MAJOR ARTICLE

A 28-aa Pneumococcal Surface Adhesin A–Derived Peptide, P4, Augments Passive Immunotherapy and Rescues Mice from Fatal Pneumococcal Infection

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Background. P4, a 28-aa peptide derived from pneumococcal surface adhesin A, is a multilineage cell activator in vitro. We hypothesized that P4-mediated activation of phagocytic cells could rapidly and substantially increase opsonophagocytosis of bacteria, which could be translated in vivo to reduced mouse morbidity from fatal pneumococcal infection.

Methods. Reference in vitro opsonophagocytic killing and uptake assays were used with suitable effector cells and pathogen-specific antibodies. P4 peptide solution was added at the preopsonization stage. ND4-SW mice were infected intranasally with *Streptococcus pneumoniae* serotype 3 (WU2). At 72 and 96 h, infected mice received intraperitoneal or intravenous injection of gamma globulin, followed by an injection of P4.

Results. P4 treatment enhanced in vitro opsonophagocytosis of bacterial pathogens by many fold, and this effect was dependent on complement, P4, and antibody concentrations. Treatment of highly virulent WU2-infected mice with the combination of P4 and serotype-specific antiserum resulted in 100% remission of bacteremia and rescued 80% of the animals ($P < .05$).

Conclusion. P4 peptide in combination with pathogen-specific antibodies and complement enhances specific opsonophagocytosis and rescues mice from life-threatening pneumococcal infection. P4 peptide provides a fresh direction for therapeutic intervention through augmented passive immunotherapy.

P4 peptide, a 28-aa putative functional epitope of pneumococcal surface adhesin A, is a multilineage cell activator [1]. This peptide has activated various cell lines in vitro, including Detroit 562 human nasopharyngeal epithelial cells, undifferentiated and differentiated human promyelocytic leukemia cells (HL-60), mouse macrophages (RAW 264.7), and human leukocytes [1].

The immune response is a series of well-orchestrated cellular events involving a variety of effector cells, such as polymorphonuclear leukocytes, macrophages, dendritic cells, and T and B cells; tissue and serum components; and opsonizing agents, such as immunoglobulins [2]. The immunological stimulation of the effector cells is greatly influenced by the quality and quantity of the antigen-specific antibodies. Failure to mount an efficient immune response to microbial infection may lead to a poor prognosis. This is further challenged by microbial immune evasion strategies, such as encapsulation [3]. Use of antibiotics can be complicated by the emergence of drug-resistant clones. Passive immunization, a clinical strategy to provoke the effector cells with preformed antibodies to target pathogen, has been successful in some studies [4]. This effort is influenced by the time effector cells take to respond to the antibodies. Expediting the response time could lead to more rapid and efficient elimination of the target pathogens and better outcome. Our premise was to exploit the cellular activation potentials of P4 peptide for this purpose.

METHODS

Peptide synthesis. The amino acid sequences of the peptides designated P4, P6, and P7 have been described elsewhere [1, 5]. The pure peptides with a free N- and

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C-terminus were synthesized and lyophilized at the Centers for Disease Control and Prevention (CDC) and the Emory University Microchemical Facility. Peptides used in this study were synthesized in an Advanced ChemTech 396 multiple peptide synthesizer by means of standard and modified 9-fluorenylmethoxycarbonyl protocols [6–8]. Peptides were analyzed for fidelity of synthesis, using protocols described elsewhere [9]. Lyophilized peptide was resuspended in diethylpyrocarbonate (DEPC) water, sonicated for 3 min for dissolution, and stored at −70°C. We derived 2 peptides, P6 and/or P7, from the P4 sequence, and these peptides had no activation effect on the eukaryotic cells [1]. These peptides were used as negative controls in all in vitro experiments.

Species-specific antibodies used in this study. Gamma globulin (Gamunex) has been used as a source of pneumococcal serotype–specific polysaccharide (Ps) antibodies [10, 11]. QC2, QC5, and QC268 are in-house quality-control human sera with titers assigned to target organisms [12, 13]. A monoclonal antibody with specificity for pneumococcal surface adhesin A [14], 8G12G11B10 (8G12), was also used as a source of anti–streptococcal protein antibody. These sera were selected to ensure the presence of specific antibodies and demonstrate the specificity of P4-mediated immune enhancement. Our in vitro experiment design involved direct comparison of changes in opsonophagocytic killing (OPK) or uptake in the presence or absence of P4.

OPK assay. In this study, we used the reference OPK assay, as described elsewhere by Romero-Steiner et al. [15], with human promyelocytic leukemia cells (HL-60) differentiated into granulocytes. Gamma globulin was used as the source for serotype-specific antibodies for *Streptococcus pneumoniae. S. pneumoniae* serotype 3 (WU2) was propagated, stored, and used in this assay as described elsewhere [2, 10]. P4 peptide solution (100 μg/mL) was added to the OPK assay mixture at the preopsonization stage, and the control wells received 10 μL of DEPC water instead. P4-mediated enhancement of OPK was also assessed with *S. pneumoniae* serotypes 6B, 15B, 15C, and 19A, using gamma globulin or 8G12.

Flow cytometric opsonophagocytic uptake assay. The flow cytometric opsonophagocytic assay (fOPA) was performed as described elsewhere, with HL-60 cells differentiated into granulocytes or monocytes [12, 13, 16, 17]. In-house quality-control sera (QC5 and QC268) were used as a source for serotype-specific antibodies against the capsular Ps of *S. pneumoniae* serotype 14 and *Neisseria meningitides* A, respectively. Polystyrene beads were covalently linked to *S. pneumoniae* and non–*S. pneumoniae* antigens, as described elsewhere [12, 13], and used in fOPA. P4 peptide solution (100 μg/mL) was added to the fOPA mixture at the preopsonization stage, and the control wells received 10 μL of DEPC water instead.

OxyBURST labeling of* S. pneumoniae *isolate. To demonstrate the enhancement of the intracellular respiratory burst in the effector cells in response to P4-mediated activation, we used an OxyBURST-labeled *S. pneumoniae* isolate. A loopful of the frozen stock of *S. pneumoniae* serotype 23F was grown overnight (37°C in 5% CO_2) in Todd-Hewitt broth (Difco) supplemented with 0.5% yeast extract (THYE). A loopful of the overnight culture was transferred to 1 mL of fresh THYE broth and incubated for 3 h. From this, 200 μL was transferred to 5 mL of THYE broth and incubated for 3 h, after which 1 mL was transferred to 5 mL of THYE broth and incubated for another 3 h (all incubations were done at 37°C in 5% CO_2). After the third passage, the bacterial suspension was centrifuged at 6000 *g* for 10 min and resuspended in 1 mL of 0.01 mol/L PBS. OxyBURST stain (Invitrogen) was reconstituted with 1 mL of deionized water, and 50 μL was added to the 1-mL bacterial suspension. This was allowed to mix thoroughly in a rotary shaker overnight at 4°C. After that, the OxyBURST-labeled bacterial suspension was washed twice in PBS and used as a source of antigen instead of polystyrene beads in fOPA. In-house quality-control serum (QC2) was used in this assay.

Isolation of polymorphonuclear leukocytes from human blood. Heparinized venous blood was obtained from the Emory Blood Donor Services. A leukocyte separation kit, Histopaque-1119 (Sigma), was used to separate granulocytes from the blood, in accordance with the method recommended by the manufacturer.

Mouse strains. Mice *(Mus musculus)* of strain Swiss Webster (ND4-SW) were obtained from Charles River Laboratories. Mice used in this study were 6–10 weeks old. All experiments were approved by the institutional committee and were conducted according to institutional ethical guidelines for animal experiments and safety guidelines.

Bacterial strains. *S. pneumoniae* WU2 (serotype 3) was used for mouse infections. This *S. pneumoniae* isolate was selected from the Streptococcal Reference Laboratory, CDC. Briefly, *S. pneumoniae* isolate (frozen stock) was streaked on a blood agar plate (blood agar base plus 5% sheep blood) and incubated (at 37°C in 5% CO_2) for 18–24 h. *S. pneumoniae* colonies on blood agar plates were scraped with an inoculation loop and grown in 5 mL of THYE broth for ~4 h (at 37°C in 5% CO_2) until midlog phase (optical density read at 492 nm, 0.5–0.6). This culture (1.5 mL) was centrifuged in a 2-mL polypropylene screw-cap tube at 10,000 *g* for 5 min, and the wet pellet was resuspended in 1 mL of 0.1 mol/L PBS (pH 7.2). The 1-mL bacterial suspension was placed on ice and used for infections. It was also diluted 10^{-6} with PBS, and the viable bacterial load was enumerated on blood agar plates. The average viable bacterial load was 4×10^7 cells/mL.

Intranasal infection. Mice were intranasally infected with *S. pneumoniae* by means of methods described elsewhere [18]. Briefly, a mouse was injected intraperitonally with 20 μL of Ketaset (100 mg/mL ketamine hydrochloride; Wyeth). Once the mouse was lethargic, 40 μL of the previously prepared bacterial

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suspension was dispensed drop by drop close to the nose, allowing the mouse to inhale the infection.

Intraperitoneal (ip) and intravenous (iv) therapy. Infection with 4×10^7 cells of *S. pneumoniae* WU2 per mouse resulted in moribund characteristics at 48 h in 50%–60% of animals. At 72 h, all the infected mice were moribund ($n = 60$; moribund score, 2–3 [see below]). At 72 and 96 h after infection, 40 animals were passively immunized with gamma globulin (100 μL/mouse; iv, $n = 20$; ip, $n = 20$). After a time lapse of 20 min, allowing for possible preopsonization in vivo, 20 of the passively immunized mice (iv, $n = 10$; ip, $n = 10$) received P4 (100 μg; 100 μL/mouse) through an iv or ip route. Control mice were given DEPC water (100 μL) or P4 alone. Initially, P4 peptide was tested for toxicity in mice at 1, 10, 100, and 1000 μg. P4 was injected ip into 10-week-old ND4-SW mice at a constant volume of 100 μL. P4 had no apparent toxic effect on mice, even at doses of 1000 μg/mouse.

Scoring of moribund characteristics. Mice were monitored and visually scored twice daily for moribund characteristics. Mice were ranked on a scale of 5 to 0, in which 5 indicated healthy with normal coat, skin, eyes, breathing, and activity/movement; 4, healthy but beginning to look sick, ruffled coat; 3, sick, ruffled coat, decreased activity; 2, very sick, ruffled coat, decreased activity, eye secretions; 1, near death, ruffled coat, little or no activity, eye secretions, decreased breathing (these animals were, hence, euthanized); and 0, dead.

Cytokine analysis. P4-treated and control mice were killed and decapitated. The blood was quickly collected in cryovials from the base of the neck and allowed to stand at 4°C for 30 min. The tube was then centrifuged at 1000 *g* for 10 min. Serum samples were collected and used immediately for cytokine analysis. Cytokines were analyzed in mouse serum by means of the Luminex-based LINCOplex mouse 22-plex cytokine kit (MCYTO-70K-PMX22; LINCO Research), using the manufacturer-recommended protocol.

Statistics. All in vitro experiments were performed in triplicate on 3 separate assay days, unless specified otherwise. The in vivo challenge experiments were repeated >5 times. The number of moribund animals after treatment was recorded for 166 h, and the data were analyzed for significant differences among various groups by use of a *t* test with paired samples for means (Microsoft Excel 2003).

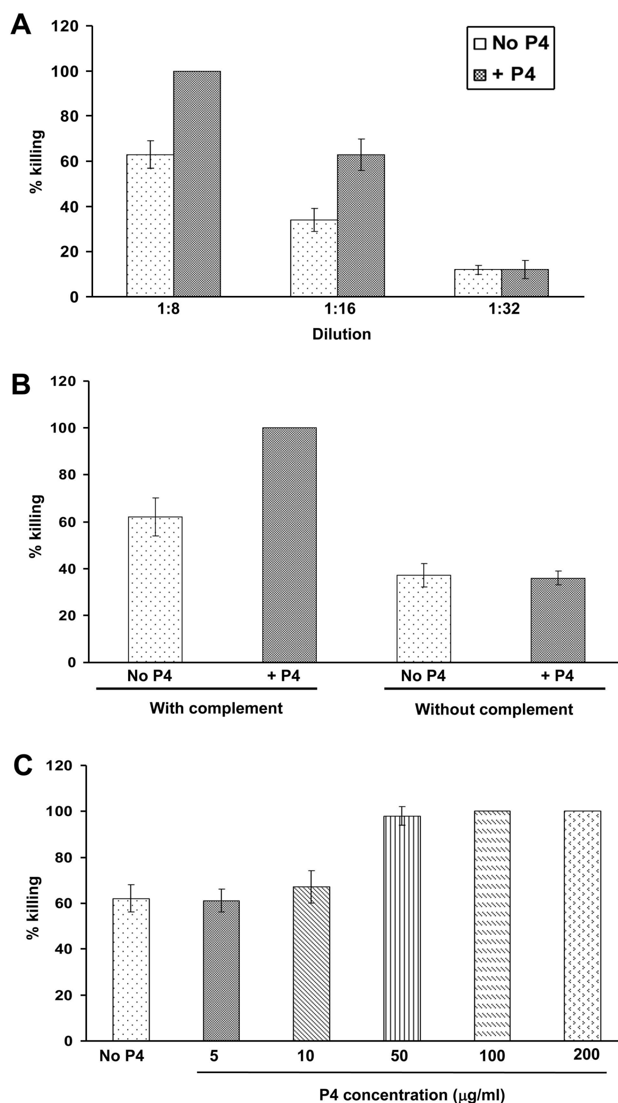

Figure 1. P4-mediated enhancement of opsonophagocytosis in vitro. *A,* Effect of antibody concentration. At a 1:8 dilution of gamma globulin, opsonophagocytic killing (OPK) of *Streptococcus pneumoniae* serotype 3 (WU2) increased by 35% compared with control; this effect titrated out with dilution, and findings paralleled those for controls at a 1:32 dilution. *B,* Effect of complement. Baby rabbit complement was required for OPK, irrespective of the presence or absence of P4. The without-complement assay group received heat-inactivated complement (56°C for 30 min). *C,* Effect of P4 concentration. A gradual increase in the OPK of *S. pneumoniae* serotype 3 (WU2) over control can be seen with the increase in P4 concentration. Gamma globulin at a 1:8 dilution was used as a source of serotype-specific IgG.

RESULTS

OPK assay. P4 peptide was tested for its potential to enhance opsonophagocytosis in vitro, and the data are given in figure 1. Data presented in figure 1*A* show that the P4-mediated increase in OPK of *S. pneumoniae* serotype 3 (WU2) was dependent on the antibody concentration. Although a 35% increase in OPK is seen at a 1:8 dilution of gamma globulin, the effect titrates out with the dilution of the antiserum (figure 1*A*). The P4-mediated increase in OPK was complement dependent, because no increase in OPK with P4 over control was observed in the absence of a complement source (figure 1*B*). The P4-mediated enhancement of OPK was dependent on the concentration of P4 in the reaction mixture. Although no change in OPK was seen with P4 supplementation at 5 μg/mL, a gradual increase was seen with an increase in P4 concentration (for 10 μg/mL, 8%; for 50 μg/mL, 30%; for 100 μg/mL, 35%). At a concentration of 100 μg/mL,

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the P4-mediated increase in OPK plateaued (figure 1*C*). A similar P4-mediated increase in OPK was also recorded with *S. pneumoniae* serotypes 6B, 15B, 15C, and 19A, using gamma globulin or 8G12 as the source of specific antibodies (data not shown).

Opsonophagocytic uptake (OPU) assay. P4-mediated enhancement of opsonophagocytosis was tested for changes in the intraphagocytic respiratory burst by means of OxyBURST-labeled *S. pneumoniae* serotype 23F (DS3848–03). P4-mediated increase in OPU was characterized by an increase in the intraphagocytic respiratory burst that titrated out with the antibody dilution (figure 2*A*). Data presented in figure 2*B* and 2*C* show P4 as being pluripotent in activating different effector cells and enhancing OPU in the presence of antigen-specific antibodies and complement. There was a ⩾50% increase in OPU of *S. pneumoniae* serotype 14 Ps beads by the granulocytes isolated from fresh human blood, which titrated out with the antibody dilution (for 1:64, 52%; for 1:128, 48%; for 1:256, 25%; for 1:512, 5%) (figure 2*B*). Figure 2*C* shows that P4 can enhance in vitro opsonophagocytosis of non–*S. pneumoniae* antigens in the presence of specific antibodies and effector cells. A P4-mediated increase in the OPU of beads coated with *N. meningitides* A Ps was recorded, with HL-60 cells differentiated into monocytes (figure 2*C*).

In vivo studies. Mice infected with *S. pneumoniae* WU2 were passively immunized with gamma globulin and/or P4 at 72 and 96 h after infection. Although the untreated mice had 10% survival (1 in 10), mice treated with gamma globulin alone (both iv and ip) had 30% survival. On the other hand, 8 (80%) of 10 mice ($P < .001$) treated with iv and 6 (60%) of 10 mice ($P < .001$) treated with ip gamma globulin and P4 exhibited complete remission of bacteremia and moribundity (figure 3). Cytokine analysis of mouse serum samples showed no consistent pattern or changes in cytokine levels in the rescued animals (data not shown).

DISCUSSION

P4 peptide, a 28-aa putative binding domain of pneumococcal surface adhesin A, is a multilineage cellular activator [1]. Here, we have demonstrated that this eukaryotic cellular activation potential in P4 peptide can be exploited for therapeutic benefits. We tested P4 peptide for its potential to enhance opsonophagocytosis in vitro.

The OPK assay is one of the classic in vitro serological assays used to demonstrate the presence of functional antibodies in serum [11]. The addition of P4 to the reaction mixture increases the OPK of target bacteria compared with the control. The requirement for target-specific antibodies and complement supports the hypothesis that P4 enhances opsonophagocytosis of the target bacteria by the effector cells. P4 peptide has no direct effect on the bacterium and no deleterious effect on human cells

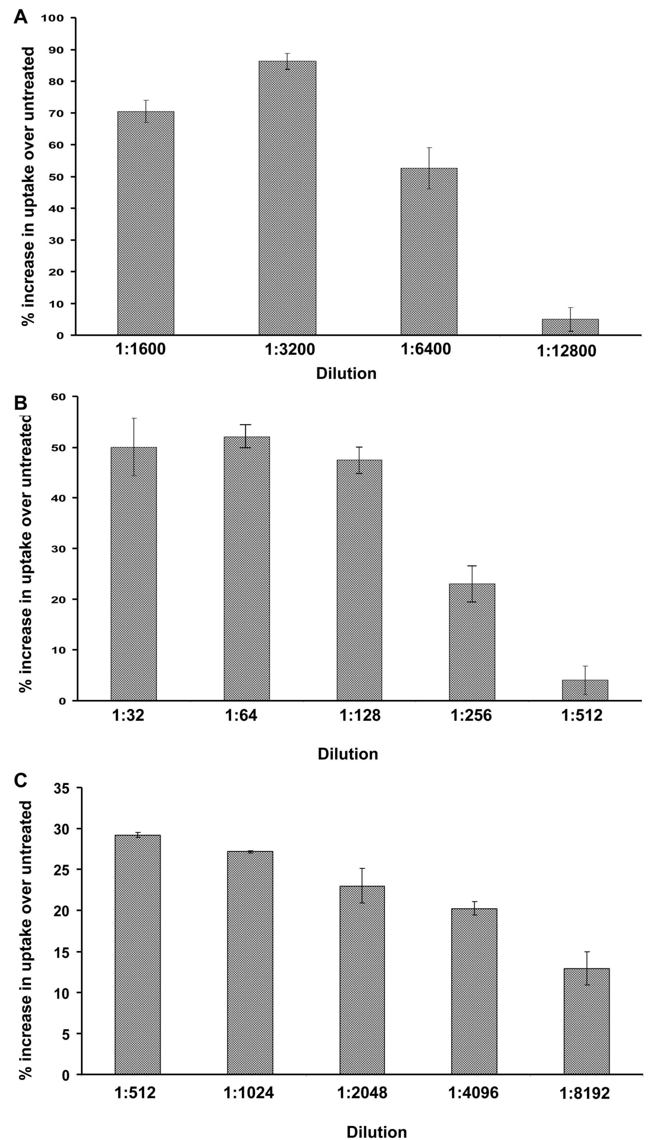

Figure 2. P4-mediated enhancement of flow cytometric opsonophagocytosis in vitro. *A,* Respiratory burst. P4 (100 μg/mL) increased the respiratory burst in the HL-60–derived granulocytes. In-house quality-control serum (QC2) was used to test the opsonophagocytic uptake (OPU) of OxyBURST-labeled *Streptococcus pneumoniae* serotype 23F capsular polysaccharide (Ps)–coated beads by granulocytes. The OxyBURST signal peaked at a serum dilution of 1:3200. *B, S. pneumoniae* OPU. P4 (100 μg/mL) increased the OPU of *S. pneumoniae* serotype 14 Ps–coated beads by freshly isolated granulocytes from human blood in the presence of QC2. *C, Neisseria meningitidis* OPU. P4 (100 μg/mL) increased the OPU of *N. meningitidis* A Ps–coated beads by HL-60–derived monocytes in the presence of in-house quality-control serum QC268.

[1]. The P4-mediated enhancement is dependent on the concentration of P4. This result suggests that a threshold level of P4 needs to be available to either trigger cell activation or activate a minimum number of effector cells for an observable increase in OPK. fOPA revealed that this enhancement was due to an increase in the number of active phagocytes in response to P4 treatment. No OPK or OPU was recorded with P4 in the absence

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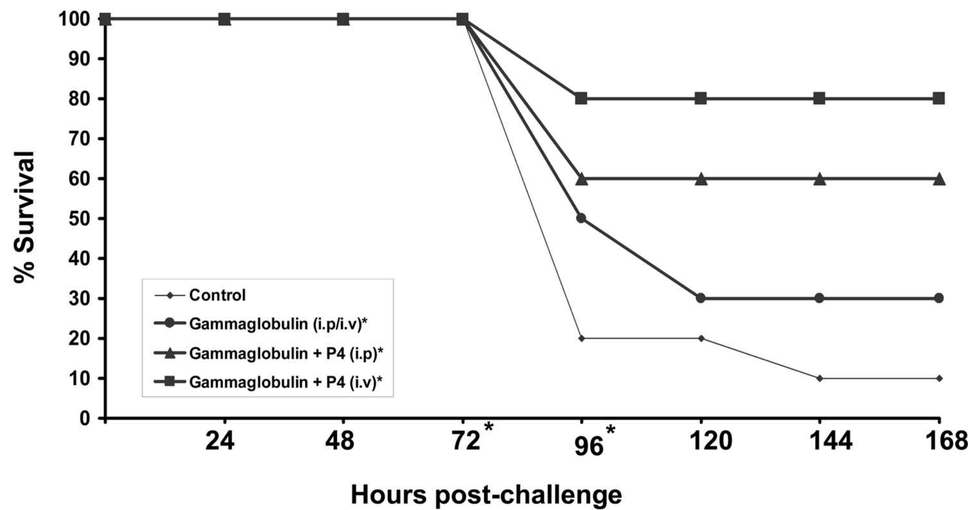

Figure 3. Conferring of protection against lethal intranasal *Streptococcus pneumoniae* serotype 3 (WU2) challenge by P4 with serotype-specific IgG. Intravenous (iv) injection of P4 (100 μg/mouse) with gamma globulin (100 μL/mouse) at 72 and 96 h after challenge provided highly significant protection (80%; $P < .001$) against lethal *S. pneumoniae* WU2 infection, followed in effectiveness by the intraperitoneal (ip) route of administration (60%; $P < .001$).

of effector cells or without specific antibody and complement, even in the presence of effector cells (data not shown). The mechanism for P4-mediated enhancement of opsonophagocytosis may be P4 peptide binding to the cadherin receptors of effector cells and activation of those cells [1, 19]. Such activation increases the number of active phagocytes participating in opsonophagocytosis, thereby increasing the uptake and/or killing of the target antigen.

To ascertain whether the P4-mediated enhancement of opsonophagocytosis could generate or translate into a therapeutic benefit in vivo, mouse rescue studies were designed. Mice tolerated P4 at $\geqslant$10 times its effective dose with no symptoms of toxicity (data not shown). Mice treated with the combination of P4 peptide and gamma globulin recovered from *S. pneumoniae* infection, compared with the untreated mice or those treated with gamma globulin alone ($P < .05$). Administration of combined P4 and gamma globulin through an iv route was most effective in rescuing the animals from fatal *S. pneumoniae* infection, with an 80% survival rate ($P < .001$), followed by 60% for those that received the combination ip ($P < .001$). The P4-mediated rescue of mice probably results from activation of circulating effector cells, which leads to an increase in the number of phagocytic cells participating in the opsonophagocytic clearance of the bacterial pathogen. P4 is less likely to cause immunological stimulation, given that we recorded no consistent changes in cytokine levels among the rescued animals (data not shown). Additionally, when P4 alone was administered to moribund animals, ~30% of animals were protected. We have tested naive mice for antibodies to *S. pneumoniae* and observed that 20%–40% of mice have natural antibodies (data not shown). We concluded that mice with natural antibodies can be rescued, but mice without antibodies will not recover in the absence of exogenous antibodies.

Opsonophagocytosis is a major immunological event triggered in response to the presence of antigen-specific antibodies and the activation of complement. Passive immunization or serum therapy uses pathogen-specific immune serum to treat infectious diseases [20, 21]. The success of serum therapy is limited to neutralizing antibodies that interfere with bacterial toxins, such as diphtheria toxin [22], or with viral colonization [23]. Antibiotics kill or interfere directly with the microbes, but antibodies trigger a cascade of immunological events in the host, including phagocytosis, complement activation, and antibody neutralization [24]. A major limitation of passive immunization is the lack of an effective broad-spectrum antiserum [4]. Furthermore, the efficacy of an antiserum depends largely on the immune status of the individual. Hence, even with fast, repeated administration of specific antiserum at an adequate dose, it is difficult to jump-start immunological events predictably, which is especially important in critically ill patients. Several strategies are being evaluated to address this problem, including the combination of recombinant antibodies [25], polyclonal antibodies [26], and single-domain antibody fragments [27]. In this study, we provide a novel strategy to potentiate the therapeutic effect of antibody with P4 peptide—"P4 therapy" for rapid protection against fatal infection. With P4 and pathogen-specific antibodies, it may be possible to initiate "rescue therapy" to save critically ill patients. P4 therapy followed by an antibiotic supplement may be effective in treating bacteremia or septicemia. In light of the cellular activation characteristics of P4 peptide and the data presented in this study, the application potentials of P4 peptide should be explored in clinical interventions that warrant cellular activation.

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To our knowledge, this is the first bacterial peptide that has been shown to augment opsonophagocytosis in vitro and rescue mice from fatal microbial infection. The molecular mechanism of P4 activation is under investigation. With the emergence of multidrug-resistant bacterial and viral pathogens, P4 peptide provides a fresh direction for therapeutic intervention through augmented passive immunotherapy.

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