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Glycolipoprotein from *Pseudomonas aeruginosa* as a Protective Antigen Against *P. aeruginosa* Infection in Mice

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After primary subcutaneous immunization of rabbits with glycolipoprotein from *Pseudomonas aeruginosa* BI, indirect hemagglutinating and bacterial agglutinating activities appeared in the antiserum 6 days after immunization and reached a peak between 15 and 20 days. Both these in vitro activities paralleled in vivo antipseudomonas-induced leukopenia and mouse passive-protection activities. Further experiments indicated that a functional association exists between the hemagglutinating and passive-protection activities, and that passive protection depends on activity levels in the plasma rather than in the peritoneum. After intraperitoneal injection in mice, in vitro and in vivo activities of antiglycolipoprotein serum declined in the peritoneal cavity as the plasma levels increased. After intravenous injection of the antiglycolipoprotein serum, initially high levels of in vitro and in vivo activity declined at approximately equal rates. Immunoglobulin G (IgG) and immunoglobulin M (IgM) fractions from 15-day antiglycolipoprotein serum were assayed for biological activity. Most of the hemagglutinating and bacterial agglutinating activity and all of the mouse passive-protection activity were found in the IgM fraction. Assay of antiglycolipoprotein serum after 2-mercaptoethanol inactivation of IgM showed that most of the in vitro and all of the passive-protection activities had been destroyed, again locating these activities principally in the IgM fraction of the original antiserum.

In recent years, antibiotic-resistant pseudomonas infections have occurred with increasing frequency in patients afflicted with seriously debilitating conditions (10). Despite extensive investigation, the mechanisms of pathogenesis for this organism remain poorly understood, and the existing means for controlling it clinically are of uncertain effectiveness. Besides improved antibiotic therapy, granulocyte replacement and passive immunization are currently under study as methods of treatment that may prove useful (10).

The extracellular slime glycolipoprotein (GLP) of *Pseudomonas aeruginosa* BI exhibits several biological characteristics that merit serious consideration in the attempt to clarify the process of infection and to provide immunologically based alternatives to antibiotic treatment. In mice, highly purified preparations of GLP produce toxic, leukopenic, and lethal responses that resemble the effects of infection initiated with viable organisms (11). The production of GLP has been detected in vivo and it appears to enter the peripheral circulation rapidly (5). Once circulating, the GLP associates with leukocytes, which are then sequestered in the liver (8). In vitro, GLP-mediated inhibition of phagocytosis has been demonstrated (11). Active or passive immunization with GLP protects mice against the toxic, leukopenic, and lethal effects of *P. aeruginosa* in experimental infection (11).

This paper presents experimental results defining the relationships among the biological activities of antibodies produced to GLP. The in vitro activities of rabbit anti-GLP serum are correlated with its in vivo capacity to prevent pseudomonas-induced leukopenia and to protect mice against a lethal challenge of *P. aeruginosa*. Additional results demonstrate that immunoglobulin M (IgM) is the immunoglobulin fraction of immune serum that is responsible for nearly all these biological activities.

MATERIALS AND METHODS

Organism. The bacterium used in this study, *P. aeruginosa* BI, was originally isolated from a clinical specimen and has been previously described (3).

GLP. GLP fraction was obtained from the extracellular slime layer of strain BI and purified by methods described previously (2, 11). Slime was extracted in 0.15 M NaCl from 18-h bacterial cultures grown on sheets of cellophane overlaying Trypticase soy agar (BBL, Cockeysville, Md.). The extract was precipitated with ethanol, clarified by centrifugation, and dialyzed against distilled water. The dialysate was

centrifuged at 105,000 × *g* for 3 h and the supernatant fluid, which contained the GLP fraction, was lyophilized. The GLP was then filtered through gels and subjected to anion-exchange chromatography, eluting at a KCl molarity of 0.3 to 0.4. Purity and homogeneity were demonstrated by chromatography, sedimentation pattern, and immunodiffusion (11). Lyophilized GLP was stored in vacuo at 4°C.

Animals. White male rabbits weighing 3 to 4 kg were housed individually. White male Swiss mice weighing 18 to 20 g were housed 10 per cage. All animals were supplied with water and Purina chow ad libitum.

Active immunization. On day 0, rabbits received subcutaneous injections in four separate sites for a total of 2 mg of GLP in equal volumes of 0.01 M phosphate-buffered saline (PBS; pH 7.2) and Freund incomplete adjuvant. Groups of three rabbits were bled at various intervals of time after immunization, and the pooled sera were stored at −20°C. Indirect hemagglutination (IHA) and bacterial agglutination (BA) titers remained undiminished for more than a year.

IHA and BA. Hemagglutinating activity of the sera and serum fractions was determined indirectly as described previously (5). In brief, formalinized sheep erythrocytes were sensitized with GLP (200 μg/ml) in PBS at room temperature for 30 min. After several washes in PBS, the sensitized erythrocytes were resuspended in PBS to a concentration of 5%, and were added in 0.05-ml amounts to serial dilutions (0.5 ml) of the appropriate serum preparation. The titer is expressed as the reciprocal of the highest dilution producing a positive hemagglutination pattern after 2 h at room temperature. BA titers were determined by mixing 1 drop of a turbid suspension of strain BI in PBS with 1 drop of the antibody dilution and then observing for gross agglutination.

Fractionation of immune serum. Immune rabbit serum obtained at 15 days was fractionated into IgM and immunoglobulin G (IgG) by sequential passage through Sephadex G 200 in a 31-cm jacketed column, at 5 to 8°C, with 0.1 M tris(hydroxymethyl) aminomethane-hydrochloride (pH 8.0) in 0.2 M NaCl, at a flow rate of 10 drops/min, as reported by Flodin and Killander (7). Refrigerated fractions were monitored by ultraviolet light absorption and dialyzed against saline before lyophilization. Lyophilized fractions were stored in vacuo at 4°C, and IHA and BA titers remained undiminished for the experimental period of 3 months. Weighed samples to a final concentration of 10 mg/ml were dissolved in PBS for experimental use.

Immunodiffusion. The Ig fractions were analyzed by immunodiffusion in Special Noble agar (Difco). The fractions were tested against goat anti-rabbit IgG heavy-chain serum (Cappel Laboratories, Downington, Pa.) and goat anti-rabbit globulin serum (Sylvania Laboratories, Millburn, N. J.) by allowing diffusion overnight at room temperature. The resulting patterns were photographed without staining.

2-ME treatment of serum. The destruction of IgM activity by means of 2-mercaptoethanol (2-ME) is based on the method of Deutsch and Morton (6). Equal volumes of immune serum and 0.2 M 2-ME in PBS (pH 7.2) were mixed and incubated at 4°C for 1 h. This was followed by dialysis for 2 h against 0.02 M iodoacetic acid in PBS (pH 7.2) and further dialysis against saline at 4°C overnight.

Passive protection. Unless otherwise stated, mice were passively immunized with an 0.5-ml intraperitoneal or intravenous injection of 15-day rabbit anti-GLP serum or Ig fractions 2 h before bacterial challenge. Bacterial cells were prepared for challenge as described previously (5). Briefly, organisms were washed in PBS from 18-h cultures grown on Trypticase soy agar slants, washed, and suspended in PBS at a turbidity equivalent to 1×10^{10} to 5×10^{10} viable cells per ml. A 0.2-ml injection of this suspension contained 5×10^{9} viable cells and was 100% lethal in unprotected mice. The protective capacity of a serum or Ig fraction is expressed as the percent survival 7 days after bacterial challenge. All experiments were repeated three times.

Leukocyte counts, peritoneal aspirates, and plasma. Leukocyte counts were made on peripheral blood collected retro-orbitally as reported previously (11), and the results are presented as averages for a group of five mice. Peritoneal aspirates and plasma were sampled as described previously (5).

RESULTS

Correlation of the biological activities of anti-GLP serum. The immunogenic capacity of GLP was demonstrated by monitoring the in vitro appearance of IHA and BA activities of serum samples collected at selected time intervals after primary subcutaneous immunization of rabbits. The results (Fig. 1) show that both IHA and BA activity first appeared at 6 days and steadily increased up to 15 days. Between 15 and 20 days the activity diminished and, by the end of the observation period at 30 days, the titers had decreased to approximately the 6-day level. Further experiments compared the results of these in vitro assays with an in vivo determination of mouse passive protection (Fig. 1), and the results indicate that the appearance of the protective capacity of anti-GLP serum against lethal infection by strain BI in mice parallels the development of the in vitro biological activities.

Leukopenia has been reported as one of the effects following infection by strain BI (11), and evidence has been reported relating this event to mortality (8, 13). Therefore, it was of some interest to study the relation of the anti-leukopenic activity to the other activities of the antiserum. The results of such a study show a correlation between the appearance of this activity and that of the other three (Fig. 1).

Dissemination of biological activities from the peritoneal cavity to plasma. The correlation of in vitro and in vivo activities during the immune response of the rabbit to subcutaneous GLP injection raised the question of

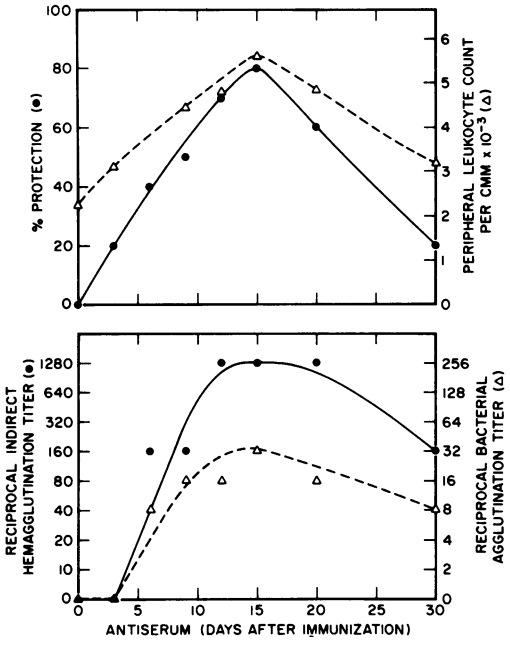

FIG. 1. *Time course of the appearance of various biological activities of rabbit antiserum after primary immunization with the GLP of P. aeruginosa. (A) Five mice per group were passively immunized with anti-GLP serum collected from rabbits on the days indicated and then were challenged with approximately 5 × 10⁹ cells. Protection was calculated as described in the text. Leukocyte counts were made 5 h after the bacterial challenge, and the mean leukocyte count for control mice receiving only normal rabbit serum followed by PBS only was 8,000 cells per cm³. The bacterial challenge for control mice receiving only normal rabbit serum was 100% lethal. Mean values of three experiments are presented. (B) IHA and BA activities were also determined as described in the text.*

whether a similar correlation between IHA levels and resistance to *P. aeruginosa* infection could be demonstrated in mice passively immunized with anti-GLP serum. Figure 2 shows that, after intraperitoneal injection of mice with anti-GLP serum, the IHA activity in the peritoneal cavity declined rapidly from a high initial level, while at the same time the level of activity in the plasma increased. Furthermore, the increased activity in the plasma corresponded to the increasing resistance of the mice to a lethal intraperitoneal injection of *P. aeruginosa*. Peak resistance to the bacterial challenge occurred at 3 h after passive immunization, precisely the period required for the maximum transfer of IHA activity from the peritoneal cavity to the blood stream. This observation supports the view that the passive-protection activity of anti-GLP serum depends on the activity levels in the plasma and not on those in the peritoneum. Furthermore, the simultaneous dissemination of both activities from the peritoneal cavity to the blood stream indicates a close association of IHA and passive-protection activities.

These observations were tested further by passive immunization of mice by the intravenous route followed by intraperitoneal infection (Fig. 3). In this case, resistance of the mice to infection was maximal immediately after passive immunization and declined steadily until at 3 h no protection could be demonstrated. As expected from earlier results, the IHA activity followed very closely the curve of protective activity; furthermore, neither activity was detectable in the peritoneal washings during the observation period. This verifies the previous observations: (i) the activity levels of the plasma, and not of the peritoneum, determine the effectiveness of the passive protection conferred by anti-GLP serum; (ii) an intimate association exists between IHA and passive-protection activities of the anti-GLP serum.

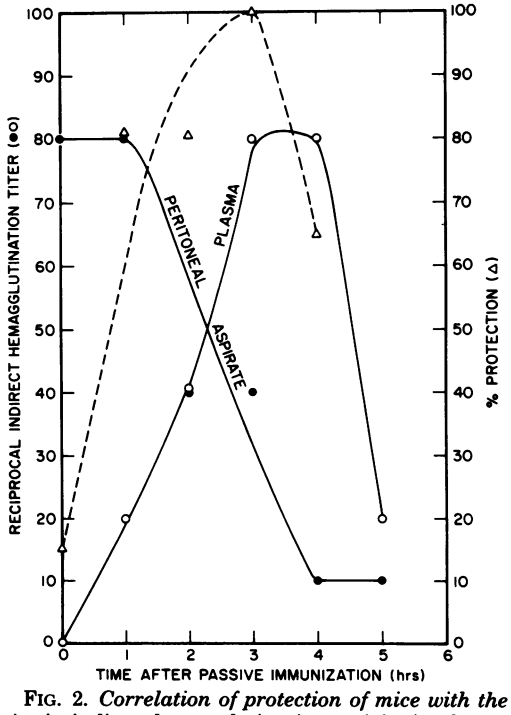

FIG. 2. *Correlation of protection of mice with the rise in indirect hemagglutinating activity in plasma after intraperitoneal passive immunization with 15-day rabbit anti-GLP serum and intraperitoneal challenge with P. aeruginosa. Five mice per group were passively immunized and challenged intraperitoneally at the times indicated as described in the text. The bacterial challenge for control mice receiving only normal rabbit serum was 100% lethal. Mean values of three experiments are presented.*

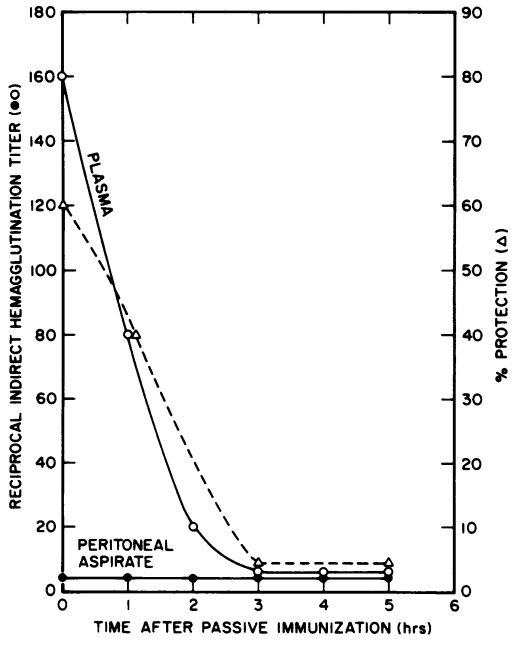

FIG. 3. *Comparison of IHA activity in peritoneal washings and plasma with protection of mice after passive intravenous immunization with rabbit anti-GLP serum followed by intraperitoneal P. aeruginosa challenge. Experimental details were as for Fig. 2.*

Biological activity of the IgG and IgM fractions of anti-GLP serum. The identity of the Ig class responsible for protective activity of antiserum against pseudomonas infection remains in dispute (14). The close correlation observed in these experiments between in vitro and in vivo activities offered a means of investigating the biological activities of Ig fractions of anti-GLP serum, with the possibility of assigning each of the activities to either IgG or IgM. Accordingly, a pool of anti-GLP serum was obtained from rabbits 15 days after primary subcutaneous immunization, the time of peak biological activity as previously demonstrated. Double passage on Sephadex G 200 separated chromatographically pure IgG and IgM. An analysis of the preparations by immunodiffusion shows that immunoglobulin was present in both the IgG and the IgM preparations (Fig. 4A), and that IgG heavy chains were present only in the IgG preparation, not in the IgM preparation (Fig. 4B). This confirms the chromatographic evidence that no structurally intact IgG contaminates the IgM preparation.

The biological activity of these fractions was compared individually with the activity of the 15-day anti-GLP serum pool from which they had been obtained. Table 1 shows that most of the IHA and BA and all of the mouse passive-protection activity resided in the IgM fraction. Only a low level of IHA and BA activity was associated with an equivalent concentration of IgG.

The association of IgM with the IHA, BA, and protective activities of 15-day anti-GLP serum was confirmed by means of IgM sensitivity to 2-ME. Anti-GLP serum pooled from rabbits 15 days after subcutaneous immunization was treated with 2-ME under conditions known to destroy IgM. The results (Table 2) were as expected from the previous observations: essentially all the IHA and BA activity, except the small amount attributable to IgG, and all the mouse passive-protection activity had been destroyed. This supports the conclusion that the IHA, BA, and mouse passive-protection activity observed in 15-day rabbit anti-GLP serum resides primarily in the IgM fraction.

DISCUSSION

The evidence relating the in vitro and in vivo biological activities of antipseudomonas serum has been inconclusive (14). In addition, the rel-

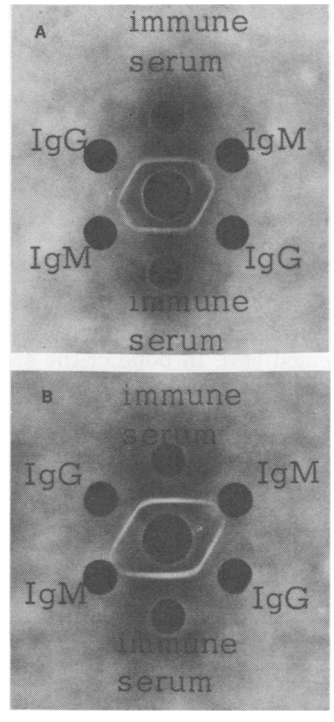

FIG. 4. *Analysis of rabbit anti-GLP serum Ig by immunodiffusion. The immune-serum well contains rabbit anti-GLP serum obtained 15 days after subcutaneous immunization; wells labeled IgG and IgM contain the purified fractions in saline; the center well contains (A) goat anti-rabbit globulin serum or (B) goat anti-rabbit IgG heavy-chain serum.*

TABLE 1. *Biological activities of rabbit anti-GLP serum and its IgG and IgM fractions*

Serum or Ig fraction	IHA titer[a]	Passive protection[b]	BA[c]
Anti-GLP serum[d]	1,280	60	16
Anti-GLP IgM[e]	1,280	60	16
Anti-GLP IgG[e]	20	0	2
Normal	10	0	2

[a] Reciprocal of the highest dilution hemagglutinating GLP-coated, formalinized sheep erythrocytes.

[b] Mice were passively immunized intraperitoneally 2 h before intraperitoneal challenge with a lethal inoculum of *P. aeruginosa* BI; protection is expressed as the percentage of surviving mice after 7 days.

[c] Reciprocal of the highest dilution agglutinating strain BI.

[d] Pooled serum from rabbits 15 days after subcutaneous immunization with GLP.

[e] Prepared from anti-GLP serum pool as described in the text.

TABLE 2. *Biological activities of rabbit anti-GLP serum treated with 2-ME*

Serum	IHA titer[a]	Passive protection[a]	BA[a]
Anti-GLP[b]	640	80	16
Anti-GLP + 2-ME[c]	20	0	2
Normal[b]	10	0	2

[a] See Table 1, footnotes *a* to *c*.

[b] Prepared as described in Table 1, footnote *d*, diluted 1:2 in PBS, incubated, and dialyzed as 2-ME-treated serum.

[c] Equal volumes of serum incubated with 0.2 M 2-ME in PBS and dialyzed as described in the text.

ative importance of the IgM and IgG fractions in protection against pseudomonas infection is still undecided (14). In none of the studies attempting such correlations of biological activity is a chemically analyzed and biologically defined antigen employed.

The extracellular GLP has been isolated from the slime of *P. aeruginosa* BI and has been purified and analyzed chemically. Furthermore, it has been shown to act as a toxin and a protective antigen (11), two biological activities of importance in experimental infection. However, the antibody response to this antigen has not yet been characterized. In the experiments reported in this paper, the primary antibody response of rabbits to GLP is characterized. Measured in vitro, the appearance of IHA and BA activity in antiserum followed very closely the appearance of the anti-leukopenic and mouse passive-protection activities measured in vivo. All activities reached a peak titer 15 to 20 days after immunization.

A close association of IHA and passive-protection activities was observed in vivo after intraperitoneal passive immunization with anti-GLP serum: passive protection increased at the same rate as did the IHA activity of the mouse plasma, accompanied by a decrease in the IHA activity of peritoneal washings. This observation was verified by passively immunizing mice intravenously: IHA and passive-protection activities declined simultaneously from initially high levels, while peritoneal washings remained inactive in either capacity. Based on these experiments, we conclude (i) that the passive-protection activity of anti-GLP serum depends on activity levels in the plasma, not in the peritoneum, and (ii) that a functional association exists between the IHA and passive-protection activities of anti-GLP serum.

Besides its intrinsic interest, the identification of the Ig class responsible for each biological activity has important applications in the augmentation of specific antibodies for treatment of pseudomonas infection. These considerations prompted an investigation of the relative contribution of IgM and IgG to the observed responses. Chromatographically distinct preparations of anti-GLP IgM and anti-GLP IgG were prepared from 15-day rabbit anti-GLP serum and their functional purity was demonstrated by immunodiffusion. Comparison of IHA, BA, and passive-protection activities located each principally in the IgM fraction of the original antiserum, whereas the IgG fraction contained only low levels of IHA and BA activity. This observation was confirmed by treating the anti-GLP serum with 2-ME, which, in appropriate concentration, destroys IgM without affecting IgG activity. The experimental results conformed to the expectations based on the previous experiments: the activities attributable to IgM were destroyed after treatment, whereas the low levels of IHA and BA activity with IgG remained unchanged. The identification of passive-protection activity with IgM may offer a partial explanation of the rapid decline of the activity from its peak values (Fig. 2 and 3): in addition to an expected dilution effect after distribution by the blood circulating system, a further factor in the decline may be the half-life of rabbit IgM in the mouse.

This report has not presented direct evidence linking the anti-leukopenic activity of anti-GLP serum with IgM. However, based on inferences from the close association of the anti-leukopenic and passive-protection activities, one may conclude tentatively that the anti-leukopenic activity also resides primarily in the IgM fraction.

Our results tend to support McCall's findings for IgM-deficient patients with pseudomonas infection (C. McCall, L. Bartlett, D. Qualliotine-

Mann, L. DeChatelet, and R. Cooper, Clin. Res. 21:607, 1973) that IgM may provide the decisive Ig component of a successful defense against pseudomonas infection of the lungs. However, the McCall study used only in vitro measures of opsonization to support its conclusions.

The results of Bjornson and Michael (4) are not readily reconciled to ours. Although they determined that in vitro activities, such as passive hemagglutination and opsonization of the bacterial particle, were much more efficiently performed by IgM, they found IgM to their mucopolysaccharide antigen afforded less protection against pseudomonas infection than IgG to the same antigen. An immediate explanation of the differences in observations may lie simply in the times at which the immune sera were collected: whereas our study deals with the primary immune response during the 30 days after a single injection of GLP, Bjornson and Michael have studied serum from rabbits immunized repeatedly over a period of several months.

It is worthwhile considering other possible reasons for our differences with Bjornson and Michael. The fact that we compared equal concentrations of Ig in vivo whereas they compared equivalent in vitro activity in vivo is not an adequate explanation of the observed differences, since essentially no IgM protection over the controls was observed when either the concentration of IgM was increased or the numbers of *P. aeruginosa* were decreased. One difference that may exert an unidentified effect is their use of mucin, which was not used in our experiments. The authors note that Olitzki (9) has reported an anticomplementary effect attributable to mucin. However, they demonstrate that mucin has no effect on phagocytosis of IgM-opsonized bacteria. Nevertheless, the effect of mucin on opsonization itself has not been determined and, therefore, cannot be discounted.

Perhaps the most likely explanation of the differences observed by Bjornson and Michael and us depends on the relative chemical composition of the antigens used in each study. The antigen isolated from the Bjornson and Michael strain of *P. aeruginosa* is a mucopolysaccharide composed of mannuronic and guluronic acids. The GLP antigen used in the experiments reported in this paper contains protein and lipid in addition to a carbohydrate moiety. Biologically active fragments have been prepared from the purified GLP; the lipid moiety was associated with leukopenia and lethality, and the carbohydrate moiety was associated with antigenic specificity and inhibition of phagocytosis (12). Possibly, after interaction of IgG with GLP, an exposed lipid portion of the GLP molecule remains toxic, whereas the larger IgM molecule, after interaction with GLP, reduces toxicity through masking or steric effects.

Differences such as these illustrate the deficiencies in our knowledge of the chemical determinants of pathogenesis in the pseudomonas infection. The importance of further investigation in this area for the development of a protective antigen that yet does not induce leukopenia can hardly be overstressed.

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