH 7.5), under argon, for 2 h at RT. During this time, the anti-pathogen mAb was reacted with sulfosuccinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (sSMCC; Pierce) at a ratio of 14 μg sSMCC/mg mAb for 2 h at RT. At the end of these incubations, both the SH-mAb and the sSMCC-mAb were subjected separately to gel filtration in HP buffer on gravity flow 10DG columns (Bio-Rad, Hercules, CA). Gel filtration of the SH-mAb was performed under a stream of argon, and the SH-mAb-containing fractions were stored under argon for a minimum period of time until coupling. The SH-mAb and sSMCC-mAb were combined at a 10–20% (by weight) excess of SH-mAb, mixed gently by inversion, flushed with argon, and reacted for 16 h at RT with gentle shaking. The coupling reaction was stopped by incubation with 1 mg iodoacetamide/10 mg mAb for 1 h at RT and then stored at 4°C. The coupling reaction mixture was subjected to gel filtration in borate saline buffer on Superose 6 (Pharmacia, Peapack, NJ) which was calibrated with monomeric IgG mAb and human IgM. Cross-linked product eluting between, but fully excluding the positions of these two markers, was pooled and used for experiments. Pooled product was stored at 4°C .

*Construction of GFP-PA01 and GFP-*E. coli

Plasmid pSMC2 coding for GFP (26) and β-lactam resistance was kindly provided by Dr. G. O'Toole (Dartmouth Medical School, Hanover, NH). The plasmid was transferred to *P. aeruginosa* PAO1 and the clinical isolate of *E. coli* by electroporation using standard procedures (27, 28). Cultures were maintained on standard agar supplemented with 350 μg/ml carbenicillin and 100 μg/ml ampicillin, respectively.

Monkey antibacterial Abs

The titers were determined by incubating GFP-PAO1 with varying dilutions of monkey plasma for 15 min at 37°C. Opsonized bacteria were washed three times and probed with PE-labeled anti-monkey IgG or a PE-labeled mAb specific for human IgM which cross-reacts with monkey IgM. The IgG titers are reported as the reciprocal dilutions of plasma that caused 50% of the bacteria to register as FL2 positive by flow cytometric analysis. IgM titers (data not shown) gave similar trends.

*In vitro binding of GFP-PAO1 and GFP-*E. coli *to primate E*

Measurement of HP- and/or serum-mediated binding of bacteria to E followed methods reported by Kuhn et al. (11). In brief, 10 μl (70 ng) of specific or irrelevant HP was added to 50 μl of a 50% E dispersion in either 1% BSA in PBS (BSA-PBS) or in a blood group-matched serum. After 5 min at 37°C, 5×10^6 GFP-transformed bacteria were added, giving an E:bacterium ratio of ~50:1 (Fig. 1*A*). Mixtures were incubated for 5–60 min at 37°C, and an aliquot was diluted into iced BSA-PBS. Samples were analyzed by flow cytometry (FACSCalibur; BD Biosciences, Mountain View, CA) by gating for FL1-positive events. Percent of bacteria either bound to E or free was determined by examination of the forward and side scattering profiles of FL1 events for the two populations. In some assays, HP were added to mixtures of whole blood and bacteria at much higher E:bacterium ratios (>500:1), and after incubation and centrifugation ($100 \times g$, 5 min), the number of bacteria free in the supernatant (SN) was determined by flow cytometry and compared with matched samples in which either an irrelevant HP or no HP was added (Fig. 1*B*). Equal volumes of the samples were examined in each case. We found no evidence for serum-mediated killing of GFP-PAO1 for an incubation period of 1 h at 37°C based on CFU assays (data not shown).

In vivo protocol

All animal experiments were supervised by a qualified veterinarian in accordance with approved protocols of the University of Virginia Animal Care and Use Committee and the Institutional Biosafety Committee. Cynomolgus and rhesus monkeys weighed between 2.3 and 8.5 kg. RIA (21, 22) conducted with anti-CR1 mAbs demonstrated 1000–3000 CR1 epitopes per E, with the exception of one monkey (see Fig. 2*C*). Some monkeys were pretreated i.v. with cobra venom factor (CVF, 70 U/kg; Quidel, San Diego, CA) 24 h before GFP-PAO1 infusion to consume complement (29). Hemolytic complement activity (CH50) determinations revealed little to no residual complement activity 24 h later. Before the bacterial infusion, the monkey was anesthetized (ketamine, 10 mg/kg i.m.; atropine, 0.04 mg/kg s.c.), intubated, and maintained under anesthesia with isoflurane and 100% oxygen. Blood pressure was monitored through a catheterized femoral artery (MicroMed, Louisville, KY).

An i.v. infusion of lactated Ringer's solution was established during the first hour of the experiment at a rate of 10 ml/kg per h. Hypotension is a common hemodynamic effect associated with isoflurane anesthesia in macaques (30) and was also seen as a direct response to the bacterial infusion. When indicated, phenylephrine was infused in boluses or at a calculated dosage of 0.5–3 μg/kg per min, with the goal of maintaining mean blood pressure above 50 mm Hg.

An overnight agar culture of GFP-PAO1 was suspended in PBS, washed three times, suspended in sterile saline (at $\sim 1 \times 10^9$ CFU/ml), and infused into the cephalic vein over a period of 1–4 h (see Figs. 2–4) at infusion rates corresponding to $\sim 10^9$ CFU/kg per h. Blood samples were drawn through an arterial catheter, and HP preparations or mAbs were infused as a bolus, 3–5 ml over 60 s, through the opposite cephalic vein. At the end of the experiment all animals were humanely euthanized under anesthesia. Samples of liver, lungs, and spleen were fixed in 10% Formalin and submitted, blinded, for examination by a pathologist. In addition, in selected experiments, samples of organs were homogenized as 20% dispersions in sterile-filtered 0.1% Triton X-100 in PBS, and diluted aliquots of these dispersions were analyzed for CFU.

Processing of blood samples

Blood samples were anticoagulated with EDTA, held on ice, and processed within 15 min. Plasma SNs were taken after centrifugation ($100 \times g$, 5 min). The pellet was washed once at $200 \times g$ and twice at $1800 \times g$, and the buffy coat was removed during washing. A 10-μl aliquot of this washed E pellet was lysed by dilution into 0.5 ml distilled water followed by vigorous vortexing and 0.5 ml of 2× PBS was added. Both the plasma and lysed E pellet were analyzed for GFP-PAO1 by flow cytometry based on a series of in vitro calibrations. The method for analysis of the E pellet is based on the controlled acquisition of an identical volume (500 μl of 1 ml) of each sample at constant flow. An FL1 threshold was set to record fluorescent events and then a forward/side scattering window of sufficient size was selected to include all bacteria in the cell lysate, including those that may have been associated with E membrane fragments. In all experiments, the E were in far excess over bacteria, and therefore the likelihood of undercounting of bacteria due to their coincident binding to the same E was minimized. The number of E in the lysed preparation was determined by measurement of the absorbance at 541 nm of the residual uncounted sample. A hematocrit (HCT) of 0.4% corresponds to an absorbance of 0.81 after lysis. Based on these determinations and the HCT, we calculated the concentration of GFP-PAO1 bound to E (designated Particles, Pellet in Figs. 2–4) and free in the plasma (designated Particles, SN). The plasma SNs were diluted into sterile-filtered BSA-PBS and measured volumes of 0.5 ml were counted for fluorescent bacteria using the same FL1 cutoff and a similar light scattering gate. Replicate samples of whole blood, plasma SN, and pelleted E were analyzed for CFU (designated CFU, Whole Blood; CFU, SN; CFU, Pellet, respectively, in Figs. 2–4). When levels were so

low that bacteria could not be detected by CFU assay in the most concentrated samples, the value is reported as 100 CFU/ml in Figs. 2–4.

Separate aliquots of blood were washed three times, the buffy coat was removed, and isolated E were reconstituted in BSA-PBS and probed with either a ^{125}I-labeled anti-CR1 mAb (the same as used to prepare the HP) or with ^{125}I-labeled goat anti-mouse IgG. Approximately 10^8 E were incubated with 0.2–1 μg ^{125}I-labeled probe for 30 min at 37°C, and after three washes or centrifugation through oil (21, 22) the amount of ^{125}I bound to the E was determined. The E concentration in these samples was determined as described above. Additional aliquots of blood were centrifuged at $3000 \times g$ to generate plasma SNs which were stored at −80°C for cytokine determinations. A total of 10–15% of the blood volumes of the animals was taken, and as much as 150 ml lactated Ringer's solution was infused; the HCT of both the control and HP-treated monkeys showed comparable decreases.

Cytokine assays

An ELISA sandwich assay was used to measure cytokines (TNF-α, IL-1β, IL-6) in the plasma of monkeys. Plates were coated with the appropriate anti-cytokine capture mAb (BD PharMingen, San Diego, CA), incubated with diluted plasma, and then with a biotinylated mAb which did not compete with the capture mAb. Development was accomplished by addition of neutralite-avidin coupled to HRP (Southern Biotechnology, Birmingham, AL). Standards included recombinant rhesus monkey TNF-α (BioSource International, Camarillo, CA), human IL-1β, and human IL-6 (BD PharMingen).

Results

In vitro assays

HP specific for binding of GFP-PAO1 to CR1 on primate E were tested in vitro with human and monkey E in preparation for in vivo studies in monkey models. Fig. 1*A* shows the degree of GFP-PAO1 binding to human E under a variety of conditions at a ratio of ~50 E:bacterium. In BSA-PBS, <15% of GFP-PAO1 bacteria were bound to E. Addition of specific HP promoted >90% binding of GFP-PAO1 to E in BSA-PBS, and this binding is rapidly attained and stable over 60 min. In the presence of serum (no HP added), where complement activation should lead to deposition of C3b on GFP-PAO1, E binding reaches 68% in 20 min, but then binding decreases, presumably as C3b decays to C3bi and C3dg. It is likely that E binding is mediated by activation of the classical pathway of complement because binding is lower during the first 20 min in serum containing Mg-EGTA, which only allows for alternative pathway activation and binding is abrogated if serum is treated with EDTA (Fig. 1*A*). Preadsorption of serum with bacteria on ice greatly reduces the ability of serum to facilitate GFP-PAO1-E binding, suggesting that the sera contain complement-fixing Abs specific for the bacteria. mAb 1B4, which blocks the C3b binding site on human E (31, 32), inhibits binding as does heat inactivation of serum (data not shown). We find that in whole serum it is difficult to demonstrate HP-mediated binding (Fig. 1*A*), because the natural process of complement-mediated immune adherence leads to a high level of binding in the absence of HP. However, as noted above, serum-mediated E binding decreases by 60 min, and at this time point the differences between the HP-treated and control samples in serum achieve modest statistical significance, 61 ± 11% vs 39 ± 19%, $p = 0.042$, unpaired t test (Fig. 1). If the bacteria are suspended in BSA-PBS, in serum EDTA, or in adsorbed sera, then HP-mediated binding is demonstrable (Fig. 1*A*). However, the level of binding in BSA-PBS (>90%) was always higher than in samples that contained plasma, even if complement was inhibited.

We next tested for HP-mediated binding of GFP-PAO1 to E in whole blood anticoagulated with EDTA, and to more closely simulate physiological conditions expected in the bloodstream, where E would be in great excess over bacteria (33, 34), we used a ratio of 500 E:bacterium. The results (Fig. 1*B*) demonstrate HP-mediated binding of GFP-PAO1 to both monkey and human E; at least two log units of bacteria were bound to E in these experiments. Finally, under similar experimental conditions, both serum and specific HP mediate substantial binding of another Gram-negative bacterium, *E. coli*, to primate E. In BSA-PBS HP-mediated binding of GFP-*E. coli* to human and monkey E averaged >90%; binding in serum (no HP added) averaged 80 and 95% for human and monkey E, respectively, based on 3–6 independent determinations.

Complement-depleted monkeys: in vivo HP-mediated binding of GFP-PAO1 to E

In view of the natural, physiologic effects of complement in facilitating binding of bacteria to E in the primate system, we tested HP in a monkey model in which animals were pretreated with CVF to consume complement. After CVF treatment, we waited 24 h to infuse bacteria, with the expectation that by this time most complement activation products would be cleared from the bloodstream and complement receptors, especially E CR1, would be available for ligation. As shown in Fig. 2*A*, continuous infusion of GFP-PAO1 led to negligible binding of bacteria to E over the first hour of the experiment. When a bolus of HP was infused, the number of GFP-PAO1 that circulated freely in the plasma decreased by ~100-fold, the number of bacteria bound to E increased by a factor of ~500, and the total number of bacteria in the bloodstream increased. The initial effect of the HP was observed within a few minutes of infusion and persisted for the remainder of the experiment. After the bacterial infusion was stopped at 160 min, the levels of both E-bound and free bacteria decreased. GFP-PAO1 bound to E and free in plasma was analyzed by flow cytometry and by determination of CFU (see *Materials and Methods*). In this experiment and those described below (Figs. 3 and 4; Table I), there was generally good agreement between the flow cytometry measurements (Particles) and the CFU assays with respect to the number of bacteria determined as either bound to E or as free in the plasma.

In the next experiment (Fig. 2*B*), we modified the experimental paradigm and infused the same dose of bacteria over a shorter time period. We observed no binding of GFP-PAO1 to E in the CVF-treated animal before administration of HP; after HP infusion, >99% of the bacteria were bound to E and the total number of bacteria in the circulation increased. After the GFP-PAO1 infusion was terminated at 120 min, levels of GFP-PAO1 decreased substantially. A second bolus of GFP-PAO1 was infused at 260 min, and >70% of the GFP-PAO1 immediately bound to E and >90% of the GFP-PAO1 was removed from the circulation after 60 min. The results from a control experiment (CVF treatment, but no HP), shown in Fig. 2*C*, indicate a more rapid disappearance of bacteria from the circulation after the GFP-PAO1 infusion was stopped at 120 min. In the absence of HP, there was little binding of GFP-PAO1 to E throughout the experiment for this control monkey. The steady-state level of bacteria in the circulation was lower than that observed in Fig. 2*B* after HP infusion, suggesting that bacteria free in the bloodstream (not bound to E) leave the circulation more rapidly.

Finally, we found that pretreatment of a complement-depleted monkey with HP before infusion of bacteria led to a very high level of E-associated binding when bacteria were infused (Fig. 2*D*). Fluorescence microscopy confirmed that in the presence of HP the bacteria were bound to E; however, the vast majority of the E had no bound bacteria (data not shown), as expected for ~10^7 bacteria/ml vs 4×10^9 E/ml.

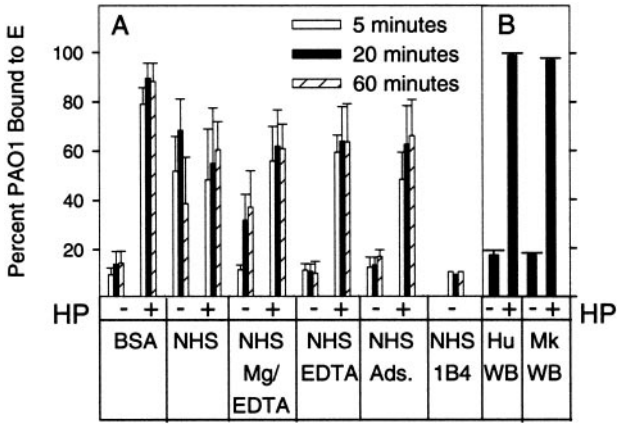

FIGURE 1. *A*, Flow cytometric determinations of the percent binding (mean and SD) of GFP-PAO1 to human (Hu) E mediated by HP or serum as a function of time and incubation medium at 37°C. In most experiments, the results represent the average for independent determinations on sera and E from six or more donors. The ratio of E:bacteria was ~50:1. The difference in percent bound for serum-mediated binding at 20 min vs 60 min (68 ± 13% vs 39 ± 19%) was statistically significant ($p = 0.003$, paired t test). *B*, HP-mediated binding of PAO1 to either human or monkey (mK) E in whole blood (WB) anticoagulated in EDTA. The E:bacterium ratio was ≥500:1, and incubations were conducted for 15 min at 37°C.

Complement-replete monkeys: in vivo HP-mediated binding of GFP-PAO1 to E

We next investigated whether HP could be used to unambiguously bind GFP-PAO1 in complement-replete monkeys (Fig. 3, *A* and *B*) which represent a more physiologically relevant condition. We found that continuous infusion of bacteria into either cynomolgus or rhesus monkeys leads to a steady state in which a variable fraction of the bacteria in the circulation is bound to E. This observation, coupled with the absence of immune adherence in CVF-treated animals, argues that complement activation must play a role in binding GFP-PAO1 to E. When HP was infused, the substantial changes in the distribution of E-bound and free bacteria observed in the CVF-treated monkeys were again demonstrable (Fig. 3, *A* monkeys and *B*). The number of bacteria free in the circulation decreased precipitously while the number bound to E increased and the total number of bacteria in the bloodstream increased 2- to 4-fold. After HP infusion, >99.9% of bacteria in the bloodstream were associated with E. These rapid changes in the distribution of E-bound and free bacteria were not observed in a control monkey in which HP was not infused (Fig. 3*C*).

Both the in vitro and in vivo results (Figs. 1 and 3) suggest that, in the absence of HP, the binding of the bacteria to primate E is facilitated to a great extent by anti-PAO1 Abs which promote complement activation (3, 5, 16). The isotype of anti-PAO1 mouse

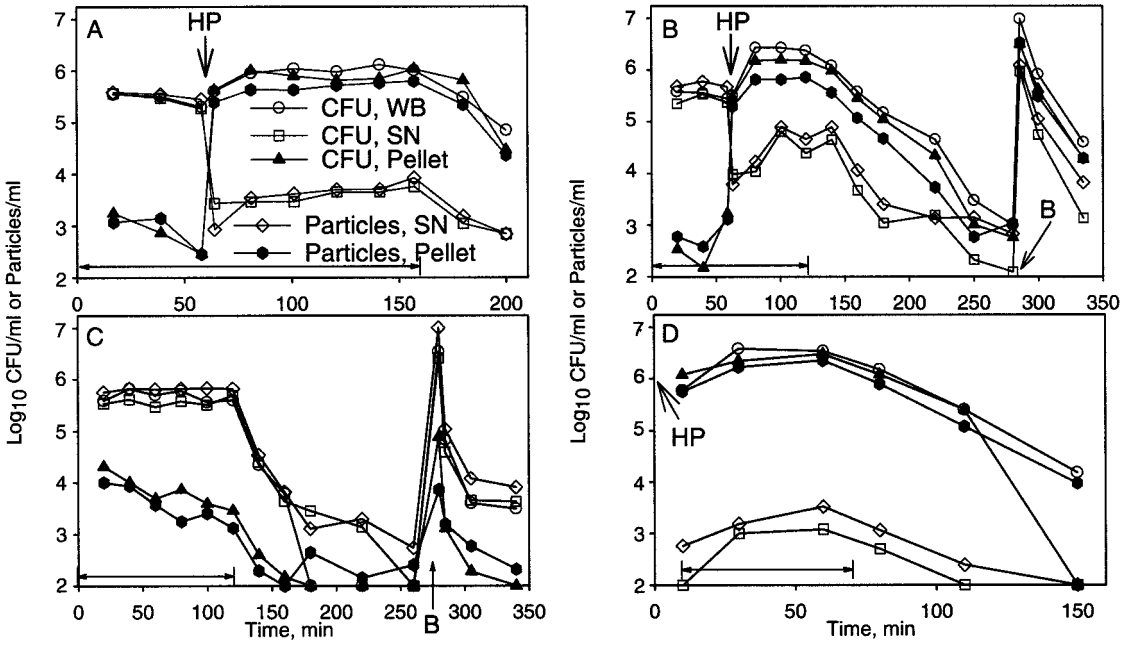

FIGURE 2. HP-mediated binding of GFP-PAO1 to E in CVF-treated cynomolgus monkeys. *A*, HP mediates binding of GFP-PAO1 to E in the circulation of a cynomolgus monkey (2A, 4.2 kg) treated with CVF 24 h previously. The CH50 of the monkey was 0 on the experimental day; 480 before CVF treatment. GFP-PAO1 was infused for 160 min at a rate of 4×10^8 CFU/kg per h (total dose, 1×10^9 CFU/kg), and HP (9H3 X 2H4, 140 μg/kg) was infused over 1 min at the 59-min point. Initial:final HCT: 23.8:17.3. *B*, As in Fig. 2*A*, except the monkey (2B, 2.7 kg) was infused with 5×10^8 CFU/kg per h of GFP-PAO1 over 120 min (total dose, 1×10^9 CFU/kg). The CH50 was 17 on the experimental day; 305 before CVF treatment. At 60 min, HP (9H3 X 2H4, 78 μg/kg) was infused. At 275 min an additional bolus of 9×10^8 CFU/kg GFP-PAO1 (denoted B) was infused over a few minutes. Initial:final HCT: 32.5:24.4. *C*, As in Fig. 2*A*, except the monkey (*C*, 5.1 kg) was not treated with HP. The CH50 of the monkey was 2 on the experimental day; 232 before CVF treatment. GFP-PAO1 was infused continuously at 4×10^8 CFU/kg per h over 120 min (total dose, 8×10^8 CFU/kg) and then a bolus (B) of GFP-PAO1 (8×10^8 CFU/kg) was infused at 270 min. This monkey had 90 CR1 epitopes/E. Initial:final HCT: 36.0:24.6. *D*, As in Fig. 2*A*, except the monkey (2D, 3.5 kg) was treated with HP (9H3 X 2H4, 117 μg/kg) at $t = 0$ before infusion of bacteria. The CH50 of the monkey was 1 on the experimental day; 394 before CVF treatment. GFP-PAO1 was infused at a rate of 8×10^8 CFU/kg per h for 1 h (total dose, 8×10^8 CFU/kg). Initial:final HCT: 43.5:33. The reciprocal titers of IgG anti-PAO1 Abs in monkeys 2A, 2B, 2C, and 2D were ND, >100, 14, and 7, respectively. CFU, whole blood (WB); CFU, SN; and CFU, Pellet are the CFU measured in the whole blood, plasma SN, and E pellet, respectively. Particles, SN and Particles, Pellet are the fluorescent events detected in the plasma SN and the E pellet, respectively (see *Materials and Methods*). The duration of the continuous bacterial infusion is denoted by the double-headed horizontal arrow in Figs. 2–5.

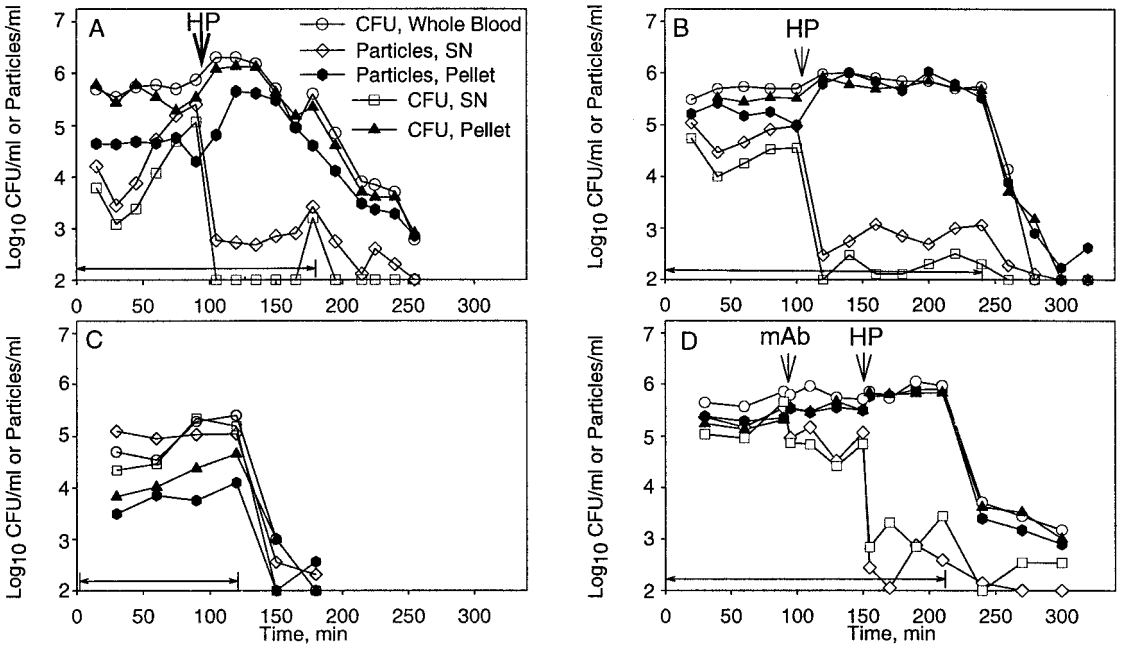

FIGURE 3. HP-mediated binding of GFP-PAO1 to E in complement-replete monkeys. *A,* HP mediates binding of GFP-PAO1 to E in the circulation of a complement-replete cynomolgus monkey (3A, 2.3 kg, CH50 = 136). GFP-PAO1 was infused for 180 min at a rate of 1×10^9 CFU/kg per h (total dose, 3×10^9 CFU/kg), and HP (7G9 X 2H4, 120μg/kg) was infused at 91 min. A liver biopsy was taken from the animal at 75 min and therefore HCT are not reported. *B,* As in *A,* except the rhesus monkey (3B, 8.5 kg, CH50 = 420) was infused with GFP-PAO1 for 240 min at a rate of 1×10^9 CFU/kg per h (total dose, 4×10^9 CFU/kg), and HP (7G9 X 2H4, 127 μg/kg) was infused at 115 min. Final CH50, 364. Initial:final HCT: 38:33. *C,* As in *A,* except the cynomolgus monkey (3C, 3.4 kg, CH50 = 550) was infused with GFP-PAO1 for 2 h at a rate of 3.5×10^8 CFU/kg per h (total dose, 7×10^8 CFU/kg). Final CH50: 600. Initial:final HCT: 43.4:36.1. *D,* Control experiment with anti-PAO1 mAb 2H4 alone to test for binding of GFP-PA01 to E in the circulation of a cynomolgus monkey (3D, 4.8 kg, CH50 = 212). GFP-PA01 was infused for 210 min at a rate of 1×10^9 CFU/kg per h (total dose, 3.5×10^9 CFU/kg), and mAb 2H4 was infused at 91 min (60 μg/kg) followed by HP (7G9 X 2H4, 120 μg/kg) at 151 min. Final CH50, 177. Initial:final HCT: 34:29. The reciprocal titers of IgG anti-PAO1 in the four monkeys (3A–D) were, respectively, 20, 40, 33, and 20. See Fig. 2 for symbol definitions.

mAb 2H4 is Ig2a, which is capable of fixing complement, and therefore it could be argued that the enhanced HP-mediated binding of GFP-PAO1 to E in the bloodstream of the monkeys might be due to complement activation after mAb 2H4 binds to the bacteria. To examine this possibility, mAb 2H4 was infused into the circulation of a cynomolgus monkey during a continuous infusion of GFP-PAO1 (Fig. 3*D*). Before mAb treatment, E binding was ~50%. Infusion of the mAb alone led to an increase in E-bound PAO1 and to a decrease in PAO1 in plasma, consistent with enhanced immune adherence (Fig. 3*D* and Table I). However, when an equimolar amount of HP was later infused, the number of bacteria free in the plasma decreased substantially, the total number of bacteria in the bloodstream increased 2-fold, and >99% of the bacteria in the bloodstream were bound to E (Fig. 3*D* and Table I). This result is in agreement with our previous findings which indicate that at equivalent doses, mAbs alone are not as effective at promoting in vivo binding of the target pathogens to primate E compared with the same mAbs when they are formulated into the HP (15).

There was no evidence that HP infusion caused E destruction. Total bilirubin levels remained low (<0.3 mg/dl; data not shown) before and after HP infusion for all monkeys. The decreased HCT at the end of some experiments (see figure legends) are expected after withdrawal of 10–15% of the total blood volume and infusion of fluids. We have previously demonstrated negligible loss of autologous E when ^{51}Cr-labeled E were opsonized with ^{125}I-labeled substrates (both proteins and *E. coli*) via HP and infused into a monkey (16).

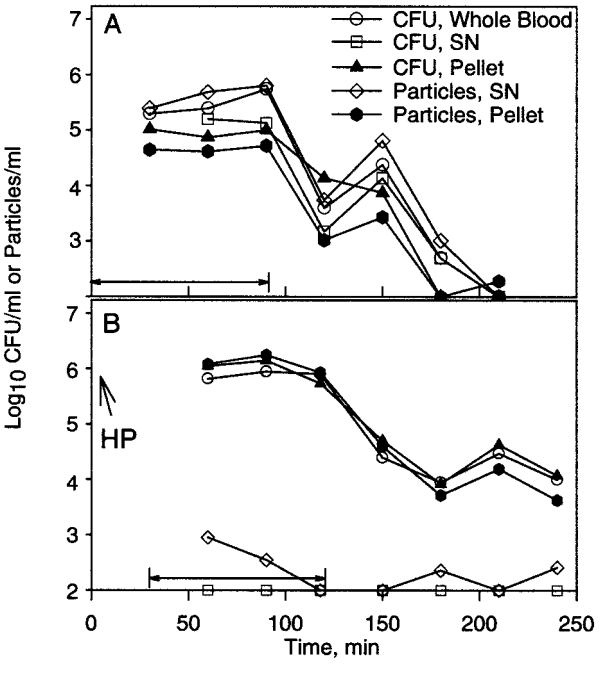

FIGURE 4. Handling of GFP-PAO1 in the circulation of two cynomolgus monkeys (4A, 3 kg; 4B, 3.3 kg), one of which (4B) was treated with HP (7G9 X 2H4, 125 μg/kg) 30 min before infusion of the bacteria. Both monkeys were infused with GFP-PAO1 at a rate of 1–1.2 $\times 10^9$ CFU/kg per h for 90 min (total dose, 4A, 1.5×10^9 CFU/kg; 4B, 1.8×10^9 CFU/kg). See Tables II and III for data on HCT and CH50. The reciprocal titers of IgG anti-PAO1 in the two monkeys were 5 and 17, respectively. See Fig. 2 for symbol definitions.

Table I. *Steady-state PAO1 distributions in monkey 3D before mAb, after mAb, and after HP treatment*[a]

	Particles SN [b,c]	Particles Pellet [b,c]	% Bound[d] (particles)	CFU SN [c,e]	CFU Pellet [c,e]	% Bound[d] (CFU)
Before mAb (30–90 min)	240,000 ± 100,000	220,000 ± 23,000	49 ± 9	220,000 ± 200,000	170,000 ± 34,000	51 ± 17
After mAb, before HP (95–150 min)	97,000 ± 48,000	320,000 ± 31,000	77 ± 11	60,000 ± 22,000	350,000 ± 78,000	85 ± 7
After HP (155–210 min)	400 ± 300	690,000 ± 120,000	99.9 ± 0.1	1,600 ± 1,100	660,000 ± 27,000	99.8 ± 0.2

[a] See also Fig. 3*D* legend for experimental details.
[b] Particles per milliliter whole blood, determined by flow cytometry, see *Materials and Methods.*
[c] Average ± SD of time points, $n = 3$ before mAb; $n = 4$ after mAb; $n = 4$ after HP.
[d] % Bound (particles) = 100 × (pellet, particles)/(pellet, particles + SN, particles). % Bound (CFU) = 100 × (pellet, CFU)/(pellet, CFU + SN, CFU). % Bound was calculated for each individual time point and then averaged.
[e] CFU per milliliter whole blood.

Complement-replete monkeys: treatment with HP before infusion of bacteria

We next investigated how pretreatment of monkeys with HP would affect the short-term responses of the animals to bacterial challenge. The experiment was designed to examine several clinical parameters, in particular lung damage, over a 24-h time period after GFP-PAO1 infusion in the presence of HP but in the absence of antibiotics. A naive animal was compared with a HP-treated monkey for three different infusion doses of bacteria. The results indicate that for each GFP-PAO1 dose, more bacteria were free in the plasma in the naive animals compared with the HP-treated animals (Table II and Fig. 4). However, binding of PAO1 to E was clearly evident in the untreated monkeys. The levels of immune adherence roughly correlated with the titers of monkey IgG Abs for PAO1. For example, monkey 4A (Fig. 4) had only moderate binding (32%, Table II) and had a reciprocal titer of 5. More than 60% of the infused bacteria bound to the E of monkeys 3B (before HP infusion), 5A, and 5C (Fig. 3 and Table II), and these monkeys had reciprocal titers of IgG for PAO1 of 40, 50, and >100, respectively.

The clinical condition of the HP-treated monkeys was better than that of the naive animals based on subjective criteria and clinical analyses. For example, elevation of liver enzymes in the HP-treated animals was lower than in the untreated animals (Table II). Organs were analyzed for CFU (Table II), and although no statistically significant conclusions can be drawn from such a small number of animals, the trend is toward lower levels of viable bacteria in the organs of the HP-treated animals. Table III summarizes the findings from the necropsy/pathology reports. Particularly striking was the level of protection from lung damage of the HP-treated animals at the higher doses of bacteria. Although there was no evidence for bacterial growth in the lungs of the control animal (monkey 5A) treated with 3×10^9 CFU/kg (Table II), postmortem evaluation of the lungs revealed congestion, fluid in the airways, and histopathologic confirmation of the gross observations. At the highest dose of 6×10^9 CFU/kg, there was a much greater difference between the naive and HP-treated animals (monkeys 5C and 5D, respectively) at the 24-h point. Although only two very small foci of infection were detected on the lungs of the HP-treated monkey (monkey 5D, Table III), severe pathology was evident in the lungs of the control animal (monkey 5C), which also presented with infection of the lungs, heart, and kidneys (Table II).

Reduction of inflammatory cytokine levels by HP

Septic shock, one of the most severe consequences of infection by Gram-negative bacteria, is mediated by LPS (35–37). HP-mediated binding of GFP-PAO1 to E reduced substantially the level of free bacteria in the bloodstream, and it seemed reasonable that

Table II. *Clinical parameters and CFU assays for selected monkeys*

	Monkey					
	4A[a]	4B[a]	5A[b]	5B[b]	5C[c]	5D[c]
HP dose	Control	125 μg/kg	Control	107 μg/kg	Control	216 μg/kg
PAO1 dose (CFU/kg)	1.5×10^9	1.8×10^9	3×10^9	2.7×10^9	5×10^9	6×10^9
% PAO1 binding to E[d]	32	>99.9	98	>99.9	89	99.7
AST/ALT[e]	248/91	135/98	195/150	75/99	421/185	222/89
HCT, initial:final	37:29	33:28	31:26	40:22[f]	37:36	34:36
CH50, initial:final	120:180	369:415	204:170	363:342	450:290	453:344
	CFU assays on organs (CFU/20 mg tissue)[g]					
Liver	300	50	200	105	3	5
Spleen	1.9×10^4	330	65	130	370	30
Lung	ND[h]	ND	ND	ND	1.6×10^7	63
Heart	90	ND	ND	ND	8×10^3	ND
Kidney	400	ND	ND	ND	800	ND

[a] Cynomolgus monkeys, see Fig. 4. Monkey 4A was lethargic, depressed, and withdrawn, and was euthanized at the 12-h mark. All other animals were euthanized at 24 h. Reciprocal IgG anti-PAO1 titers: 4A, 5; 4B, 17.
[b] Cynomolgus monkeys. Reciprocal IgG anti-PAO1 titers: 5A, 50; 5B, >100.
[c] Rhesus monkeys. Both 5C and 5D had reciprocal IgG anti-PAO1 titers > 100. Monkey 5C was weak and flushed at the 24-h mark. Except for 4A and 5C, no other monkeys had observable symptoms at euthanasia.
[d] Based on CFU determinations on whole blood and pelleted E 60 min after start of bacterial infusion.
[e] AST, aspartate aminotransferase, normal 32 ± 8 U/L; ALT, alanine aminotransferase, normal 35 ± 7 U/L.
[f] Leakage from arterial access site postoperatively. Further blood loss minimized by pressure wrap application.
[g] CFU assays performed on tissue samples homogenized in 0.1% Triton X-100 in PBS at 5 g tissue/20 ml PBS.
[h] ND, Not detectable.

Table III. *Necropsy and pathology findings for selected monkeys*

PAO1 Dose	Control	HP-Treated
1.5×10^9 CFU/kg	4A: Diffuse congestion in lungs, liver, and spleen	4B: Diffuse congestion and neutrophilia in spleen and liver; lungs normal
3×10^9 CFU/kg	5A: Mild acute reactive hepatitis; mild to moderate acute congestion and edema in the lungs; minimal lymphoid necrosis in the spleen	5B: Mild acute reactive splenitis; no evidence of tissue necrosis
6×10^9 CFU/kg	5C: Substantial consolidation in the lungs; large numbers of hemorrhagic lesions throughout; mild focal acute hemorrhagic pneumonia; minimal to mild reactive splenitis	5D: Lungs, healthy and pink; two very small (pencil point) hemorrhagic lesions; mild leukocytosis, small blood vessels, liver; minimal acute reactive splenitis

redirection to a clearance pathway which includes E binding might also affect the inflammatory potential of bacterial LPS. The mechanisms by which LPS interacts with plasma proteins and cell surface receptors to initiate inflammation are complex. However, it is well established that one of the earliest events in the inflammatory pathway is the appearance in the bloodstream of inflammatory cytokines such as TNF-α, IL-1β, and IL-6 (19, 37–39). Several groups have used primate models to delineate the kinetic profile of cytokine appearance in the circulation upon challenge with *E. coli* (18, 19, 38, 39). We find that the cytokine release pattern after challenge with GFP-PAO1 is quite similar (Table IV and Fig. 5). TNF-α levels increase in the circulation ~1 h after the bacterial infusion is initiated, peak after 90–120 min, and decrease thereafter. The increase in TNF-α is followed by an increase in the levels of IL-1β and IL-6. Our results indicate that use of HP dampens significantly the increase in cytokines promoted by the bacterial infusion. This finding strongly suggests that HP facilitate redirection and clearance of the bacteria by a pathway that may substantially reduce inflammation (see *Discussion*).

Some of the HP and control animals were treated with phenylephrine (Table IV and Fig. 5) to control blood pressure. It is unlikely that this treatment is responsible for the reduced cytokine levels in the HP-treated animals because phenylephrine is an α-adrenergic agonist and does not block TNF-α production (40–42). Moreover, in the animals not treated with phenylephrine the same trends are evident.

E-HP and E CR1 levels

Isolated and washed E were examined by RIA for bound HP and relative CR1 levels. The results indicate that the HP rapidly binds to E, because infusion of HP led to a substantial increase in the amount of E-bound ^{125}I-labeled anti-mouse IgG (Table V). As the experiment progressed, the amount of this probe which could bind to the E decreased, suggesting that HP were being removed from the E, as we have previously demonstrated in similar systems (14, 21). E probed with anti-CR1 mAb 7G9 used to prepare the HP evidenced only small decreases in mAb binding immediately after HP infusion, which would be expected since the HP infused into the monkeys was sufficient to occupy ~30% of total CR1, and some re-equilibration between free mAb and E-bound HP might have occurred during the in vitro incubations. It is noteworthy, however, that at later time points the amount of anti-CR1 probe that bound to the E further decreased, and these results follow the same patterns we have reported previously, in which clearance of E-bound HP occurs concomitantly with loss of E CR1 (14, 16, 21).

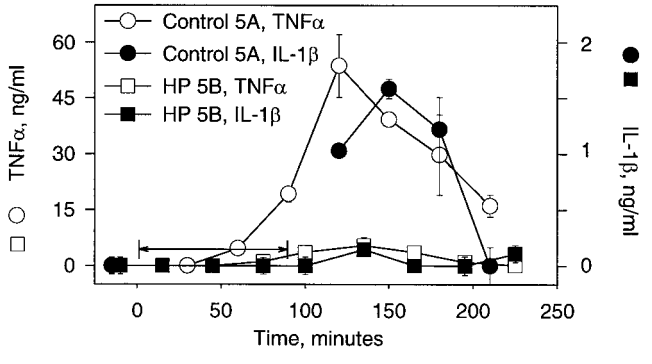

FIGURE 5. Effect of infusion of GFP-PAO1 on the levels of TNF-α and IL-1β in the circulation of two cynomolgus monkeys (5A, 3 kg; 5B, 2.3 kg), one of which (5B) was treated with HP (7G9 X 2H4, 115 μg/kg) 15 min before the start of the bacterial infusion. To control blood pressure in the control (no HP, 5A) monkey, phenylephrine was infused continuously starting at the 15-min point. The HP-treated monkey did not receive phenylephrine until the 2-h mark.

Discussion

In vivo evidence for HP-mediated binding

Our goal in this study was to determine the ability of the HP system to target GFP-PAO1 in the bloodstream and bind the bacteria to E during an i.v. challenge. HP were able to facilitate a very high level of binding of GFP-PAO1 to human and monkey E in BSA-PBS (Fig. 1*A*) and in anticoagulated whole blood at higher E:PAO1 ratios (Fig. 1*B*). At lower E:PAO1 ratios (Fig. 1*A*), in the presence of normal human serum (NHS), there was a variable level of immune adherence, and HP-mediated binding to E could not easily be distinguished from natural complement-mediated binding. When complement activation was blocked, HP clearly promoted E binding, but the presence of the plasma proteins may have reduced HP-mediated binding in vitro. However, experiments in both complement-depleted and complement-replete monkeys (Figs. 2–4) clearly demonstrate the very high level of efficiency by which HP promotes binding of the bacteria to E in vivo.

When the anti-PAO1 mAb 2H4 was used alone, an increase in complement-mediated immune adherence of bacteria to E was observed (Fig. 3*D*; 91 min, Table I). However, subsequent use of the HP containing this mAb at equal concentrations was far more effective in promoting E binding (Fig. 3*D*; 151 min, Table I). It is likely that this enhanced E binding mediated by the HP occurs because the anti-CR1 mAb in the HP, which acts as a surrogate for C3b (16), binds to CR1 with a higher avidity than C3b, and therefore substantially increases ligation to CR1. We have found that a concentration of 0.13 μg/ml anti-CR1 mAb 7G9 is sufficient to achieve 50% saturation of E CR1, which corresponds to an association constant in excess of 10^9 M^{-1} (43), whereas the avidity of monomeric C3b for CR1 is >100-fold less (44, 45). Therefore, successful immune adherence requires that multiple C3b molecules deposit on a substrate and engage clusters of CR1 on the E to assure multivalent binding (32). The present work, including the

Table IV. *Summary of cytokine levels after infusion of PAO1*[a]

Monkey/Treatment	PAO1 Dose (CFU/kg/h)	Infusion Time (h)	TNF-α at Peak		IL-6 at Peak		IL-1β at Peak	
			ng/ml	Time (min)	ng/ml	Time (min)	ng/ml	Time (min)
2C/CVF	4×10^8	2	50 ± 5	100	55 ± 18	180	0.13 ± 0.02	180
2D/CVF, HP	8×10^8	1	10 ± 4	120	19.1 ± 0.1	160	0.29 ± 0.01	160
4A/None	1×10^9	1.5	55 ± 7	150	131 ± 22	210	0.81 ± 0.10	150
4B/HP	1.2×10^9	1.5	8 ± 3	150	44 ± 8	210	0.075 ± 0.01	240
5A/None[b]	2×10^9	1.5	41 ± 5	120	133 ± 6	210	1.58 ± 0.08	150
5B/HP[b]	1.8×10^9	1.5	5 ± 1	135	66 ± 13	165	0.15 ± 0.02	150
3B/None[c]	1×10^9	4	42 ± 13	120				
3C/None	3.5×10^8	2	14 ± 2	90				

[a] Monkeys 2C, 2D, 4B, 5A, and 3B were treated with phenylephrine during the first 15 min of the experiments. Other monkeys were either not treated or were treated after the TNF-α peak.
[b] Values for TNF-α for monkeys 5A and 5B differ slightly from those shown in Fig. 5, which were determined as a time course in a different assay batch.
[c] HP administered at 115 min; TNF-α was 24.5 ng/ml at 100 min.

findings in both the CVF-treated and complement-replete monkeys, confirms earlier studies which indicate that the HP construct functions quite effectively in promoting in vivo binding of substrates to E CR1 (12, 14, 15).

We used mAb 2H4 alone in the in vivo immune adherence test (Fig. 3*D*) rather than an irrelevant HP (i.e., 2H4 × IgG) because the HP themselves do not activate complement when bound to a variety of substrates. For example, of relevance to the present work, flow cytometry experiments indicated that incubation of the 7G9 X 2H4 HP with E and NHS in solution, or incubation of preformed E-HP complexes with NHS, gave negligible deposition of C3b on the E (data not shown). These observations are in agreement with the work of Meri and colleagues (46, 47). Synthesis of the HP makes use of *N*-hydroxysuccinimide chemistry to derivatize lysines on the mAbs (see *Materials and Methods*), and Jokiranta and Meri (46) have reported that such chemical modification of mAbs blocks classical complement activation by blocking binding of C1q.

Immune adherence

Most animals appear to develop Abs against common bacteria (5, 48, 49) and therefore it is not surprising that our in vitro and in vivo experiments demonstrate binding of GFP-PAO1 and *E. coli* to both monkey and human E under conditions allowing for activation of complement (Figs. 1, 3, and 4; Table I). Although the acute bacterial infusion model has been extensively tested and described in a variety of nonhuman primates (17–20, 38, 39), to our knowledge there have been no attempts to determine whether bacteria were bound to E or free in the plasma. In addition, although there is an extensive literature describing human clinical conditions associated with bacteremias, these reports have not revealed whether the bacteria in the bloodstream were free in the plasma or bound to E (33, 34, 50). Our results demonstrate immune adherence of bacteria to E in the nonhuman primate infusion model. Because the degree of immune adherence of bacteria to E in the circulation is undoubtedly related to several factors, including the levels of complement and anti-bacterial Abs, it is possible that quantitative measurements of immune adherence may provide important prognostic information for patients with bacteremia. Similarly, it would seem important to determine whether bacteria infused into the bloodstream of mice or rabbits are bound to platelets which contain the nonprimate immune adherence receptor (51, 52).

Effects of HP on bacterial clearance

Experiments which compared HP-treated and naive monkeys indicate that HP-mediated binding of GFP-PAO1 to E tends to maintain the bacteria in the circulation for longer periods (compare monkey 2B vs monkey 2C, Fig. 2, and monkey 4B vs monkey 4A, Fig. 4). That is, the rate of removal of GFP-PAO1 from the vasculature was faster in the untreated monkeys, since both the steady-state levels of bacteria in the circulation were lower and the bacteria left the bloodstream more rapidly after the bacterial infusion was stopped. In contrast, after a monkey was treated with HP, the new steady-state level of bacteria in the circulation increased and, when the infusion ended, bacteria bound to E were removed from the circulation at a slower rate. An important question focuses on the fate and organ distribution of the bacteria after they exit the bloodstream in the untreated vs the HP-treated monkeys. The slower rate of clearance of GFP-PAO1 bound to E via HP may reflect a different clearance mechanism due to a rate-determining step which requires scission of CR1 (14, 16, 53) by proteases associated with fixed tissue macrophages in the liver and spleen,

Table V. *Relative HP binding and CR1 levels on selected monkeys*

Monkey	^{125}I-Labeled Goat Anti-Mouse IgG[a] (cpm bound)				^{125}I-Labeled Anti-CR1 mAb[a,b] (cpm bound)			
		Post-HP				Post-HP		
	Pre-HP	<40 min	3–4 h	24 h	Pre-HP	<40 min	3–4 h	24 h
2A	58 ± 2	2300 ± 200	1800 ± 200	NA	850 ± 50	600 ± 20	500 ± 20	NA
3A	150 ± 10	1150 ± 50	800 ± 50	NA	2100 ± 100	1750 ± 100	1550 ± 100	NA
3D	110 ± 30	550 ± 50	400 ± 20	NA	530 ± 10	420 ± 10	420 ± 10	NA
4B	34 ± 3	550 ± 30	400 ± 10	300 ± 10	110 ± 5	97 ± 2	90 ± 5	91 ± 5
5D	380 ± 10	1730 ± 20	1360 ± 20	650 ± 50	2560 ± 30	2250 ± 50	2100 ± 100	1300 ± 50

[a] All results are normalized to cpm bound per 10^6 E (mean ± SD).
[b] Binding of ^{125}I-labeled anti-CR1 mAb to sheep E (lacking CR1) was <10% of the values observed for monkey E. Several different ^{125}I-labeled probes of different specific activities were used.

followed by uptake of the bacteria in these organs. Clearance through this mechanism should decrease the rate at which otherwise free (not E-bound) bacteria can invade other organs and tissues, including the lungs, which are particularly susceptible to PAO1 (54, 55). The decreased pathology associated with the lungs in the HP-treated monkeys (see above) is consistent with this hypothesis. Therefore it is reasonable to anticipate that upon HP treatment a larger fraction of the bacteria will be redirected to the liver and spleen where the bacteria will be phagocytosed and destroyed. We measured live bacteria associated with these organs (Table II); if the bacteria were indeed killed they would not register in the CFU assay. There was no evidence for increased liver pathology as a result of HP treatment, and in fact the levels of liver enzymes in the circulation tended to be lower in HP-treated animals (Table II). Finally, comparison of monkey *A* and monkey *B* of Fig. 4 suggests that in the control monkey the bacteria that bound to E by immune adherence are cleared faster than bacteria bound to E via HP in the treated monkey. It is likely that, with respect to binding of PAO1 to E, more HP (compared with C3b) engage a greater number of CR1 with higher avidity. It is therefore reasonable to expect that clearance of HP-bound bacteria would be slower, presumably because more CR1 molecules would have to be cleaved to allow transfer of the HP-bacteria complex to acceptor macrophages.

Effect of HP on cytokine release

Recognition of bacterial-associated structures such as LPS by plasma proteins and cellular receptors such as CD14/Tlr4 constitutes an important element in defense against bacterial invasion. High levels of LPS which are processed via the CD14 pathway can, however, provoke an exaggerated inflammatory response, generally signaled by an increase in cytokines in the circulation, which is ultimately damaging to the host (35, 36, 56, 57). In monkeys treated with HP, >99% of bacteria in the bloodstream were bound to E, and the reduced cytokine levels in these animals suggests that processing of bacteria in these animals may have been different from processing of bacteria in the untreated animals. It is likely that bacteria bound to E via HP are more efficiently phagocytosed and destroyed by fixed tissue macrophages via a pathway that presumably utilizes Fc receptors on the macrophages (53, 58–60). Under these conditions, the bacteria and LPS in particular could therefore be redirected away from pathways which engage the CD14 receptor and might otherwise provoke an inflammatory response mediated by cytokines such as TNF-α. We hope to examine this possibility directly in future in vitro studies. We recognize that the number of monkeys used in the present study is limited and that statistically significant comparisons cannot be made based on such a small sample size.

In summary, we have examined how treatment with HP affects handling of GFP-PAO1 in the bloodstream of monkeys. A fraction of infused bacteria bind to E via immune adherence, a complement-mediated reaction. However, infusion of a HP specific for GFP-PAO1 and E CR1 leads to a much higher level of binding of the bacteria to E (>99%), and to a substantially reduced level of bacteria free in the plasma. Based on the results presented, we propose that E-bound bacteria have less opportunity to colonize susceptible organs and in addition are cleared from the circulation by a mechanism which bypasses to a great extent the CD14/LPS inflammatory pathway. Several clinical parameters, including the degree of lung damage, cytokine levels, and liver enzymes in the circulation, indicate that the HP, besides facilitating robust and rapid binding of bacteria to E, can provide a degree of protection against the bacterial challenge.

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