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Evaluation of Type-Specific and Non-Type-Specific Pseudomonas Vaccine for Treatment of Pseudomonas Sepsis During Granulocytopenia

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The protective role of serotype-specific and non-type-specific active immunity against *Pseudomonas aeruginosa* infection was assesed in granulocytopenic dogs. Dogs were preimmunized with either specific serotype 6 vaccine (SI) or nonspecific serotype 3 vaccine (NSI) and challenged intravenously with 10^7 viable serotype 6 *P. aeruginosa* during granulocytopenia. Control dogs (C) having insignificant anti-pseudomonas antibody levels were also tested. Results showed: (i) significant increase in survival of SI dogs ($P < 0.05$) compared to C and NSI dogs, with no significant difference between C and NSI animals; (ii) lower febrile responses in SI dogs; and (iii) markedly reduced bacteremia in SI dogs compared to C and NSI animals. SI dog sera from survivor animals did not kill the infecting pseudomonas strain in vitro. The study demonstrated that type-specific immunity to *P. aeruginosa* induced by active immunization is effective in protection against pseudomonas during granulocytopenia and that non-type-specific immunity offers no cross-reactive protection. The findings suggest that the reticuloendothelial system in conjunction with specific immunity constitute an important defense against pseudomonas infections.

Pseudomonas aeruginosa is an invasive pathogen in leukopenic hosts, often causing disseminated tissue infection and sepsis. Numerous reports have assessed the protective role of anti-pseudomonas antibody in animal models (3, 4, 9, 10) and in man (1, 2, 5, 11-14). Most in vitro work has demonstrated that optimal phagocytosis and killing of *P. aeruginosa* occur when functioning granulocytes and strain-specific opsonizing antibody are combined (3, 4, 13). Active immunization with vaccine in man has shown significant protection in burn patients (1, 2) and very limited protection in cancer patients on chemotherapy (11, 14). There have been difficulties in assessing the role of antibody in cancer patients, however, because of highly variable treatment with antibiotics, steroids, and antitumor agents.

In an attempt to evaluate various therapeutic approaches to the problem of pseudomonas infection during leukopenia, our laboratory has developed a reproducible canine model of pseudomonas sepsis during transient myelosuppression. This model has been used to evaluate the effect of granulocyte transfusions alone on the course of pseudomonas infection and has shown that transfusions reduced the number of circulating pseudomonas but did not significantly prolong survival (6). More recently, we have used the model to evaluate combined preimmunization (with pseudomonas vaccine) and granulocyte transfusions on the course of infection (L. Harvath, B. R. Andersen, A. R. Zander, and R. B. Epstein, J. Lab. Clin. Med., in press). Combined therapy significantly increased survival and prevented sepsis.

The current study used the canine model and was undertaken to evaluate the role of both serotype-specific and non-type-specific active immunity in pseudomonas-infected, nontransfused, granulocytopenic animals.

MATERIALS AND METHODS

Dogs. Healthy mongrel dogs ranging in weight from 15 to 25 kg, immunized against hepatitis and distemper and dewormed, were isolated for a minimum period of 2 weeks before use.

Experimental model. Three groups of animals were studied: the control group received no vaccine, and the two experimental groups were preimmunized with either serotype 3 or serotype 6 *P. aeruginosa* lipopolysaccharide vaccine. The group receiving serotype 6 vaccine was specifically immunized (SI) against the challenging pseudomonas type; the group receiving serotype 3 *P. aeruginosa* vaccine was nonspecifically immunized (NSI) against the challenging pseudomonas strain. The vaccines were lots ×41722 and ×41595 supplied by M. Fisher and

H. Devlin of Parke-Davis Co., Detroit, Mich. Preimmunization consisted of six injections of 50 μg/kg of body weight administered intramuscularly over a 3-week period in 3- to 4-day intervals.

With the exception of preimmunization, all dogs were treated identically. Dogs were injected with a single intravenous dose of cyclophosphamide (40 mg/kg) on day 0. They were supported with Ringer solution subcutaneously during periods of anorexia after drug administration. Animals were granulocytopenic (≤600 granulocytes/mm³ of blood) 4 days after cyclophosphamide injection (day 4). On day 4 dogs were challenged intravenously with a 1-ml suspension of 10^7 viable serotype 6 *P. aeruginosa*. The bacterial suspension was prepared by the method previously described by Epstein et al. (6).

Leukopenia consistently occurred from days 4 through 6 in this model. In animals surviving infection and leukopenia, bone marrow recovery of myelocytic elements usually began by day 7, and peripheral leukocyte levels returned to normal at day 11 or 12.

Titration of antibody. Type 3 and type 6 *P. aeruginosa* antibodies were titered by the passive hemagglutination procedure of Neter et al. (8) modified by sensitizing sheep erythrocytes with the purified lipopolysaccharide vaccines. Sera were tested for total antibody (immunoglobulin [Ig] G + IgM) by titration of serial twofold dilutions of untreated samples. IgG antibody was titered after treating sera with equal volumes of 0.2 M 2-mercaptoethanol for 18 h at room temperature. All animals had serotype 6 total antibody titers of 16 or less and IgG titers of 2 or less prior to immunization.

Leukocyte counts, blood cultures, and temperatures. Leukocyte counts and rectal temperatures were measured daily. Quantitative peripheral blood cultures were performed at the time of pseudomonas challenge, at 5 min and at 1 h after challenge, and daily thereafter. During autopsy right ventricular blood samples were obtained for quantitative blood culture. All isolates were serotyped for positive identification of type 6 *P. aeruginosa*.

In vitro serum bactericidal assay. Fresh sera of survivor dogs were tested for direct killing of type 6 pseudomonas. Sera of normal dogs having no significant anti-pseudomonas antibody were also tested. Solutions containing 1, 10, or 100% serum in saline were mixed with 10^3 or 10^6 log-phase serotype 6 pseudomonas. Controls consisted of 10^3 or 10^6 pseudomonas in saline alone. All tests were run in duplicate and incubated for 60 min in a 37 C shaker water bath. After incubation, 0.1-ml samples were plated by the agar overlay technique. Colonies were counted 18 to 20 h after incubation of plates at 37 C. In some experiments, fresh normal dog serum was added as a source of complement to heat-inactivated (56 C, 30 min) immune or normal serum.

Statistical analysis. The Wilcoxin rank sum test for unpaired samples (7) was used to analyze differences in length or survival, temperatures, and granulocyte counts. Analyses were carried out using a two-tailed test.

RESULTS

Survival periods. Table 1 shows survival periods of all dogs after pseudomonas challenge. Specifically immunized (SI) dogs survived significantly longer than control (C) dogs and nonspecifically immunized (NSI) dogs, $P < 0.05$. The mean survival of C animals is 48.9 and 47.9 h for NSI animals, with no significant difference between these two groups. There were two survivor dogs and three dogs that died of pseudomonas infection in the SI group. The mean survival of the three dogs that died in the SI group (76.3 h) is significantly longer than C group survival, $P < 0.05$. All SI animals survived longer than C animals and four of five dogs survived longer than NSI dogs.

Blood cultures. At the time of pseudomonas challenge all dogs had negative cultures. Cultures of all dogs were positive 5 min after challenge (range of 100 to 800 bacteria/ml of blood) and were again negative 1 h after challenge. Blood cultures became positive on the day of death in C and NSI dogs. SI animals failed to show positive blood cultures prior to death.

Quantitative blood cultures of all animals at autopsy are shown in Table 1. Animals in C and

TABLE 1. *Survival period and quantitative blood cultures at autopsy*

Dog no.	Control		Type-specific immunized (type 6)		Non-type-specific immunized (type 3)	
	Survival[a] (h)	Culture[b]	Survival[a] (h)	Culture[b]	Survival[a] (h)	Culture[b]
1	47	3,000	Survivor	0[c]	50	17,000
2	44	19,000	65.5	0	41	100
3	64.5	7,100	86	0	47	55,000
4	30	24,000	Survivor	0[c]	35	16,000
5	59	2,500	77.5	50	66.5	8,600

[a] Survival period after pseudomonas challenge.

[b] Right ventricular blood culture within 2 h after death; represents number of type 6 *P. aeruginosa* per milliliter of blood.

[c] Persistently negative cultures through day 21 post-cyclophosphamide administration.

NSI groups had high concentrations of pseudomonas in blood specimens at death, with median values of 7,100 and 16,000 organisms/ml of blood, respectively. In comparison, SI animals had negative blood cultures with the exception of one dog, which had 50 pseudomonas/ml of blood.

Leukocyte counts and temperatures. Figure 1 illustrates the mean peripheral granulocyte values of all three groups throughout the course of cyclophosphamide treatment and infection. All groups were granulocytopenic ($\leq$600 granulocytes/mm^3 of blood) on the day of bacterial challenge and for 48 h after challenge. Granulocyte values for all three groups were not significantly different until day 6, when the SI group was significantly higher than C and NSI groups, $P < 0.05$.

Figure 1 also illustrates the mean temperature responses of dogs during the same time period. All animals had temperatures within normal range (shaded area) until day 5. On day 5, 24 h postchallenge, C and NSI dogs demonstrated marked febrile responses with mean values of 105.5 F (ca. 40.6 C). SI dogs at this time had a mean temperature of 102.3 F (ca. 38.9 C). By day 6, SI dogs became febrile, with temperatures comparable to those of C and NSI animals.

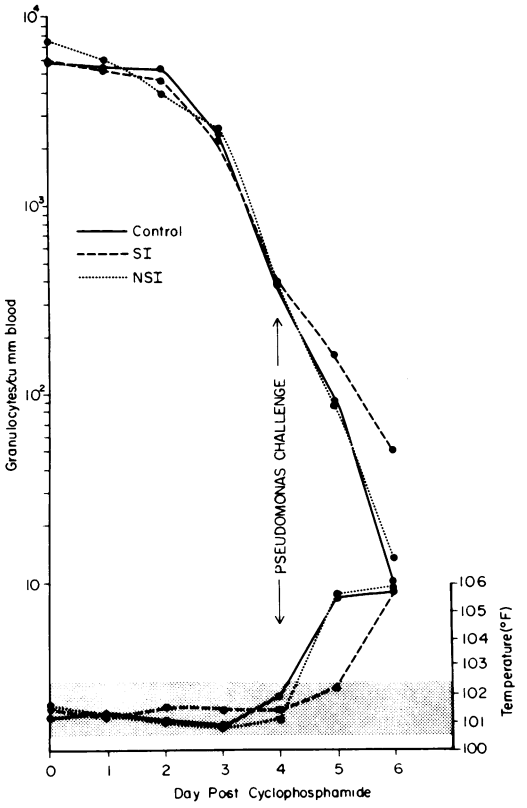

FIG. 1. *Average peripheral granulocyte counts and temperatures after cyclophosphamide administration. Symbols: (——) Control dogs received no vaccine; (- - -) specifically immunized dogs (type 6); (····) non-type-specific immunized dogs (type 3). Shaded area represents the normal temperature range of dogs.*

Antibody levels. Figure 2 illustrates type 6 anti-pseudomonas antibody titers of all dogs. SI dogs maintained high total (IgG + IgM) anti-pseudomonas antibody titers (1,024 to 2,048) prior to and during infection, with the exception of one survivor dog whose titer dropped significantly (to 16) on day 6. IgG titers of SI animals ranged from 16 to 256 prior to pseudomonas challenge, and all except the same survivor dog remained in this range during infection. NSI animals had type 6 cross-reactive total antibody titers ranging from 16 to 128 on the day of pseudomonas challenge (day 4). Two of five NSI dogs displayed significant decreases in total type 6 antibody on day 5 (to titers of 8 and 2), whereas other dogs remained in the prechallenge range. None of the NSI animals developed detectable type 6 IgG antibody. All C animals had total type 6 antibody titers of 16 or less and IgG titers of 2 or less during treatment and infection.

NSI dogs had high titers of serotype 3 antibody, with total antibody titers ranging from 1,024 to 2,048 and IgG levels ranging from 128 to 512. There was no change in total or IgG type 3 anti-pseudomonas antibody titers during infection.

Serum bactericidal activity. Immune sera of survivor dogs (total anti-pseudomonas antibody titers of 512) and normal dog sera did not kill pseudomonas in vitro. In samples containing 10^6 pseudomonas, control values ranged from 2 $\times$ 10^6 to 5 $\times$ 10^6 pseudomonas after 60 min of incubation, whereas samples containing 100% immune or normal sera ranged from 9 $\times$ 10^6 to 10^7 organisms or 5 $\times$ 10^6 to 9 $\times$ 10^6 organisms, respectively. Immune and normal sera at the three concentrations tested were not significantly different from controls containing no serum. Assays using a decreased inoculum (10^3 pseudomonas) also failed to demonstrate a difference between immune and normal sera. Experiments with fresh normal dog serum as a complement source in combination with heat-inactivated immune or normal sera demonstrated that serum with type-specific antibody was not bactericidal to the infecting pseudomonas strain.

Autopsies. All dogs were autopsied within 2 h after death and demonstrated consistent

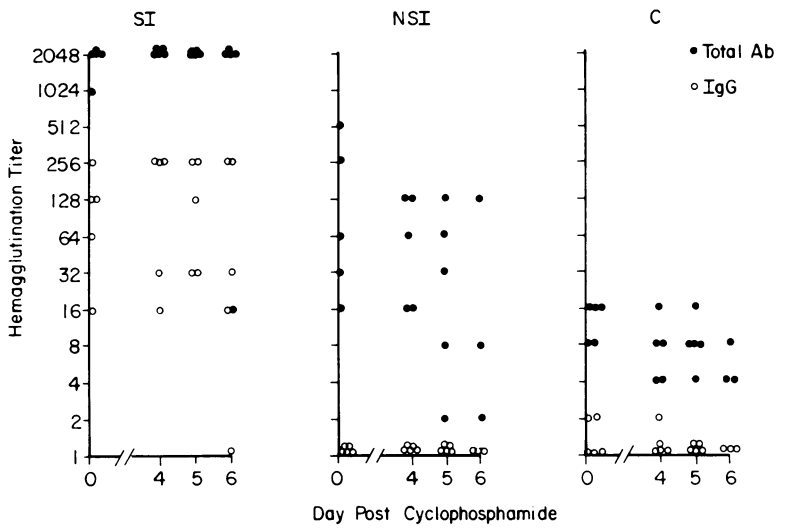

FIG. 2. *Hemagglutination titers of serum antibody against type 6 P. aeruginosa after cyclophosphamide administration. SI, Specifically immunized dogs (type 6); NSI, nonspecifically immunized dogs (type 3); C, control dogs, not immunized. Hemagglutination titer represents the reciprocal of the highest dilution giving hemagglutination.*

pathological changes. Lungs, liver, kidneys, and spleen were congested and had numerous focal hemorrhages associated with pseudomonas invasion and tissue necrosis. The lungs were most severely affected, with marked pulmonary edema, hemorrhage, and necrosis of alveolar septae.

Quantitative organ cultures from the lungs of C and NSI dogs had high concentrations of pseudomonas per gram of wet tissue weight. The data in Table 2 show the range and median values of quantitative lung homogenate cultures for the three animal groups. The C and NSI group ranges and medians were similar; however, SI dogs that died of pseudomonas infection had significantly fewer organisms in the lung, $P < 0.05$, with a median value 4 logs lower than C and NSI medians. SI animals also had lower concentrations of pseudomonas in kidneys, spleen, and liver in comparison with C and NSI dogs.

TABLE 2. *Quantitation of pseudomonas from lung tissue at autopsy*

Group	No. of serotype 6 *P. aeruginosa*/g of wet tissue wt	
	Range	Median
C	8.6×10^8–2.0×10^{11}	3.3×10^{10}
SI (type 6)	1.6×10^6–2.7×10^7	2.5×10^6
NSI (type 3)	1.5×10^{10}–1.5×10^{12}	3.1×10^{10}

DISCUSSION

This study demonstrates that type-specific active immunity offered signficant protection against pseudomonas sepsis during myelosuppression, but nonspecific immunity had no protection value. Active preimmunization with type-specific vaccine prevented bacteremia, increased survival periods, and reduced tissue infection. All animals were leukopenic during infection and, therefore, had few circulating granulocytes to phagocytize the pseudomonas. Granulocytes remaining in circulation had decreased phagocytic ability (Andersen, Debelak-Fehir, and Epstein, Proc. Soc. Exp. Biol. Med., in press) and were not observed within necrotic or infected tissue. The infecting pseudomonas strain was resistant to killing by immune sera of survivor dogs. Thus, neither granulocytes nor direct killing of organisms by immune serum was responsible for the prolonged survival in this model. This study suggests that fixed macrophages of the reticuloendothelial system in conjunction with type-specific immunity are protective against pseudomonas infections in granulocytopenic animals.

The findings of this study implicating combined activity of macrophages and specific immunity support in vitro studies of others (3, 4, 9, 10). Bjornson and Michael (3, 4) have previously demonstrated that mouse peritoneal macrophages in the presence of specific IgG and IgM anti-pseudomonas antibodies are bactericidal to pseudomonas in vitro. Studies by Reynolds (9, 10) with alveolar macrophages and anti-pseudomonas respiratory immunoglobulins have shown that IgG is an effective macro-

phage opsonin in vitro. The opsonizing activity of anti-pseudomonas antibody for macrophages, which has been demonstrated in vitro, is probably an important defense mechanism in our leukopenic animal model.

A consistent pattern of pseudomonas infection was observed in animals of this study. SI dogs had negative blood cultures, even at death. In contrast, NSI and C animals had marked bacteremia within 24 h of death. C and NSI dogs also had significantly higher concentrations of pseudomonas in tissues than SI animals. The bacteremia appeared to be a preterminal event resulting from organisms entering the blood from heavily infected tissues. The negative blood cultures of SI animals were probably the result of less severe tissue infection. Although SI dogs had significantly fewer pseudomonas in their tissues, some died of infection. The reason for death is unclear, but, since they survived longer than the other dogs, the cause may have been progressive tissue damage from persistent infection.

Previously we have demonstrated that combined therapy of active preimmunization and granulocyte transfusions significantly prolonged survival and prevented pseudomonas sepsis (Harvath et al., J. Lab. Clin. Med., in press). A comparison of combined therapy with active immunity alone indicates that granulocyte transfusions increased survival of leukopenic immune dogs. Thus, it appears that phagocytosis of pseudomonas by transfused granulocytes was therapeutically important in the clinical course of infection, but the present study suggests that it was not the only clearance and killing mechanism. Active immunity with type-specific antigen affords significant protection to leukopenic animals; however, it remains to be clarified whether the critical immune mechanism is humoral or cellular. A current study using passively administered antibody in our canine model should provide further insight into this question.

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