A STUDY OF THE ANTIBODY RESPONSE TO ANTIGENIC PREPARATIONS DERIVED FROM *PSEUDOMONAS AERUGINOSA*

by

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A THESIS SUBMITTED IN PARTIAL FULFILMENT OF

THE REQUIREMENT FOR THE DEGREE OF

MASTER OF SCIENCE

In the Department

of

Microbiology

We accept this thesis as conforming to the

required standard

THE UNIVERSITY OF BRITISH COLUMBIA

September, 1971

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Date Oct. 5, 1971

ABSTRACT

Several cellular and subcellular fractions were prepared from Pseudomonas aeruginosa strain PA-7. Those found to be immunogenic in rabbits included a heat-stable lipopolysaccharide, a protein-lipopolysaccharide complex, a cell wall preparation and a formalin-killed whole cell vaccine. However, a lipopolysaccharide preparation extracted with phenol and water was found to be a poor immunogen in rabbits. The cell wall fraction proved to be the most effective immunogen in terms of the amount of antibody evoked, and of the duration of the serum antibody response.

Hyperimmune sera produced against all four antigens were found to contain a mixed population of 2-mercaptoethanol sensitive and 2-mercaptoethanol resistant antibodies. Gel filtration and ion exchange chromatography studies established the presence of both IgM and IgG immunoglobulins in all four types of hyperimmune serum. Whole immune serum, as well as the IgM and IgG serum fractions, afforded passive protection to mice challenged with twenty or more LD_{50} of viable organisms. There was an indication that the IgG fraction of two of the four serum types provided better protection than did the IgM fraction, but precipitation studies indicated that this may have been due to greater numbers of IgG immunoglobulins. In addition, serum containing a high proportion of 2-mercaptoethanol resistant antibody was found to promote faster clearance of injected bacteria

than did serum taken earlier in the response.

Immunodiffusion studies indicated that all four antigenic preparations contained at least one common immunogen; moreover, all serum types were able to react with sheep red blood cells coated with the heat-stable lipopolysaccharide preparation in passive hemagglutination and hemolysin tests.

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ACKNOWLEDGEMENTS

I would like to thank Dr. D. Syeklocha, my research supervisor, for her help and criticism during the course of my research, and during the preparation of this thesis, and Dr. J.J.R. Campbell for his expressed interest in my research project.

I would also like to thank Mrs. Teresa Walters for the electron microscopy, and Mr. Bill Page for the drawing of the figures in this manuscript.

Lastly, I should like to express my thanks to the faculty, staff and students of this department for their interest and encouragement throughout the course of this study.

INTRODUCTION

The characteristics of the immune response to various bacteria has received a great deal of attention in recent years. A humoral antibody response, involving both IgM and IgG immunoglobulins, has been demonstrated for a large number of pathogenic bacteria (Pike and Schulze, 1964; Yoshida and Ekstedt, 1968; Smith, J.W., et al. 1970). Both active and passive immunization studies have shown that the immune serum is able to protect the host animal against infection by the homologous microorganism; one or the other of the two major immunoglobulin classes has been demonstrated to be more effective in this regard, depending upon the bacterium involved (Yoshida and Ekstedt, 1968; Dolby and Dolby, 1969). Much of the recent work has been directed toward the development of protective antigenic fractions from cell extracts of various bacteria (Alms and Bass, 1967; McGhee and Freeman, 1970).

Studies by Bass and McCoy (1971) and by Schwarzmann and Boring (1971) have shown that a fraction isolated from the slime layer of Pseudomonas aeruginosa induces the formation of antibodies which are protective against infection by Pseudomonas aeruginosa. The object of this investigation was to examine several crude fractions prepared from a strain of Pseudomonas aeruginosa with regard to their ability to stimulate the production of protective antiserum. The classes of immunoglobulin elicited, their activities in several in vitro tests,

the duration of the response, and the relative capacities of the different fractions to evoke antisera capable of protecting mice against challenge infections by homologous and heterologous strains of Pseudomonas aeruginosa were also investigated.

LITERATURE REVIEW

I. *Pseudomonas aeruginosa* as a Human Pathogen

In recent years, *Pseudomonas aeruginosa* strains have approached or replaced those of *Staphylococcus aureus* in frequency of occurrence as the causative agents of human infection in the hospital environment (Huang, *et al.* 1961; Farmer and Herman, 1969). Patients debilitated by disease processes, burns or surgery, and individuals on antibiotic therapy all seem particularly prone to infection by this organism (Kefalides, et al. 1964). Victims of cystic fibrosis have been found to be particularly susceptible to chronic infections of the respiratory tract by mucoid variants of common serotypes of *Pseudomonas aeruginosa* (Doggett, 1969; Diaz *et al.* 1970). Eye infections caused by *Pseudomonas aeruginosa* strains are not uncommon, nor are urinary tract infections involving this organism (Ayliffe, *et al.* 1966; Klyhn and Gorill, 1967).

Numerous sources of *Pseudomonas aeruginosa* have been found in hospital surroundings. The organism's ability to exist in moist environments is well documented; for example, *Pseudomonas aeruginosa* has been cultured from soap dishes, hand cream, respirators, sinks, floors and mops (Wahba, 1965: Wormald, 1970). In this context, Emmanouilidou-Arseni and Kommentaleou (1964) showed that numerous strains of *Pseudomonas aeruginosa* were able to survive in distilled water held at several different temperatures for at least three

hundred days. Findings such as these point to the great difficulties still being encountered in attempting to suppress infections in hospitals. Similarly, the discovery that Pseudomonas aeruginosa is part of the intestinal flora of a small percentage of the population has disclosed another potential source of infection, at least in the hospital environment (Wahba, 1965).

The emergence during the past thirty years of Pseudomonas aeruginosa as an increasingly frequent human pathogen is no doubt due in part to the concomitant increase in antibiotic therapy (Huang et al. 1961; Finland, 1970). Strains of this bacterium not only resist the inhibitory effects of most antibiotics, but also are unaffected by many antiseptics in present use (Emmanouilidou-Arseni and Kommentaleou, 1964; Adair et al. 1971). In vitro studies have shown that of the clinically available antibacterial agents, only polymyxin B, coliston, gentamycin and carbenicillin are effective in inhibiting the growth of Pseudomonas aeruginosa (Hedberg and Miller, 1969; Lindberg et al. 1970); although these antibiotics are used clinically to combat Pseudomonas infections, reservations have been placed upon their efficacy, due to solubility and toxicity problems, and to the development of strains which are resistant to these drugs (Smith, C.B. et al. 1970). An excellent summary of the difficulties to be encountered in antibiotic therapy of Pseudomonas aeruginosa infections can be found in reports by Smith, C.B. et al. (1970), Lindberg et al. (1970) and others in the Symposium on Carbenicillin (Kirby, 1970).

II. Nature of the Infection

The exact means by which Pseudomonas aeruginosa exerts its pathogenicity is not completely understood at present. Under certain conditions, the organism is known to be invasive; moreover, pulmonary involvement and urinary tract infections encountered subsequent to primary invasions in other parts of the body, indicate that Pseudomonas infections often become systemic (Jones, 1970; Jordan et al. 1970; Smith, C.B. et al. 1970). Support for these findings comes from animal experiments which demonstrated that the deaths of mice which had received an intravenous injection of live cells was due to renal failure caused by lodgement and multiplication of the bacteria in the kidneys (Klyhn and Gorill, 1967). However, as yet, insufficient evidence has been presented to attribute the pathological effects of Pseudomonas aeruginosa solely to invasiveness, without any consideration of the possible roles of the various cellular and extracellular factors produced by this organism.

Several extracellular enzymes are known to be produced by strains of Pseudomonas aeruginosa which may aid the organism in its establishment and subsequent growth in the infected site. These include a hemolysin, a lecithinase, one or more proteases, and several others (Berk, 1964; Carney and Jones, 1968). Several of these proteins have been partially purified and shown to be lethal for experimental animals (Berk, 1964; Johnson et al. 1967). Meinke and coworkers (1970) described localized hemmorhage and necrosis upon

autopsy of mice injected with varying concentrations of a partially purified protease with elastolytic activity. Relatively large doses of an enzyme containing fractions obtained from culture supernatant produced an eye exudate, hemmorhage and inflammation of the intestine when injected into mice (Carney and Jones, 1968). Although not all of the enzymes are necessarily produced by any one strain, qualitative and quantitative differences in enzyme production by strains of this organism may influence their pathogenicity (Liu, 1964; Carney and Jones, 1968). A further extracellular toxin has also been implicated which is believed to be separate from these enzymes, and which is also lethal for mice (Liu and Hsieh, 1969). The role of this fraction in the pathological process has yet to be evaluated.

The extracellular slime or capsular material has been shown to be toxic for mice (Liu et al. 1961; Cetin et al. 1965), especially after mild acid hydrolysis, or deoxyribonuclease degradation (Callahan et al. 1964). However, recent evidence has shown that slime exerted no effect on the viability of leukocytes in vitro at least (Schwarzmann and Boring, 1971).

The endotoxin moiety of Pseudomonas aeruginosa has been shown to be somewhat similar in chemical composition to those of the Enterobacteriaceae (Michaels and Eagon, 1969; Fensom and Gray, 1969). However, conflicting reports have been published concerning its importance in the overall infectious process (Liu et al. 1961; Klyhn and Gorill, 1967). The protein portion of the molecule is

capable of inducing a Schwartzman reaction, and a pyrogenic response; on the other hand, the intact complex appears to be relatively non-toxic, since large numbers of whole cells have been injected into mice with relatively little effect on the host (Braun and Elrod, 1941; Liu et al. 1961; Laborde and de Fajardo, 1965). An important consideration in the evaluation of the role of the endotoxin and of the other extracellular products in the disease process is the number of bacterial cells actually achieved in the infected animal. As noted by Roantree (1967) in the case of Salmonella infections, only if the host's defences are overcome sufficiently to allow the presence of a large number of bacteria within the body can one expect to achieve the concentrations of endotoxin, slime, or enzymes which have been shown necessary to produce biological effects.

III. Host Defences Against Pseudomonas aeruginosa

Bacterial infections are prevented in various ways by the human body. External and internal surfaces are protected by the fatty acid content of the skin, the lysozyme in tears, saliva, and acidic secretions, all of which are mildly bactericidal. The resident flora of the body also aids in suppressing the multiplication of potential pathogens. If organisms do penetrate these defences, and begin to multiply within the tissues, the body's inflammatory response may prevent further spread of the invading bacteria. Normal human serum is bactericidal to some bacterial species, including Pseudomonas

aeruginosa; in the latter case, this antibacterial activity has been shown to be enhanced at temperatures slightly above 37 C (Muschel et al. 1969). In addition, complement factors of the serum aid in the phagocytosis of many bacterial species.

Pseudomonas aeruginosa is known to stimulate antibody formation in humans and experimental animals. Normal human serum may contain agglutinins to the organism (Gaines and Landy, 1955); individuals who have survived an acute Pseudomonas infection, as well as those chronically infected by the organism, have been shown to possess elevated antibody titers (Gaines and Landy, 1955; Diaz et al. 1970; Young et al. 1970). This response may persist in humans for some time after recovery from infection (Young et al. 1970), although the antibody response to Pseudomonas aeruginosa antigens injected into experimental animals appears to be quite transitory (Laborde and de Fajardo, 1965; Bass and McCoy, 1971).

The importance of phagocytosis in determining the outcome of bacterial infections has been the object of a considerable study in recent years (Cohn and Hirsch, 1965; Rowley et al. 1968). Resistance to ingestion and destruction by phagocytic cells of the reticuloendothelial system seems to be related to the virulence of the bacterial strain for the animal host (Wells and Hsu, 1970; Nakamura et al. 1970; Yee and Buffenmeyer, 1970). Surface antigenic composition, as well as lytic substances which may be produced by the microorganisms, can enable bacterial cells to resist

phagocytosis and/or post-phagocytic destruction (Cohn and Hirsch, 1965; Roantree, 1967; Medearis et al. 1968). In vitro studies have shown that rabbit peritoneal macrophages phagocytose and kill Pseudomonas aeruginosa cells, although the slime produced by mucoid variants of Pseudomonas strains was found to inhibit ingestion of the bacterial cells by the leukocytes (Schwarzmann and Boring, 1971).

Phagocytic efficiency may be increased in the presence of serum antibodies specific for the microorganism. Salmonella typhimurium cells have been found to be more susceptible to phagocytosis in vitro in the presence of immune serum (Wells and Hsu, 1970); increased phagocytosis of Escherichia coli cells in previously immunized animals has also been reported (Benecerraf et al. 1959; Whitby and Rowley, 1959). Immune serum was found to counteract the antiphagocytic effect of the slime of mucoid Pseudomonas aeruginosa variants (Schwarzmann and Boring, 1971). Thus the correlation between detectable serum antibodies and protection from infection which has been observed for several bacterial species, including Pseudomonas aeruginosa (Jones and Lowbury, 1965; Roantree, 1967; Rowley et al. 1968), is in all likelihood a reflection of the increased efficiency of phagocytosis and intracellular destruction of the organism in the presence of specific antiserum.

The liver and spleen appear to be the principal organs of sequestration and elimination of bacterial cells injected intraperitoneally or intravenously as well as in natural infections which have become systemic (Benecerraf et al. 1959). Which of the two is most active

in phagocytosing the invading microorganisms seems to be determined by the amount and class of specific antibody present (Benecerraf, et al. 1959; Schulkind and Rabins, 1971). The liver appears to sequester the majority of the injected organisms in immune animals; in contrast, enhanced uptake of the organisms by the spleen is seen in non-immune animals, or those in the early stages of the immune response (Schulkind and Rabins, 1971).

IV. Therapeutic Measures

Because of the obstacles which have been encountered in attempts to combat Pseudomonas aeruginosa infections with antibiotics, much emphasis has been placed upon the development of some form of immunotherapy to supplement or replace the use of antibacterial drugs. Both active and passive immunization procedures have been considered as possible approaches. The preparation of a polyvalent vaccine or of an immunogen with a protective antigenic determinant common to all of the frequently encountered serotypes has thus far met with little success, a fact which tends to argue against the development of an active immunization program (de Fajardo and Laborde, 1968; Fisher, et al. 1969). On the other hand, antiserum specifically prepared against the infecting serotype or serotypes (Lindberg et al. 1970) could be passively administered to help counteract already established infections. A potential hazard in this type of treatment, of course, is the possibility of eliciting allergic reactions to the foreign serum or serum proteins administered.

Whole cells, viable or killed by various methods, cell extracts, culture filtrates and the slime layer of Pseudomonas aeruginosa have all been shown to be immunogenic in experimental animals. Liu and his coworkers (1961) found that the slime layer and whole cells evoked antibodies capable of protecting mice against lethal numbers of live cells. Alexander and coworkers (1966) and Alms and Bass (1967) have shown that antiserum specific for an ethanol-precipitable material in the slime layer is capable of protecting mice against an experimental Pseudomonas aeruginosa infection. Bass and McCoy (1971) recently showed that killed cells or the alcohol-precipitated fraction from the slime layer elicit cross-protection against infection by various strains which correlates well with the heat-stable "O" serotypes as defined by Verder and Evans (1961). This finding is in agreement with an earlier proposal that protection obtained by using whole cells as the immunogen is directed against a part of the slime layer which cannot be removed from the cells (Liu et al. 1961), but is in contrast to the results obtained by Fisher and coworkers (1969), who found little correlation between protective activity of an antiserum and its "O" agglutinin content. Jones (1968) has also found that the antiserum directed against an enzyme containing fraction from culture filtrates protects burned mice against colonization by lethal numbers of Pseudomonas aeruginosa cells; in this case, there was also some cross-protection when strains of different serotypes were used to infect the serum-protected mice.

Whole cells of some Gram-negative organisms, as well as their somatic antigens, have long been known to stimulate humoral antibody production. The response to a single injection of organisms or of their "O" antigen has been found to consist mainly of antibodies of the IgM class (Pike and Schulze, 1964; Landy et al. 1965; Fukazawa et al. 1970); IgG molecules appear in appreciable quantities only late in the immune response, if at all. "O" antigens from different strains of Proteus mirabilis, however, have been found to evoke either an IgM or an IgG response after a single dose (Smith, J.W. et al. 1970). Hyperimmunization with whole cells or somatic antigen preparations, on the other hand, elicits an IgM response, but also leads to earlier formation of, and, larger quantities of IgG immunoglobulin (Pike and Schulze, 1964; Landy et al. 1965; Bjornson and Michael, 1970).

The relative abilities of the two major immunoglobulin fractions to prevent infection by bacteria have been compared by a number of investigators. Yoshida and Ekstedt (1968) found that the protective activity of hyperimmune serum against infection by Staphylococcus aureus was associated with the IgM fraction. Dolby and Dolby (1969), however, demonstrated that IgG was more effective in protecting mice against a lethal intracerebral challenge of Bordetella pertussis than was the IgM fraction of the serum. Antibodies isolated early in the immune response to Salmonella typhimurium increased sequestration of the injected organisms by the spleen; "late" antibodies seemed to aid

the liver in taking up the organisms (Schulkind and Rabins, 1971). However, the protective abilities of these sera against an experimental Salmonella infection was not reported. Hyperimmunization of rabbits with the protective antigen isolated from the slime of Pseudomonas aeruginosa evoked a mixed IgM and IgG response; IgG antibodies were found to be more effective, on a weight basis, than IgM molecules in the protection of mice against a lethal intraperitoneal challenge of live organisms (Bjornson and Michael, 1970). No reports concerning the types of immunoglobulins evoked by immunization with Pseudomonas aeruginosa cells or somatic antigen are known.

In conclusion, then, there are a number of factors to consider in attempting to develop an antigen which could be used immunotherapeutically to combat Pseudomonas aeruginosa infections. The amount of antigen given in a single injection has been shown, in studies with Serratia marcescens, to be important in determining the duration of the antibody response (Field et al. 1970). The type of antigenic preparation administered is known to affect not only the length of the response, but also the class of immunoglobulin evoked (Laborde and de Fajardo, 1965; Smith, J.W. et al. 1970). Bass and McCoy (1971) have found that booster injections of Pseudomonas aeruginosa antigens fail to produce antisera of higher titer than that achieved in the original response, but do maintain the antibody level for a longer period of time. Sublethal doses of fully virulent live Pseudomonas aeruginosa cells have been shown to evoke a prolonged

antibody response (Laborde and de Fajardo, 1965; Young et al. 1970), but the potential toxicity of this type of vaccine renders it unsuitable for clinical use. Perhaps immunization with viable cells of relatively avirulent mutant strains such as the rough Salmonella typhimurium strains used in studies by Germanier (1970), might also induce a relatively long period of antibody production.

The type of immunoglobulin produced in response to a given antigen, as well as the amount of cross-reactivity of the protection induced, are also important considerations in the development of a suitable vaccine. Ideally, one would hope to find an immunogenic fraction which is relatively non-toxic, stimulates the production of high levels of protective antibody, and which exhibits at least some cross-specificity in the protection conferred.

MATERIALS AND METHODS

1. Organisms and Media

The organism used in this study was a strain of *Pseudomonas aeruginosa* designated PA-7. This strain was obtained from Dr. P.V. Liu, of the Department of Microbiology, University of Louisville, School of Medicine, Louisville 2, Kentucky, in 1968. The original stock culture was maintained in the lyophilized state; once a year, a fresh vial was reconstituted to be used as the working stock. This culture was kept at 4 C on trypticase soy (BBL) or brain heart infusion (Difco) agar slants and was transferred to a fresh slant every six to eight weeks. At intervals of approximately three months, this working stock was serially subcultured onto human blood agar plates to maintain its virulence for mice, and its slime producing capacity (personal observation).

Pseudomonas aeruginosa strains PA-1 and PA-479 were also used in some experiments. PA-1 was obtained from Dr. P.V. Liu and PA-479 was obtained as a clinical isolate at the Department of Microbiology, University of British Columbia.

For the production of the antigenic preparations, PA-7 was grown in trypticase soy broth (TSB) on a shaking water bath (Metabolyte Water Bath Shaker, New Brunswick Scientific Co., Inc., New Brunswick, N.J.) at 37 C for 12-14 hours.

II. Preparation of Antigens

1. Formalin-killed whole cell vaccine. Cells were washed three times in sterile saline, and resuspended in saline. The optical density at 660 nm was measured with a Beckman Spectronic 20 (Beckman Instruments, Inc., Fullerton, Calif.). The concentration of cells was then adjusted to 10 O.D. and formalin was added to a concentration of 0.3 percent. The vaccine was left at room temperature for at least 24 hours, after which time sterility tests were carried out. The cells were then sedimented by centrifugation, resuspended in sterile saline, and stored at 4 C until needed. This preparation will be referred to hereafter as Form Vacc.

2. Heat-stable lipopolysaccharide. The method used for the extraction of this fraction was one modified from that of Suzuki and his coworkers (1964). Broth-grown cells were washed several times in sterile saline, then resuspended in distilled water to a final concentration of 0.5 gm wet weight of cells per ml. This suspension was heated at 100 C for 90 minutes. After cooling, the cellular debris was removed by centrifugation, at 10,000 x g for 20 minutes. The volume of the supernatant was measured, and solid NaCl was added to obtain a concentration of 0.1 M. Five volumes of 95 percent ethanol were added slowly with stirring, and the resulting suspension was left at room temperature for 30 minutes. The precipitate was collected by centrifugation at 15,000 x g for 20 minutes, redissolved in 0.1 m NaCl, and reprecipitated twice more

with ethanol. The final precipitate obtained was dissolved in 0.1 M phosphate buffer, pH 7.0, containing 20 µg each of deoxyribonuclease (Worthington Biochemical Corp., Freehold, N.J.) and ribonuclease (Calbiochem, Los Angeles, Calif.); and 2 µg of $MgCl_2$ per ml. This mixture was incubated at 37 C for one hour, then centrifuged at 25,000 x g for 90 minutes. The pellet was resuspended in distilled water, washed once with distilled water, and stored in the lyophilized state in a desiccator at room temperature. This material was designated later in the text as H-S LPS.

3. Extraction of lipopolysaccharide with the phenol-water. The method for the extraction of this fraction was taken from Nowotny, 1969. TSB grown cells were washed several times in saline, then resuspended in distilled water. The cell suspension was heated to 70 C, and an equal volume of warm phenol (70 C) was added. This mixture was maintained at 70 C with stirring for 10 minutes, then cooled and centrifuged at 3,000 x g for 30 minutes. The two phases of the supernatant were separated, and the phenol phase was extracted with distilled water twice more, as described above. The three water phases were combined, and the volume was reduced by flash evaporation. Exhaustive dialysis of the remaining material against distilled water removed the remaining traces of phenol. Three volumes of cold methanol containing 0.2 percent $MgCl_2$ were added to the non-dialysable material. The resulting precipitate was collected by centrifugation at 3,000 x g for 30

minutes, redissolved in distilled water, and reprecipitated twice with cold methanol without $MgCl_2$. Repeated flash evaporations removed the methanol. The material was lyophilized, reconstituted in 0.1 M phosphate buffer, and incubated with the nucleases as described above for the heat-stable lipopolysaccharide fraction. The lipopolysaccharide material was collected by centrifugation at 25,000 x g for 90 minutes; the pellet was resuspended and washed once in distilled water, and lyophilized for storage. This preparation is designated later as P-WLPS.

4. Cell walls. The method followed was essentially that of Bobo and Eagon, 1968. Broth-grown cells were washed several times in saline, resuspended in 0.1 M phosphate buffer, pH 5.5, and passed twice through a precooled French pressure cell (Aminco, Silver Springs, Md.). The resulting suspension was centrifuged at 3,000 x g for 10 minutes, and the pellet was discarded. The supernatant was centrifuged at 15,000 x g for 20 minutes. This pellet was suspended in phosphate buffer, pH 7.0, with 20 µg of deoxyribonuclease and ribonuclease, and 2 µg of $MgCl_2$ per ml. After 1-2 hours incubation at 37 C, the cell walls were pelleted by centrifugation at 15,000 x g for 20 minutes, washed twice in pH 7.0 phosphate buffer, twice in distilled water, and lyophilized for storage.

5. Protein-lipopolysaccharide complex. The method described by Rogers et al. 1969, was followed for the isolation of this fraction. Cell walls isolated as previously described were

suspended to a concentration of 1.5 mg per ml in 0.033 M Tris-HCl buffer, pH 8.0, containing 1 µmole of ethylenediaminetetraacetate (EDTA) per ml. The suspension was incubated at 25 C with stirring for 30 minutes, then centrifuged at 37,000 x g for 60 minutes at 0 C. The supernatant was passed through a 0.45 filter (Millipore Corp., Bedford, Mass.), concentrated by lyophilization, dialyzed for 96 hours at 4 C against several changes of 0.033 M Tris HCl buffer, pH 8.0, and relyophilized for storage. This preparation is later called Pr-LPS.

III. Experimental Animals

All animals used were obtained from the Animal Unit, Faculty of Medicine, University of British Columbia, Vancouver, B.C. White, female, Swiss-bred mice weighing between 20 and 25 gm were used in all experiments. White, female rabbits used for immunization with the Pseudomonas fractions weighed 2-3 kg at the start of the immunization schedule.

IV. Immunization Procedures

Rabbit immunization schedules are shown in Table I. Two routes of injection were used. For intravenous (i.v.) injections, the antigens were diluted to the appropriate concentration in pyrogen-free saline (Baxter Laboratories of Canada, Ltd., Alliston, Ont.) and injected via the marginal ear vein. For subcutaneous (s.c.) injections,

Table I. Rabbit Immunization Schedule for the Various Antigenic Preparations from *Pseudomonas aeruginosa* Strain PA-7.

Day of Injection	Dose[a]				
	H-S LPS	P-W LPS	Pr-LPS	Cell Walls	Form. Vacc.
0	10	10	10	10	5×10^8
4	10	10	-	-	1×10^9
5	-	-	10	10	-
8	20	20	-	-	2×10^9
10	-	-	20	20	-
12	20	20	-	-	2×10^9
15	-	-	20	20	-
16	50	50	-	-	-
20	50	50	50	50	-
24	100	100	-	-	-
25	-	-	50	50	-
30	-	-	100	100	-
44	-	2×10^3[b]	-	-	-
57	50	-	-	-	-
63	-	-	-	-	2×10^9
79	-	-	-	50	-
96	-	-	2×10^3[b]	-	-

[a]- Antigen dose, in μg, except in the case of the formalin-killed vaccine, where the dose refers to the number of cells injected.

[b]- Subcutaneous injection of the antigen in Freund's complete adjuvant. All other injections were intravenous.

Abbreviations: H-SLPS, heat-stable lipopolysaccharide; P-WLPS, phenol-water lipopolysaccharide; Pr-LPS, protein-lipopolysaccharide; Form. Vacc, formalin-killed vaccine.

the saline solution was mixed with an equal volume of complete Freund's adjuvant (Difco) and 0.2 ml of the emulsion were injected into each of five sites on the shaved area of the animal's back.

V. Antisera

1. Collection and Storage

Rabbits were bled from the marginal ear vein or by cardiac puncture. Blood samples were stored overnight at 4 C to allow for clot retraction. The sera were separated, cleared of any residual red blood cells by low speed centrifugation, then heated to 56 C for 30 minutes to inactivate the complement. Sera were stored in small volumes at -20 C.

2. Determination of Antibody Titer

All sera were tested routinely for antibody activity by the passive hemagglutination and hemolysin tests; many were also tested for their bacterial agglutinin content.

Passive hemagglutination and hemolysin tests were carried out using the Microtiter dilution technique (Cooke Engineering Co., Alexandria, Va.). 25 µl samples of serum were serially diluted with 25 µl of saline in the dilution plates. 25 µl of a 2.5 percent suspension of lipopolysaccharide-coated sheep red blood cells were then added to each well in the passive hemagglutination assay; for passive hemolysis, 25 µl of a 1/30 dilution of guinea pig complement were added to each antiserum dilution, and the plate was incubated at 37 C for 30 minutes before the coated sheep red blood cells were

added. Plates were incubated at 37 C for 2 hours, and at 4 C for 18 hours before the results were read. The highest antiserum dilution showing at least 50 percent hemagglutination or hemolysis of the added red blood cells upon visual inspection was taken as the end point of the titration. Appropriate saline and normal red blood cell controls were included in all assays; normal rabbit serum controls were occasionally included.

Washed sheep red blood cells were coated with alkali-modified heat-stable lipopolysaccharide according to the method of Neter and his coworkers (1956). Lipopolysaccharide was dissolved in saline to a concentration of 500-1,000 µg per ml. The pH was adjusted to 8.5-9.0 with 0.2 N NaOH. After heating the solution at 56 C for 30 minutes, the pH was readjusted to 7.2 with 0.2 N HCl. An equal volume of 5 percent sheep red blood cells was added, and the mixture incubated at 37 C for 30 minutes with occasional shaking. The red cells were then collected by low speed centrifugation, washed twice in saline, and resuspended to a concentration of 2.5 percent in saline.

Formalin-killed vaccine was used as the antigen in bacterial agglutinin titrations. Serial two-fold dilutions in saline were made of the serum under test. The vaccine was diluted with saline to equal in opacity the third tube of the Brown opacity tube series (Burroughs, Wellcome and Co., London, Eng.); plate counts demonstrated that this opacity is equal to 2-3 x 10^9 PA-7 cells per ml. A volume of this antigen equal to that of the antiserum dilutions was added

to each tube, and to a saline control tube. The highest antiserum dilution in which visible agglutination was detectable was taken as the bacterial agglutinin titer.

3. Fractionation of Antisera

a) Dissociation of Macroglobulin with 2-Mercaptoethanol

The procedure for this step was taken from Nowotny (1969). Equal volumes of the serum and of 0.2 M 2-mercaptoethanol (in saline) were mixed and allowed to stand at room temperature for one hour. This mixture was then used in the place of serum in the passive hemagglutination and hemolysin and bacterial agglutination tests as described above.

b) Gel Filtration

Whole rabbit serum was applied in 2-3 ml samples at 4 C to a 2.5 x 45 cm Sephadex G-200 column (Pharmacia, Uppsala, Sweden). Flow was in the upward direction, at a rate of 0.15 ml per minute. The buffer was 0.05 M phosphate buffer, pH 7.3, containing 2.2% NaCl and 0.2% sodium azide. Fractions of 1.5 ml were collected and assayed for protein content by measuring the optical density at 280 nm with a Beckman DBG (Beckman Instruments Inc., Fullerton, Calif.).

In order to achieve a better separation of IgG and IgM immunoglobulins, further fractionation of the antibody-containing protein peaks were attempted. The leading front of the first peak eluted (see Figure 8) was concentrated to a volume of 2-3 ml by ultrafiltration (Amicon Ultrafiltration Cell, Amicaon Corp., Lexington,Mass.),

and refractionated on Sephadex G-200 under the conditions described above. The leading front of the protein peak thus obtained (Figure 9) was again concentrated by ultrafiltration to 2-3 ml. Ring tests and gel diffusion tests were performed on this fraction with goat anti-rabbit-γ-globulin (Meloy Laboratories, Springfield, Va.) to check for the presence of any contaminating IgG antibodies.

c) Ion exchange Chromatography

The method used was taken from the procedure of Nowotny (1969). Whole rabbit serum which had been dialyzed against 0.005 M sodium phosphate buffer for 48 hours at 4 C, was applied in 5 ml samples to a 2.5 x 45 diethylaminoethyl (DEAE)-cellulose column, also at 4 C. The serum was eluted with an 800 ml continuous gradient, in which the starting buffer was 0.005 M sodium phosphate buffer, pH 7.0 and the final buffer was 0.05 M NaH_2PO_4 in 0.05 M NaCl. The flow rate was one ml per minute. Fractions of 5 ml were collected and assayed for protein content by measuring the optical density at 280 nm with a Beckman DBG spectrophotometer. Protein peaks were pooled, each was concentrated to the original serum volume by ultrafiltration, and was tested for antibody content by the passive hemagglutination test.

VI. Passive Protection Studies

The method adopted for these studies was modified from those used by Bass and McCoy (1971) and Jones and his coworkers (1971). Mice

that had received an intraperitoneal injection of 0.2 ml of whole or fractionated rabbit serum, or of saline, were challenged by the same route 4 hours later with various numbers of viable bacteria suspended in 10 percent TSB in saline. The serum fractions were dialysed against saline before use, and all serum dilutions were made in pyrogen-free saline.. Groups of 10 mice were used for each dilution tested.

The challenge organisms were grown in TSB on a shaking water bath at 37 C for 6 hours, sedimented by centrifugation, and resuspended to the original volume in 10 percent TSB-saline. All further dilutions of the organisms were also made in 10 percent TSB-saline. A plate count was performed on the washed bacterial suspension in all experiments to determine the number of organisms injected.

Mortality was recorded in most experiments only for the 72 hour period following injection of the challenge bacteria; in some cases, however, deaths were recorded for periods of up to 2 weeks post-challenge.

VII. Clearance Studies

To determine the effect of the rabbit antiserum on the persistence of the injected organisms in passively protected mice, those animals which had survived the 72 hour test period were sacrificed at various intervals thereafter, and samples of their heart blood and peritoneal washings were plated on trypticase soy agar to check

for the presence of viable organisms. The liver and spleen of each animal were also removed aseptically, cut into small pieces, and spread on trypticase soy agar plates. The plates were incubated at 37 C for 24 hours, after which time they were inspected for typical colonial morphology. Representative colonies were tested by slide agglutination with rabbit anti-*Pseudomonas* serum to ensure that the organisms isolated were PA-7.

To confirm the validity of the assay method used, in some experiments samples taken from the mice were divided; one half was treated as described above, and the other half was added to TSB in tubes, and incubated at 37 C for at least 48 hours. Growth in these tubes was plated, and the resulting colonies were tested as above.

VIII. *In Vitro* tests

1. Bactericidal Assay

The method for this assay was developed from one described by Bjornson and Michael (1970). Cells were grown in TSB on a shaking water bath at 37 C for 6 hours, sedimented by centrifugation, washed once in sterile saline, and resuspended in saline to the original volume of the culture. The concentration of cells per ml of this suspension was determined by a surface plate count.

The cell suspension was diluted by a factor of 10^{-6}, and various volumes of the diluted material were added to mixtures of whole or fractionated antiserum and guinea pig complement (Hyland). Normal

rabbit serum and saline controls were also included. The mixtures were incubated at 37 C for one hour, then 0.1 ml volumes were plated in duplicate for counting.

2. Immunodiffusion Tests

Double diffusion plates were prepared according to the method of Campbell et al. (1964). The plates contained 0.85 percent Ionager No. 2 (Oxoid) in borate-saline, pH 8; 0.1 percent Merthiolate (Eli Lilly and Co., Indianapolis) was added to the borate-saline solution, but the trypan blue stain was omitted. Antigen preparations and antisera were diluted in borate-saline, and 0.1 ml volumes were added to each well.

The plates were incubated in a moist chamber at 37 C for at least one week, and were observed daily for the development of precipitation lines. When the development of the lines was complete, the plates were rinsed with several changes of saline to remove unreacted protein and antigen, sealed and stored at 4 C until photographed.

3. Quantitative Precipitation Test

The amount of antibody in each type of purified fraction was quantitatively determined by the precipitation test. Equal volumes of undiluted serum fractions from serum prepared against the cell wall fraction, and of saline dilutions of heat-stable lipopolysaccharide were mixed, and incubated at 37 C for 1-2 hours. The tubes were then refrigerated for 48-72 hours; after this time, the tubes were centrifuged in the cold at 2500 rpm, and the

supernatant carefully removed and tested for antigen or antibody excess by ring tests. The precipitated material was washed twice in cold saline to remove any unreacted protein, and dissolved in 0.1 N NaOH to the original volume of the mixture. The protein content of these solutions was determined by the method of Lowry and coworkers (1951).

IX. Electron Microscopy

Cell wall suspensions which had been treated with deoxyribonuclease and ribonuclease as described above were suspended in distilled water and frozen until needed for electron microscopic studies. In addition, samples of the cell wall preparation were subjected to further enzymatic treatments for comparative purposes. One set of cell walls was incubated at 37 C for 3 hours in 0.05 M sodium phosphate buffer, pH 8.0, with 500 µg/ml of trypsin (Calbiochem, Los Angeles, Calif.), sedimented by centrifugation, washed several times in distilled water, and stored as an aqueous suspension at -20 C. The other sample was suspended in 0.1 M sodium acetate buffer to which 500 µg/ml of lipase (Miles Laboratories, Inc., Elkhart, Ind.) had been added, and was incubated at 37 C for 3 hours. These cell walls were then washed as described above, and frozen.

Cell wall preparations were fixed and stained by several different methods. One set was fixed in 2.3 percent glutaraldehyde in phosphate buffer for 30 minutes, then in 1 percent OsO_4 in phosphate

buffer for 15 minutes, and lastly in uranyl acetate in sucrose-acetate buffer for 30 minutes (Pease, 1964; Silva et al. 1968). Another sample was fixed in OsO_4, and post-fixed in uranyl acetate (Silva et al. 1968). A third set was fixed in glutaraldehyde, and post-fixed in OsO_4 (Pease, 1964). Samples were dehydrated in ethanol and propylene oxide, and embedded in Epon.

Sections were stained with a saturated alcohol solution of uranyl acetate for one minute, rinsed with distilled water, and stained for 2 minutes with lead citrate (Fahmey, 1967). After drying, the preparations were examined in a Phillips 300 electron microscope at 60 KV.

RESULTS AND DISCUSSION

The purpose of this study was to prepared an antigenic fraction from PA-7 which would be capable of stimulating the production of protective antiserum in experimental animals. Although other workers have been successful, by the injection of whole cell vaccines, in stimulating an agglutinin response (Laborde and de Fajardo, 1965) and protective serum (Jones et al. 1971) in mice, we failed to evoke markedly elevated passive hemagglutinin levels in these animals. The rabbit, however, was found to be more suitable for the production of antiserum containing high levels of passive hemagglutinating antibodies.

I. Antibody Response to the Antigenic Preparations

The passive hemagglutinin and hemolysin responses to each of the preparations injected into rabbits are shown in Figures 1 - 5. All of the preparations tested were found to be immunogenic with the type of injection schedule used (see Table I of the Materials and Methods) and no toxic effects were noted in the rabbits at any time.

Humoral antibody levels elicited by all of the preparations, with the exception of the phenol-water extract, were quite high (peak passive hemagglutinin titers of 1/2048 or greater). The most

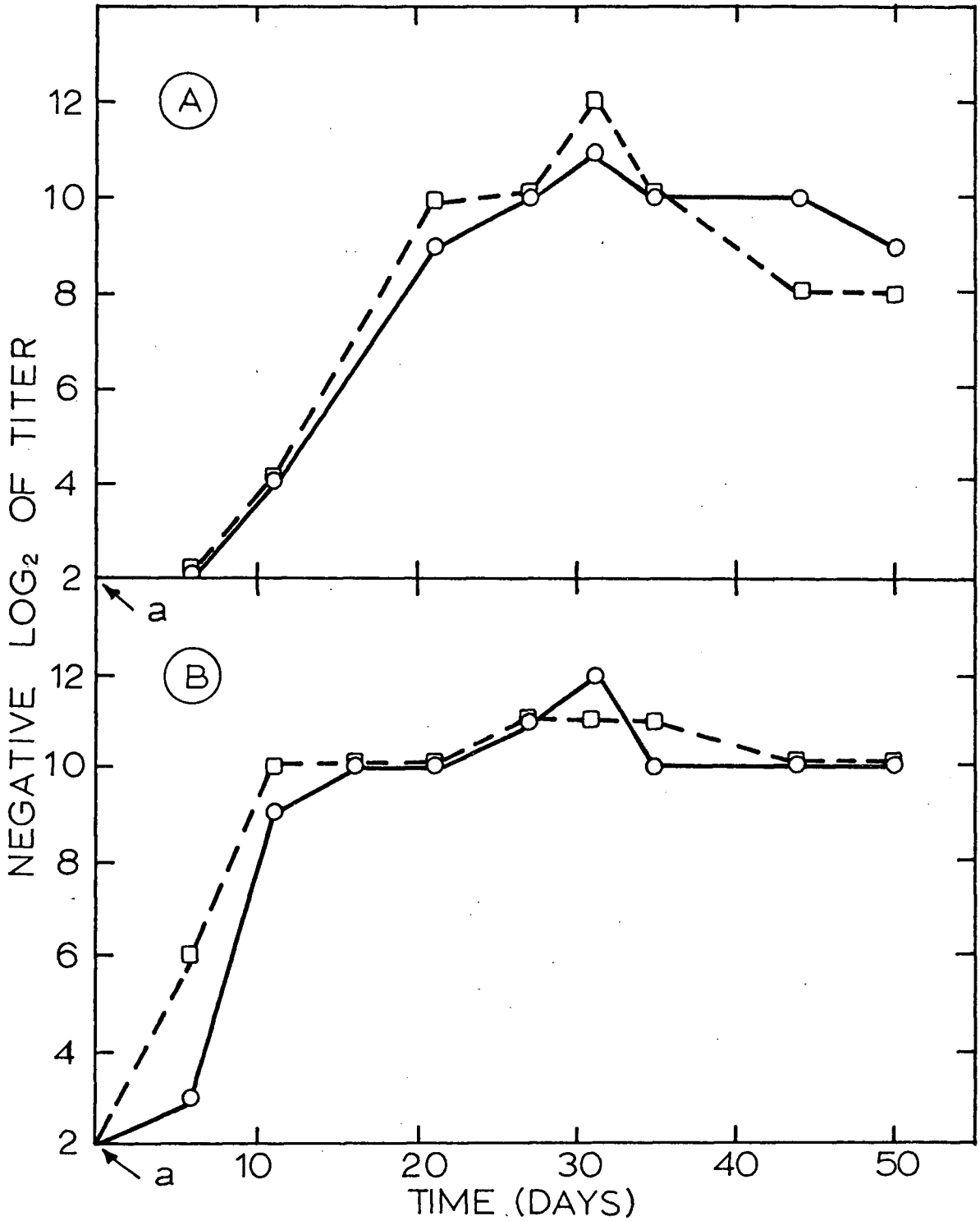


Figure 1. Rabbit antibody response to cell preparations from Pseudomonas aeruginosa strain PA-7.

Rabbits were injected with the cell wall preparation according to the schedule shown in Table I (Materials and Methods). Titers shown refer to A, passive hemolysin and B, passive hemagglutinin levels.

Symbols: □--□, rabbit 1; 0—0, rabbit 2;
a - first injection of series.

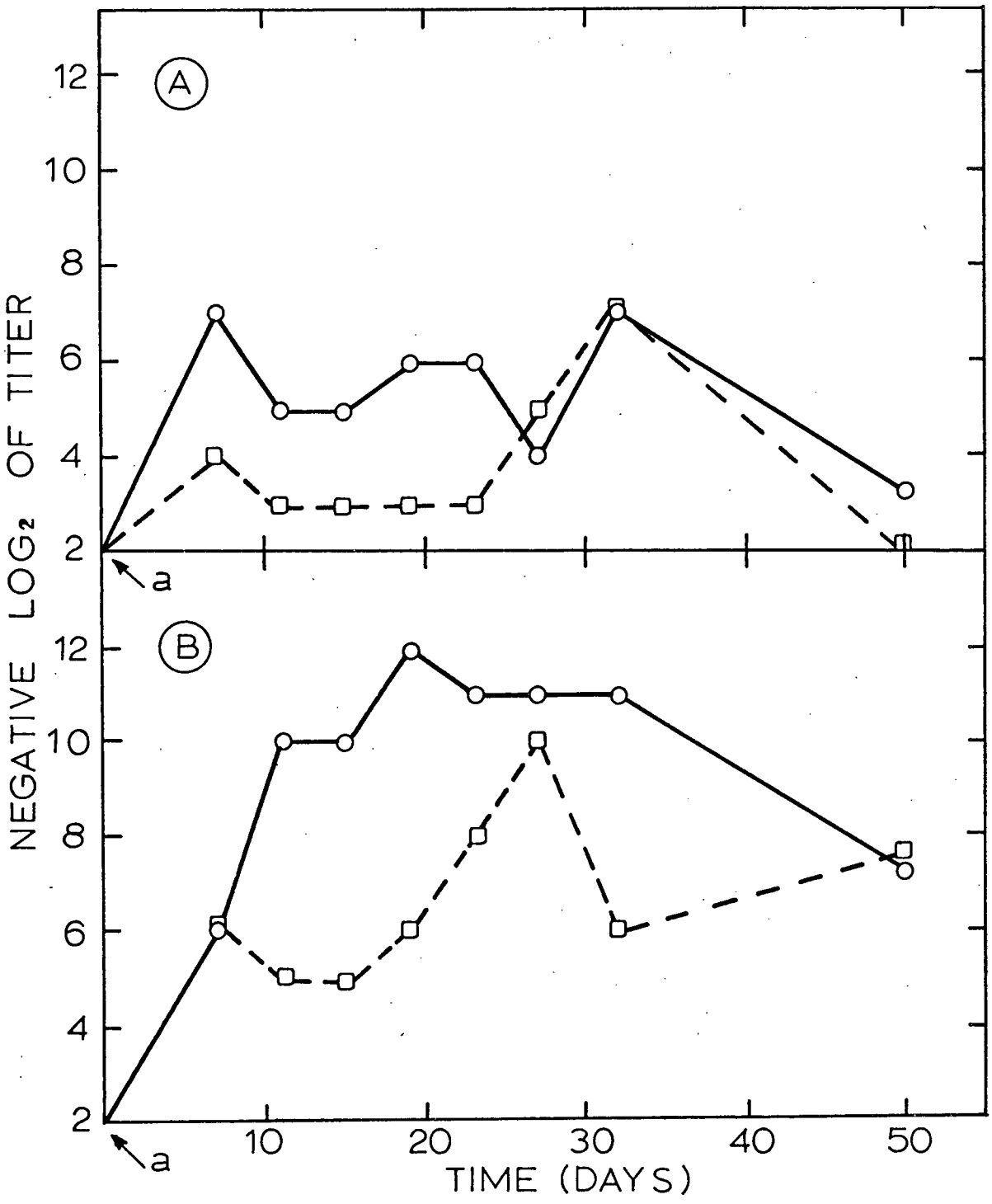


Figure 2. Rabbit antibody response to heat-stable lipopolysaccharide preparation from Pseudomonas aeruginosa strain PA-7.

Rabbits were injected with the heat-stable lipopolysaccharide preparation according to the schedule shown in Table I (Materials and Methods). Titers shown refer to A, passive hemolysin, and B, passive hemagglutinin levels.
Symbols: □--□, rabbit 1; O—O, rabbit 2;
a - first injection of series.

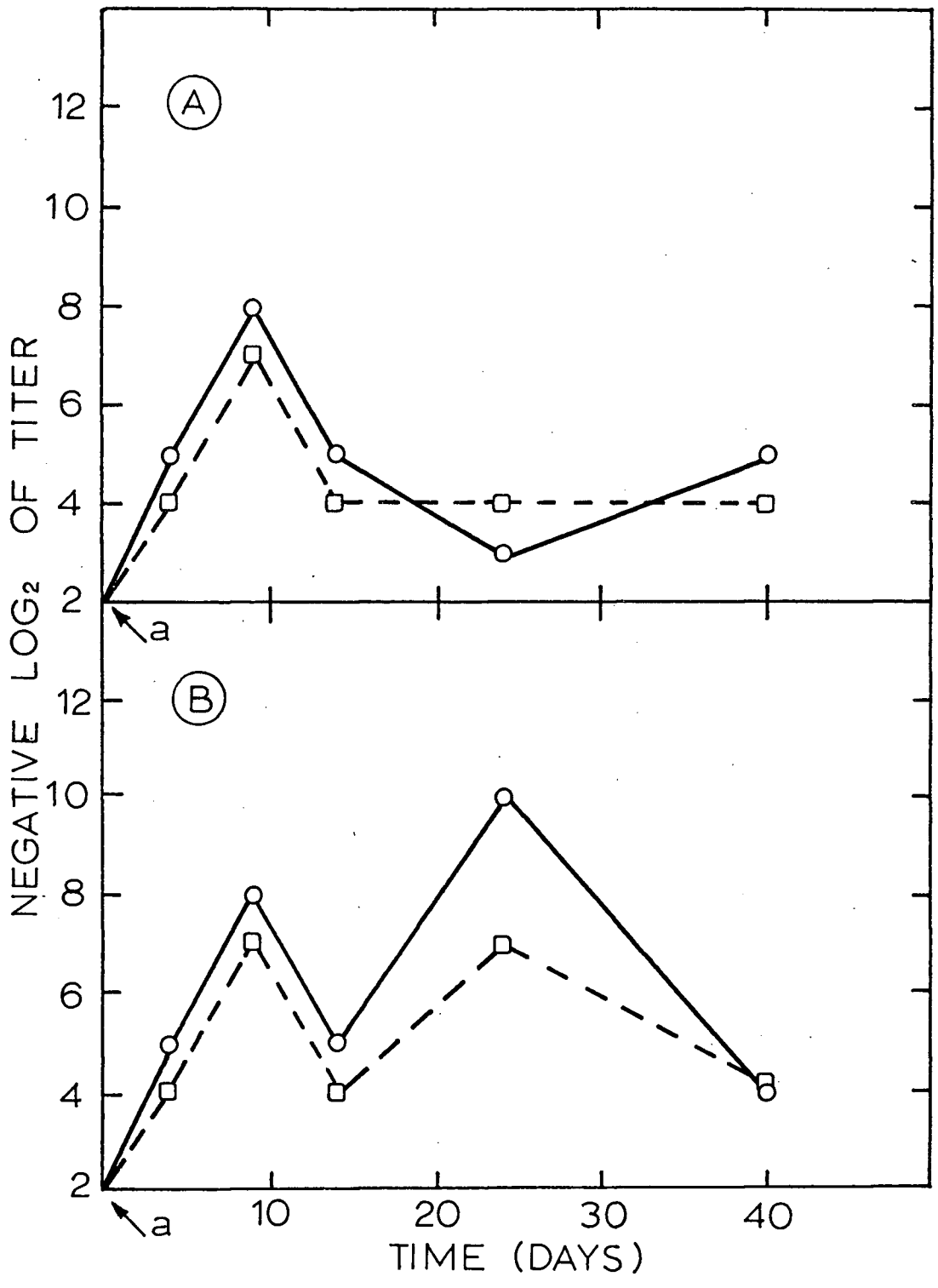


Figure 3. Rabbit antibody response to phenol-water extracted lipopolysaccharide from Pseudomonas aeruginosa strain PA-7.

Rabbits were injected with the phenol-water lipopolysaccharide preparation according to the schedule shown in Table 1 (Materials and Methods). Titers shown refer to A, passive hemolysin and B, passive hemagglutinin levels. Symbols: □--□, rabbit 1; O—O, rabbit 2;
a - first injection of series.

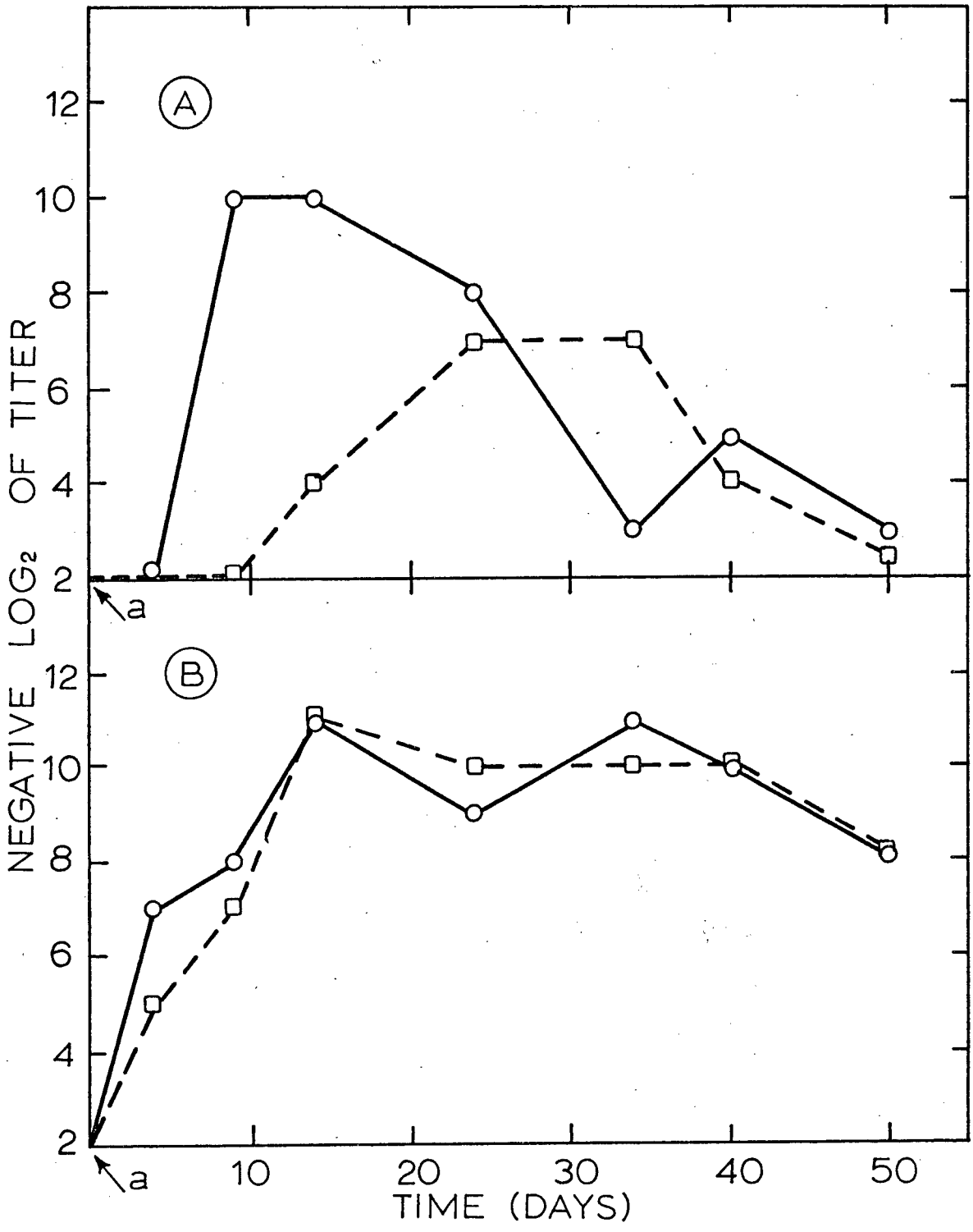


Figure 4. Rabbit antibody response to protein-lipopolysaccharide from Pseudomonas aeruginosa strain PA-7.

Rabbits were injected with the protein-lipopolysaccharide preparation according to the schedule shown in Table 1 (Materials and Methods). Titers shown refer to A, passive hemolysin and B, passive hemagglutinin levels.
Symbols: □--□, rabbit 1; 0—0, rabbit 2;
a - first injection in series.

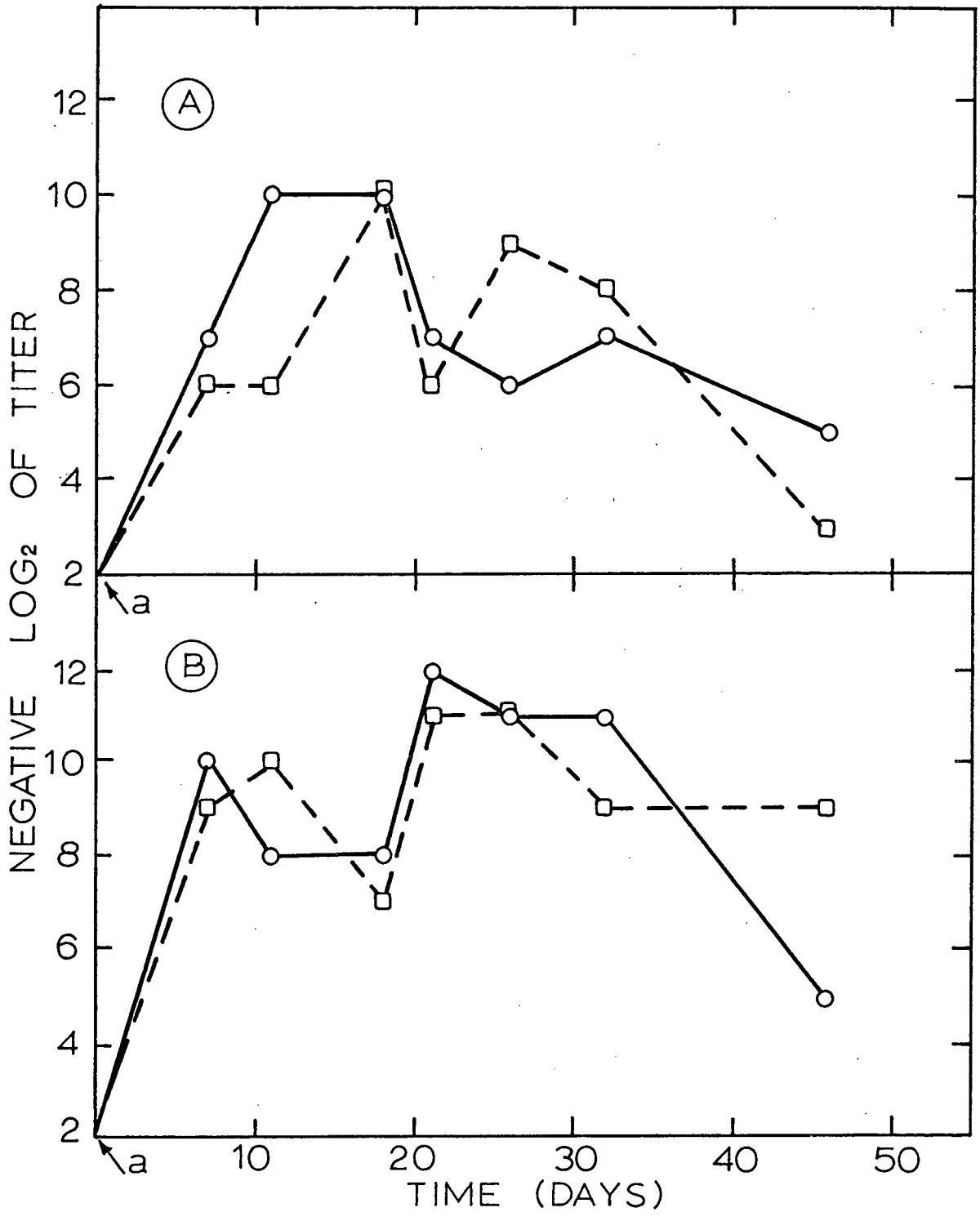


Figure 5. Rabbit antibody response to formalin-killed vaccine of Pseudomonas aeruginosa strain PA-7.

Rabbits were injected with formalin-killed cells according to the schedule shown in Table I (Materials and Methods). Titers shown refer to A, passive hemolysin, and B, passive hemagglutination levels.
Symbols: □--□, rabbit 1; 0—0, rabbit 2,
a - first injection in series.

prolonged response of high titer antibodies was stimulated by the cell wall preparation (Figure 1). The reason for the poorer response to the phenol-water extract (Figure 3) is not known, although the relatively low protein content of this preparation in comparison with that of the other lipopolysaccharide preparations may be a factor. Other workers (Whang et al. 1971) have also noted that phenol-water extracted lipopolysaccharides are poor immunogens, and have mentioned the lack of aggregation of the molecules as a contributing factor.

Although the antibody responses to the recall injections (Table I) are not shown in Figures 1 - 5, the intravenous boosters of the cell wall and heat-stable lipopolysaccharide preparations, and of the formalin-killed vaccine resulted in rapid rises in the titers of both the passive hemagglutinating and hemolysing antibodies. Peak titers equalled or exceeded those achieved during the earlier hyperimmunization regime; only the cell wall preparation, however, stimulated a response which persisted for longer than two weeks after the booster injection.

The recall injections of the phenol-water extracted lipopolysaccharide and the protein-lipopolysaccharide preparations were administered subcutaneously in Freund's complete adjuvant in order to determine the effect of this type of injection upon the response to a relatively good and a relatively poor immunogen. Neither preparation stimulated the production of detectable antibody

for approximately six weeks after the injection. After this time, the level of serum antibodies rose slowly for about a month in the rabbits which had been injected with protein-lipopolysaccharide; the level obtained was close to the peak titer of the earlier response, and remained constant for over two months. No appreciable rise in antibody titer was noted over a 2 month period, in the serum of rabbits injected with a booster dose of the phenol-water extract in Freund's adjuvant.

Because of the low level of antibody synthesis stimulated by the phenol-water extract, no further work was done on this serum. The antiserum prepared against the cell wall fraction was used in all subsequent tests, since it was felt that this fraction would contain antigens most representative of those that would be encountered in the natural situation. Antisera specific for the heat-stable lipopolysaccharide and protein-lipopolysaccharide preparations, and for the formalin-killed vaccine were also used for comparative purposes.

II. Properties of the Antigen Preparations.

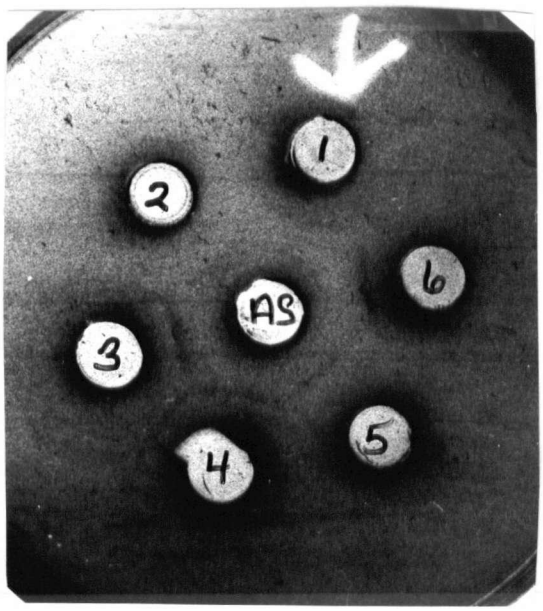
The average yield of the antigenic fractions calculated as a percentage of the dry weight of the cells used in their preparation was 2 - 3 percent for the heat-stable and phenol-water lipopolysaccharide preparations, 0.5 percent for the protein-lipopolysaccharide fraction, and 8 percent for the cell walls. Thus, not only

does the cell wall preparation appear to be the most effective immunogen, but also it is the most efficient fraction to produce in terms of yield per unit weight of cells. In addition, toxicity studies have indicated that the lethal doses for the heat-stable lipopolysaccharide, protein-lipopolysaccharide, and cell wall preparations are all greater than 8 mg per kg of mouse body weight; however, this dose level was found to be toxic in all cases, with diarrhea, eye exudates, a hunched position, and immobility of the affected animals being noted. Therefore, it was concluded from these studies that the amounts of each preparation used for the immunization did not approach the lethal dose; furthermore, no obvious adverse affects on the rabbits were noticed, even when they received the recall injections.

The reason for the low toxicity of Pseudomonas aeruginosa cells, cell walls and lipopolysaccharide preparations is not known. Fensom and Gray (1969) showed that the chemical composition of the lipopolysaccharide molecule of Pseudomonas aeruginosa is basically similar to that of the Enterobacteriaceae. The cell walls were also found to resemble, qualitatively and quantitatively,the cell walls of other Gram-negative bacteria (Clarke et al. 1967). However, for the Enterobacteriaceae, LD50 levels of 0.1 - 0.2 mg of endotoxin (lipopolysaccharide) per kg of body weight have been reported for mice (Davis et al. 1967); since our preparations are only partially purified, however, caution must be used in attempting to compare these values.

Immunodiffusion analyses of antigenic preparations are presented in Figure 6. Schematic representations are presented in addition to photographs, since some of the lines disappeared very quickly, and others are very faint.

Figure 6 A shows the reaction of antiserum prepared against the heat-stable lipopolysaccharide preparation with different concentrations of heat-stable lipopolysaccharide, phenol-water lipopolysaccharide, and protein-lipopolysaccharide; lines of identity between the heat-stable lipopolysaccharide and the phenol-water lipopolysaccharide (Wells, 1 & 2, 4 & 5); and between the heat-stable lipopolysaccharide and the protein-lipopolysaccharide (Wells, 3 & 4), indicate that there are common antigenic determinants in these preparations. Figure 6 B-D show that each of the three lipopolysaccharide preparations is able to react with antisera prepared against the heat-stable lipopolysaccharide, the protein lipopolysaccharide, and the cell walls; although antiserum prepared against the formalin-killed vaccine reacts only with the protein-lipopolysaccharide here, other studies have demonstrated that it is also able to react with the heat-stable and phenol-water lipopolysaccharide preparations (Figure 6E). The cross-reactivity seen in these figures is to be expected because of the nature of the antigen fractions; however, the lack of correlation between antigenicity and immunogenicity in the case of the phenol-water extract is interesting. It may be that the antigen shared by the



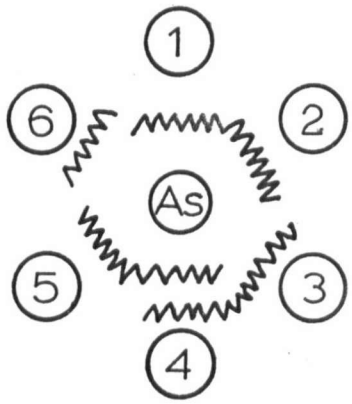


Figure 6A.

Figure 6. Immunodiffusion analysis of lipopolysaccharide preparations from Pseudomonas aeruginosa.

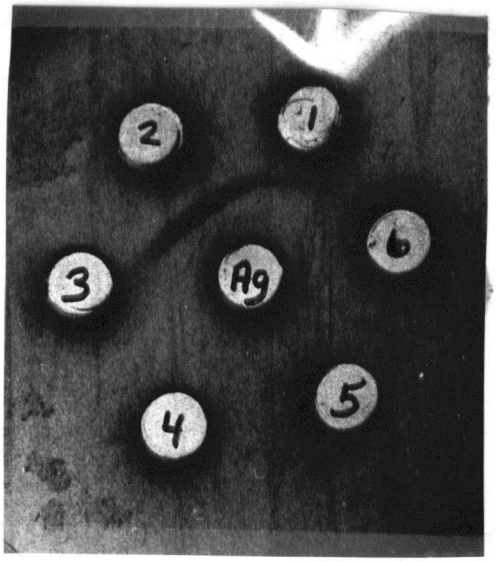
Plates contain 0.85 percent Ionager No.2 in borate-saline buffer, pH 8.0. Antigens and antisera were added to the wells in 0.1 M volumes. Plates were incubated at 37 C for 7 - 10 days.

A. Antiserum (AS) was prepared against heat-stable lipopolysaccharide. Well 1 contains 1 mg/ml of heat-stable lipopolysaccharide; well 2, 1 mg/ml of phenol-water lipopolysaccharide; well 3, 1 mg/ml of protein-lipopolysaccharide; wells 4-6, 0.5 mg/ml of heat-stable lipopolysaccharide, phenol-water lipopolysaccharide, and protein-lipopolysaccharide respectively.

B-D. Well 1 contains anti-heat-stable lipopolysaccharide antiserum; well 2, anti-cell wall serum; well 3, anti-protein-lipopolysaccharide serum; well 4, anti-formalin-killed vaccine antiserum; well 5, normal rabbit serum; well 6, saline. Antigens (Ag) are heat-stable lipopolysaccharide (B), protein-lipopolysaccharide (C), and phenol-water lipopolysaccharide (D), at 1 mg/ml

E. Antiserum (AS) was prepared against the formalin-killed vaccine. I. Antigen (Ag) is heat-stable lipopolysaccharide at a concentration of 1 mg/ml. II. Antigen (Ag) is phenol-water lipopolysaccharide at a concentration of 1 mg/ml.

Symbols: ~~~ , Diffuse line; ——— , sharp line.



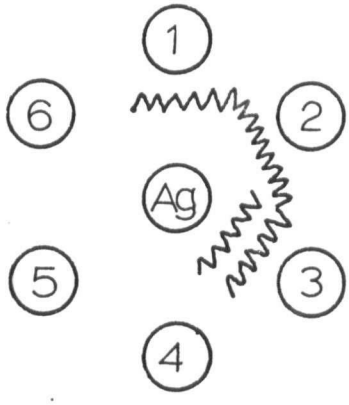
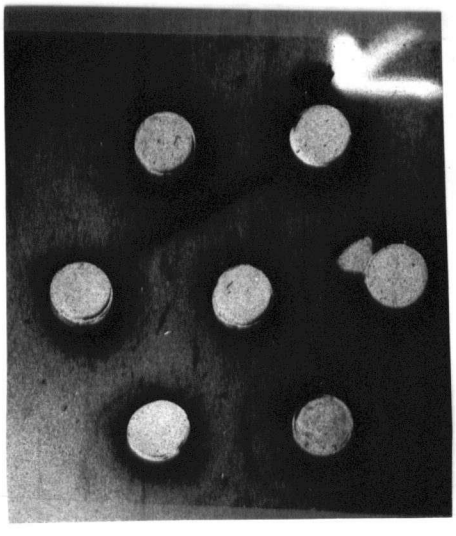


Figure 6B.



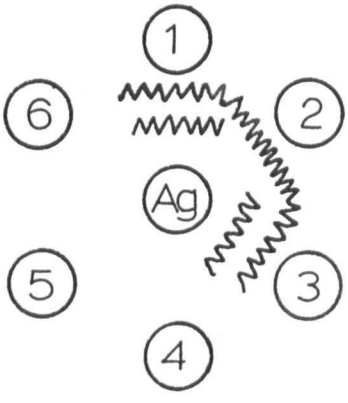


Figure 6C.

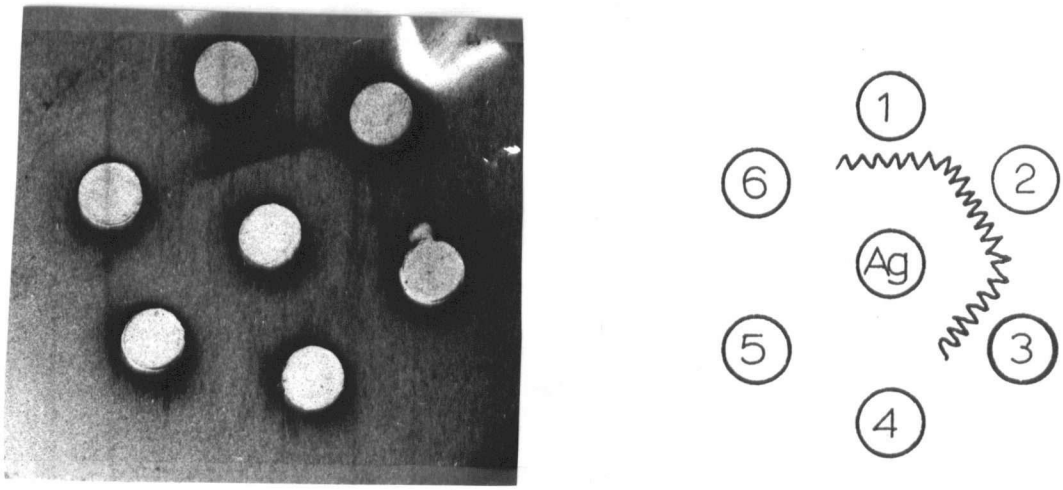


Figure 6D.

(i) (ii)

Figure 6E.

protein-lipopolysaccharide and the heat-stable lipopolysaccharide, but missing from the phenol-water preparation (Figure 6 A, Wells, 3 - 5) is the immunogenic moiety. It is of course, also possible that the two lines at well 4 of Figure 6 A merely represent different degrees of aggregation of the molecule, and that only the more aggregated species of the heat-stable lipopolysaccharide molecule (outer line) is capable of acting as an immunogen (see Whang et al. 1971).

Electron micrographs of the cell wall preparations are shown in Figure 7. Hubert and his coworkers (1971) have published electron micrographs showing the appearance of the cell wall and cell membrane in cross-sections of whole cells of Pseudomonas aeruginosa. They showed the external triple layered cell wall and the triple layered plasma membrane which are typical of Gram-negative organisms. Kellogg and coworkers (1971), in a study of the particulate fractions of Neisseria gonorrheae, show cell wall fragments which resemble closely those shown in Figure 7 B of our preparations.

A triple layered structure with two electron dense layers (as described by Hubert et al. 1971) is seen only in those preparations in which the cell walls were treated with the nucleases, followed by lipase digestion (Figures 7 E & F). Nuclease treatment alone resulted in the isolation of a structure containing three electron dense layers (Figures 7 A & B) while trypsin appeared

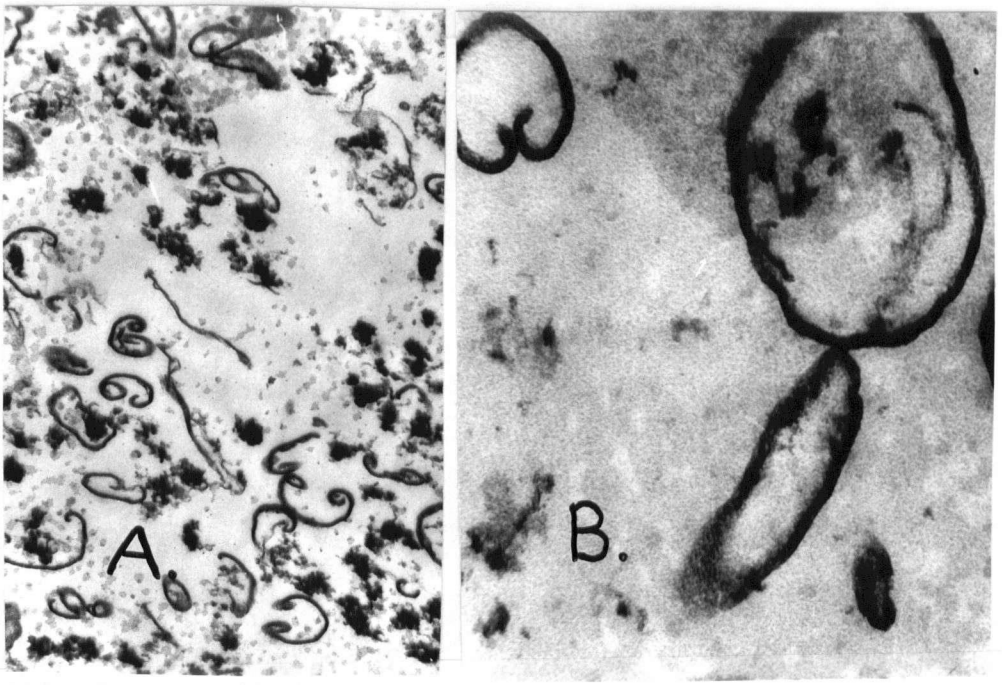


Figure 7. Electron micrographs of cell wall preparations from Pseudomonas aeruginosa.

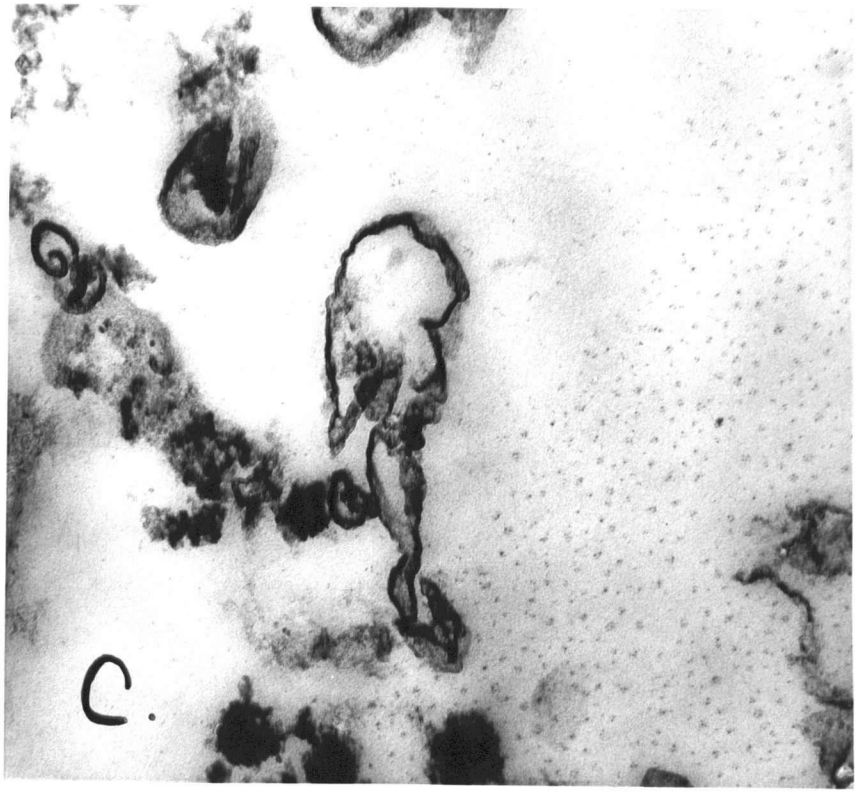
Isolated cell wall preparations were fixed and stained as outlined in the Materials and Methods.

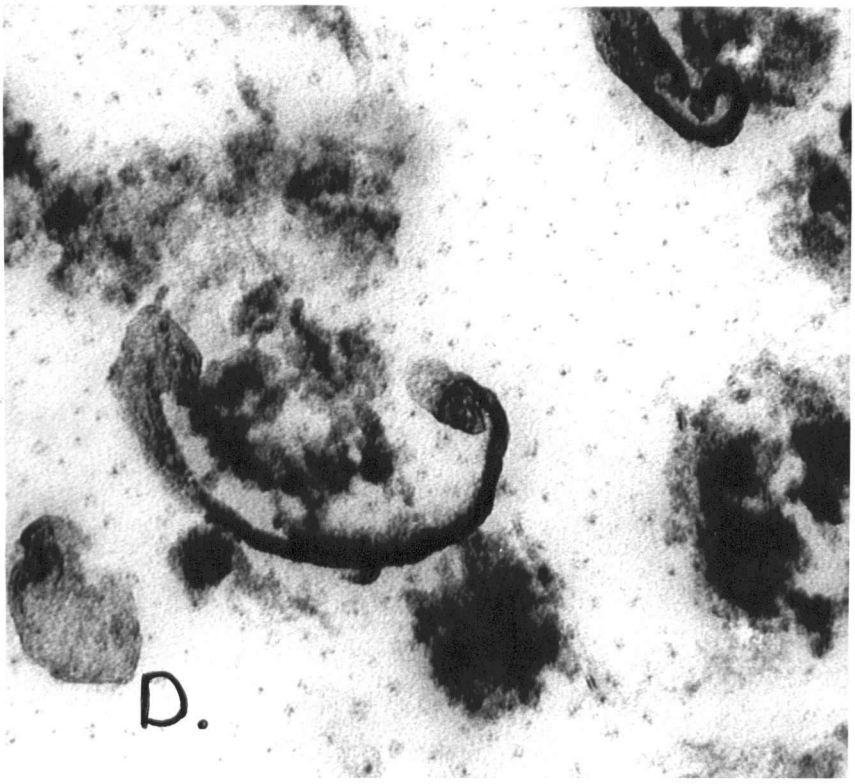
A. Fixed in osmium tetraoxide, post-fixed in uranyl acetate. The various sizes of cell wall fragments can be noted. x 15,250.

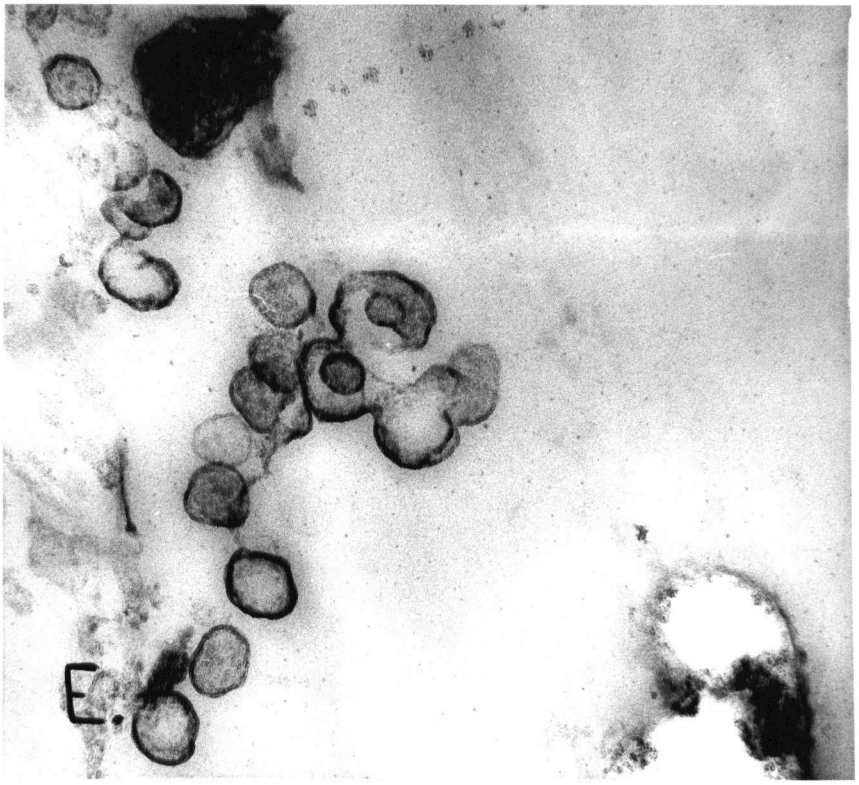
B. Fixed in osmium tetraoxide, post-fixed in uranyl acetate. Note the three electron dense layers. x 58,000.

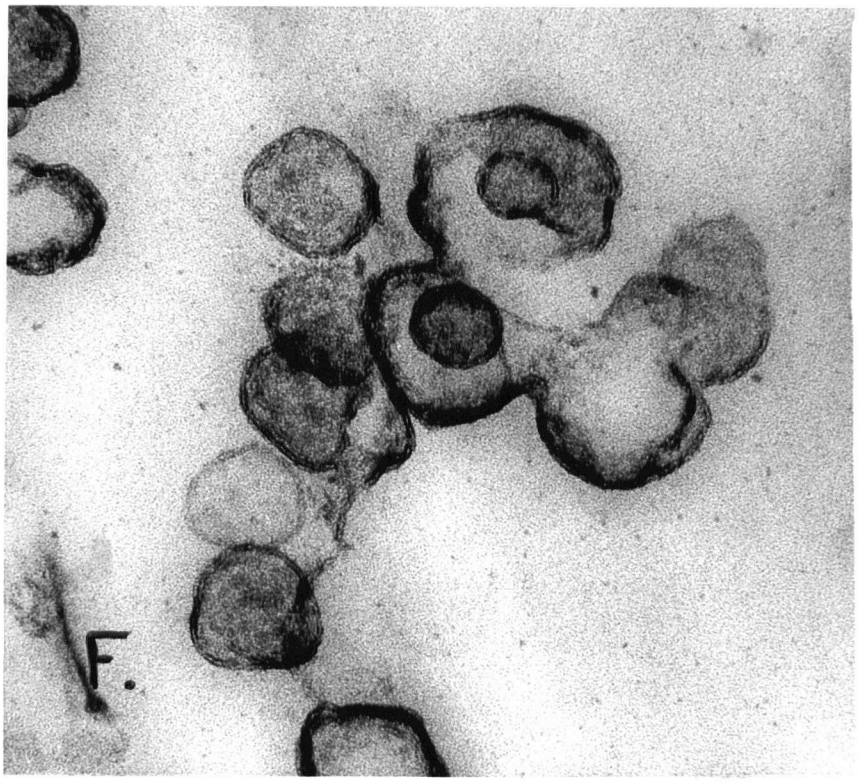
C-D. Fixed in 2.3 percent glutaraldehyde, then post-fixed in one percent osmium tetraoxide. Note the loss of organized structural layers. C- x 73,200; D- x151,000.

E-F. Fixed in 2.3 percent glutaraldehyde, post-fixed in one percent osmium tetraoxide. Note the loss of one electron dense layer. E- x 73,200; F- x 151,000.


C.


D.


E.


F.

to have simply caused a disorganization of the structural layers (Figures 7 C & D).

It was decided, on the basis of these photographs, to employ in further studies the cell wall preparation, which had undergone only nuclease digestion. This was felt to be the most representative antigenic sample, since trypsin appeared to disorganize the natural structure of the cell envelope, while it was not possible, without further chemical analysis, to determine whether the electron-dense layer removed by the lipase digestion, was part of the cell membrane or of the cell wall.

III. Fractionation of Rabbit Antisera

a) Sensitivity to 2-Mercaptoethanol

The sequence of appearance of 2-mercaptoethanol sensitive (2-MES) and 2-mercaptoethanol resistant (2-MER) passive hemagglutinins in the serum of rabbits immunized with the cell wall preparation (Table I) is shown in Figure 8. Aliquots of the serum samples taken during the course of the response (Figure 1) were treated with 2-mercaptoethanol as described in the Materials and Methods in order to determine the proportion of 2-mercaptoethanol resistant antibody at various stages during the response. The 2-mercaptoethanol resistant activity was found in appreciable quantities only late in the response to the hyperimmunizing series of injections; in this regard, the results are very similar to data

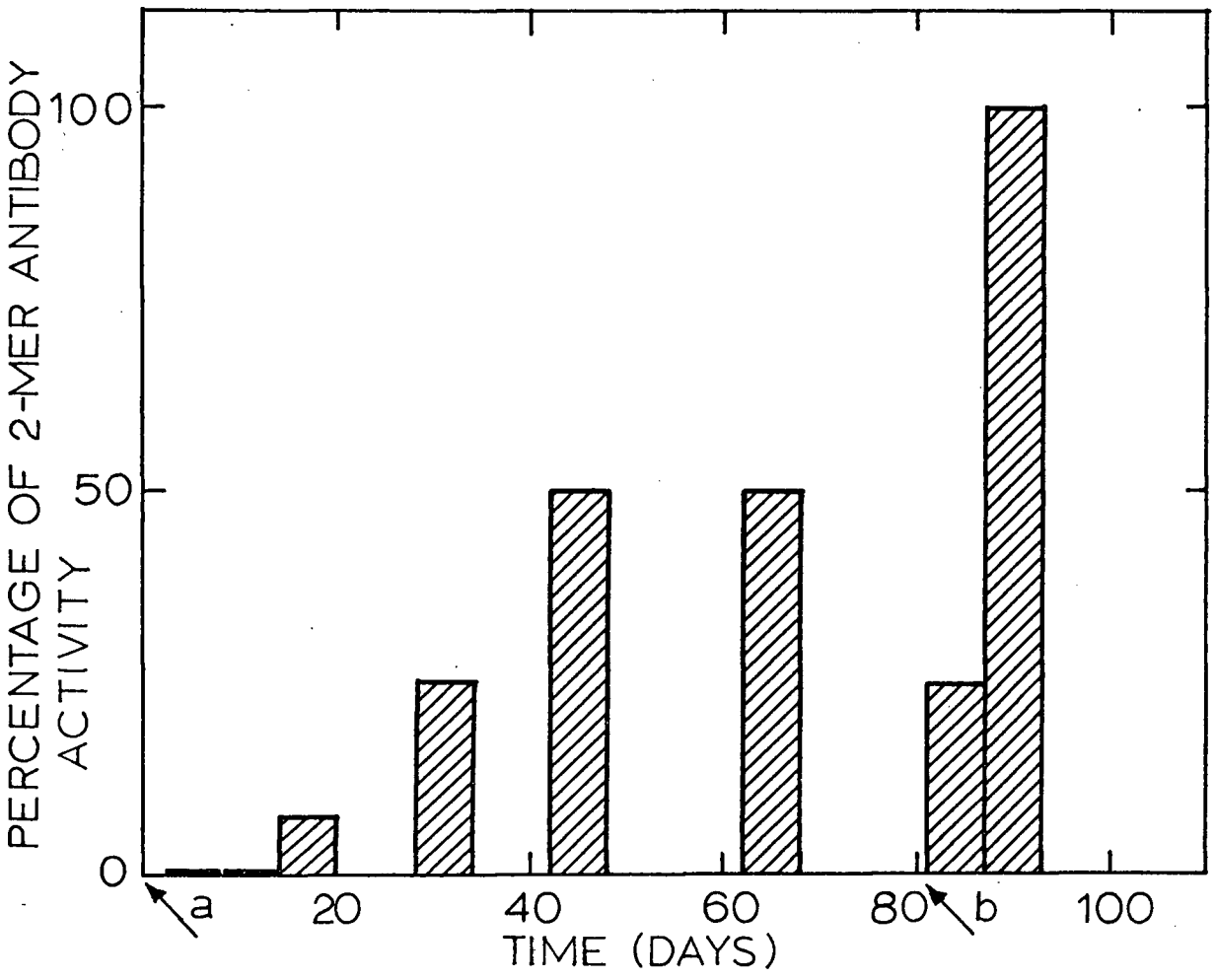


Figure 8. Detection of 2-mercaptoethanol resistant antibody activity in rabbit serum.

Aliquots of antiserum from rabbits injected with the cell wall preparation according to the schedule shown in Table I (Materials and Methods) were mixed with equal volumes of 0.2 M 2-mercaptoethanol, incubated for one hour at room temperature, and used as the serum source in the passive hemagglutination test. Original titers are shown in Figure 1.

obtained by Landy and his coworkers (1965) and by Pike and Schulze (1964) in analogous studies with other Gram-negative organisms. The recall injection, on the other hand, was found to stimulate not only an increase in the rate of overall antibody synthesis, but also in the rate of increase of the 2-mercaptoethanol resistant fraction of the antibody activity.

In addition to the above studies, antisera directed against the other three antigenic fractions were also treated with 2-mercaptoethanol to determine the percentage of 2-mercaptoethanol-sensitive and 2-mercaptoethanol resistant antibody activity. A large percentage of the passive hemagglutinating activity in sera obtained late in the response to all of these antigenic fractions was also found to be resistant to 2-mercaptoethanol treatment. Although sensitivity and resistance to 2-mercaptoethanol may not be a completely valid measure of the IgM or IgG content of an immune serum, the results obtained in these studies were helpful in estimating the periods during the antibody responses which would yield the most IgM or IgG antibody upon fractionation of the sera.

b) Fractionation of Rabbit Serum Proteins by Gel Filtration and Ion Exchange Chromatography

Attempts were made to separate the IgM and IgG immunoglobulin fractions of whole rabbit serum by passage of serum samples through Sephadex G-200. A typical elution profile, expressed as the optical

densities of the fractions measured at 280 nm is shown in Figure 9. Each of the three protein peaks, when pooled and concentrated to the original serum volume, was found to contain some passive hemagglutinating activity. A large proportion of this activity was located in the first protein peak, and was mainly 2-mercaptoethanol sensitive antibody.

Interfacial tests performed with goat anti-rabbit γ-globulin and the concentrated material from the shaded area of the first protein peak (Figure 9) demonstrated that there was some IgG globulin contaminating this fraction. Accordingly, this material was refractionated on Sephadex G-200, and the elution profile illustrated in Figure 10 was obtained. The leading front of the protein peak (shaded area) was again concentrated, and ring tests with goat anti-rabbit γ-globulin did not detect any remaining IgG in the fraction. Passive hemagglutination tests performed on samples of this fraction, with and without prior treatment with 2-mercaptoethanol, indicated that not all of the antibody activity was sensitive to the action of 2-mercaptoethanol (Table 4); however, this material was used as the IgM fraction in subsequent tests.

Since the IgG immunoglobulins appeared to be eluted from the Sephadex G-200 column in a broad band, whole rabbit serum which had been shown to be rich in 2-mercaptoethanol resistant antibody activity was chromatographed on DEAE-cellulose, and the IgG was eluted by means of a continuous gradient. A typical elution pattern

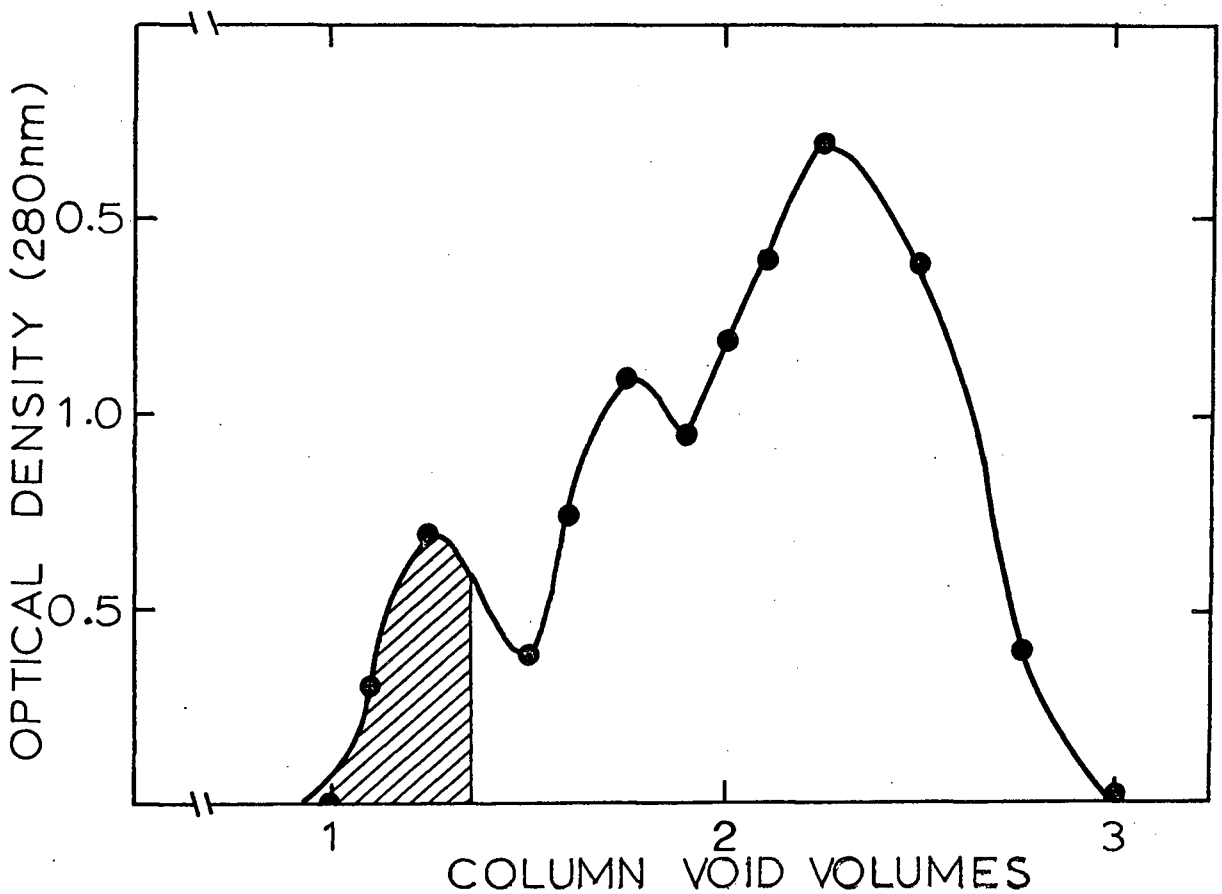


Figure 9. Elution profile of whole rabbit serum from Sephadex G-200.

A 2 - 3 ml sample of whole rabbit serum, taken early in the response, was applied to a 2.5 x 45 cm Sephadex G-200 column at 4 C. The flow rate was 0.1 ml per minute and the column void volume was 70 ml. 3 ml fractions were collected, and the optical density at 280 nm measured with a Beckman DBG-spectrophotometer. Fractions within the cross-hatched area were pooled and concentrated for refractionation.

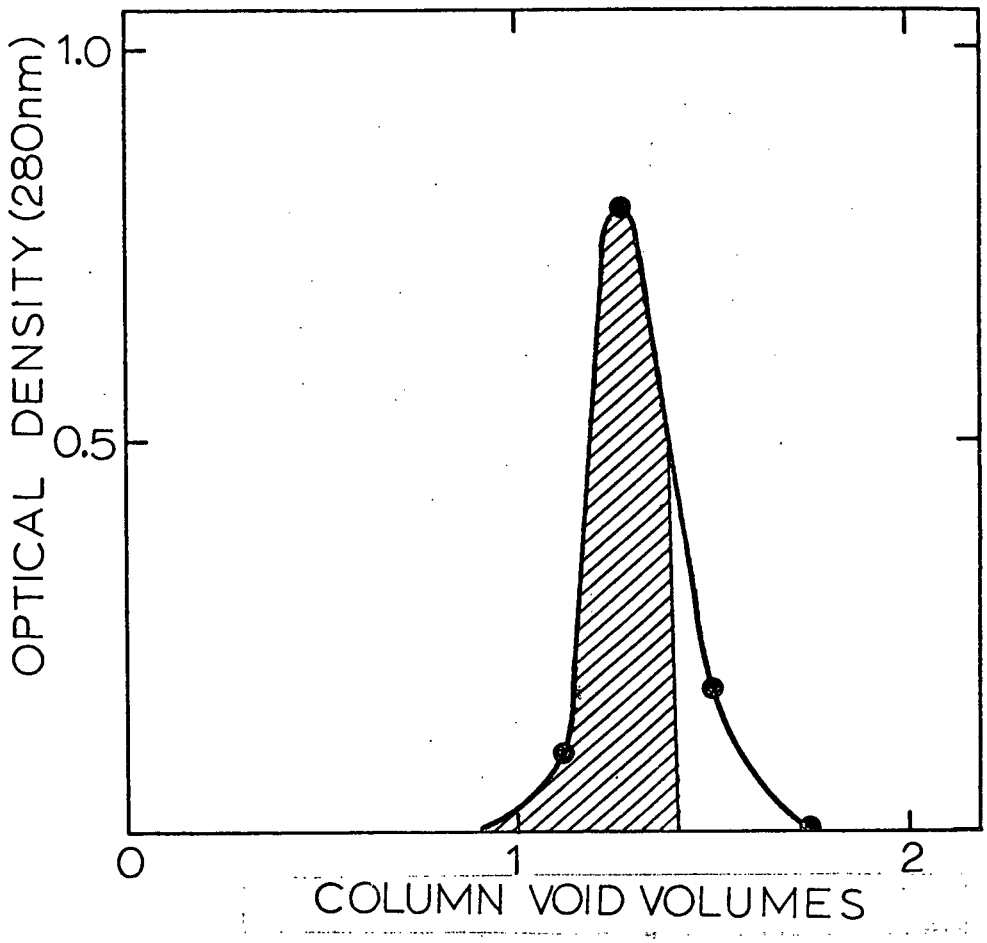


Figure 10. Elution profile of IgM-containing serum fraction from Sephadex G-200.

The first protein peak eluted when whole serum was passed through Sephadex G-200, was concentrated by ultrafiltration and repassaged through Sephadex G-200 at 4 C. The column void volume was 70 ml. Fractions within the shaded area of the protein peaks were pooled, concentrated to a volume of 2 - 3 ml, and used as the IgM fraction.

is shown in Figure 11. Material under the first protein peak was pooled and concentrated to the original serum volume; interfacial tests with goat anti-rabbit γ-globulin demonstrated the presence of IgG molecules in the fraction, and passive hemagglutination tests showed that all of the antibody activity was resistant to 2-mercaptoethanol treatment. This material was used as the IgG fraction in the subsequent tests.

IV. Passive Protection Tests

a) Whole Serum

Passive protection tests were performed with mice, using rabbit antisera prepared against the four antigenic preparations (heat-stable lipopolysaccharide, protein-lipopolysaccharide, cell walls and formalin-killed vaccine) to determine their capacity to elicit antisera protective against a lethal challenge of PA-7. The challenge dose of 20 LD50 (3×10^8 cells) was chosen after preliminary experiments showed that this dose was lethal to all mice injected with normal rabbit serum, whereas at least 50 percent of groups of mice protected by low dilutions of immune rabbit serum survived the challenge. The data presented in Table II shows that each of the four types of whole antiserum was able to protect mice against this challenge dose. In most cases, experiments were terminated after 72 hours, since simultaneous virulence tests demonstrated that all of the unprotected animals died within 48 hours

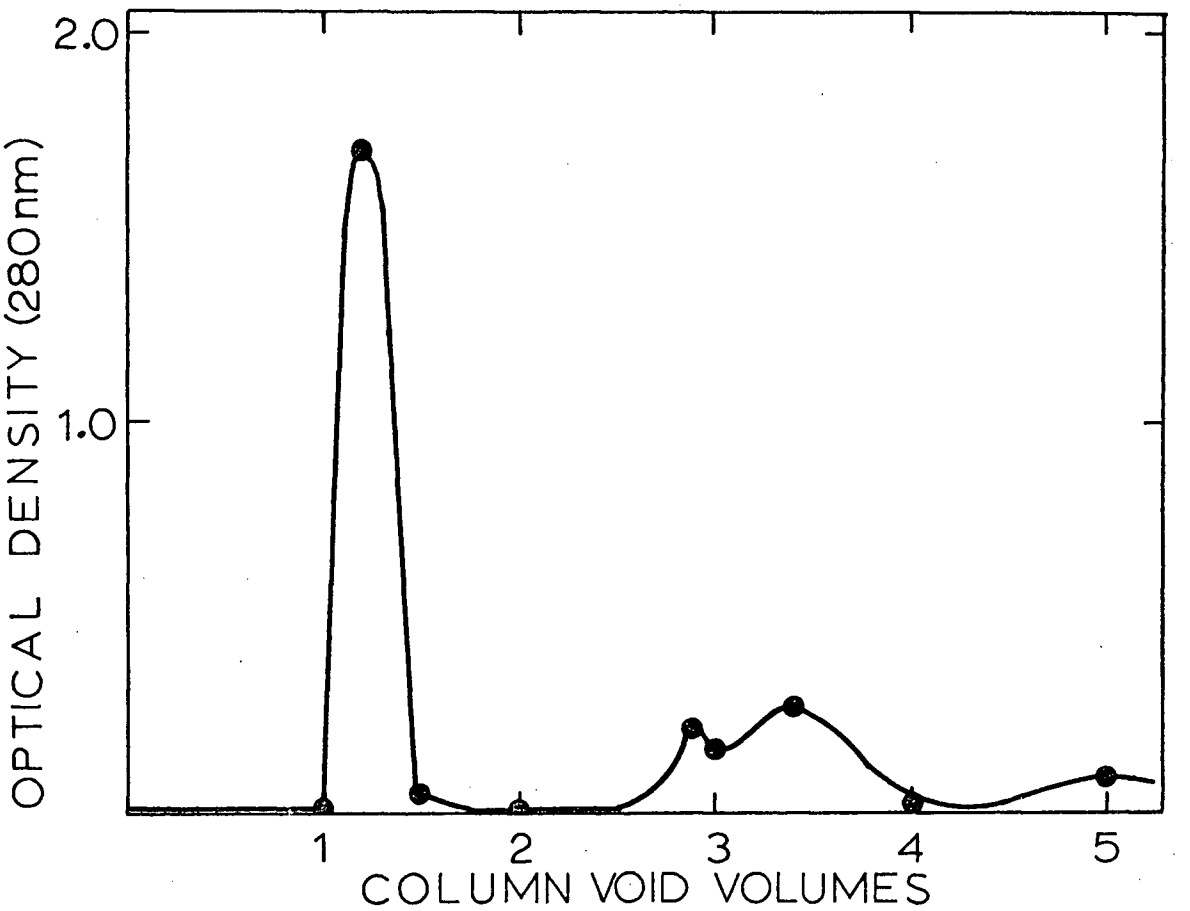


Figure 11. Elution profile of whole rabbit serum from the DEAE-cellulose column.

A 5 ml sample of whole rabbit serum was applied to a 2.5 x 45 DEAE-cellulose column at 4 C. The serum was eluted with an 800 ml continuous gradient from 0.005 M sodium phosphate buffer, pH 7.0, to 0.05 M NaH_2PO_4 in 0.05 M NaCl. The column void volume was 120 ml; 6 ml fractions were collected and monitored at 280 nm for protein content.

Table II. Protection conferred by the passive immunization of mice with rabbit antisera directed against various fractions from Pseudomonas aeruginosa.[a]

Immunizing Agent	Antiserum Titer		Antiserum Dilution	Challenge Dose (LD 50)	Percent Survival
	PHA	BA			
Heat-stable Lipopolysaccharide	1/1024	1/640	1/2	20	67
			1/10	20	58
Protein-lipopolysaccharide (serum from day 30)	1/4096	1/640	1/2	20	92
			1/10	20	80
Protein-lipopolysaccharide (serum from day 160)	1/512	1/5120	1/2	20	85
			1/10	20	69
			1/50	20	23
			1/100	20	8
Cell Walls (serum from day 11)	1/1024	1/640	1/2	20	93
			1/10	20	73
			1/20	20	66
Cell Walls (serum from day 31)	1/4096	1/640	1/2	20	77
			1/50	20	64
Formalin-killed vaccine	1/2048	1/1280	1/1	20	100
			1/10	20	43
	1/1024	1/256	1/10	20	30
-[b]	-	-	1/1	20	0

[a] Mice were injected intraperitoneally with 0.2 ml of rabbit serum diluted in sterile, pyrogen-free saline. Four hours later, viable PA-7 cells suspended in 10 percent TSB-saline were injected intraperitoneally. Deaths were recorded for a 72 hours period post-challenge.

[b] Normal rabbit serum was injected in place of the immune serum as described above.

of challenge; moreover, very few deaths in the groups of serum-protected animals occurred after the initial three-day period post-challenge.

The results show that the degree to which an antiserum sample can be diluted before a significant loss in the protection afforded is incurred, can be estimated on the basis of the passive hemagglutinating antibody content of the serum (with the exception of the antiserum prepared against the formalin-killed vaccine). In addition, the time during the response from which the antiserum was obtained did not seem to affect the relationship between passive hemagglutinin content and passive protection ability. This is evident from the data for serum prepared against cell walls and protein-lipopolysaccharide. The lower protective capacity of serum directed against the formalin-killed vaccine is puzzling; it might be suggested that formalin adversely affects the immunogenicity of the protective antigen alone, without altering other immunogenic structures in the cell responsible for the stimulation of agglutinating and passive hemagglutinating activities. This seems unlikely, however, in view of the relationship between passive hemagglutination and protection noted above.

Some experiments also involved the injection of different sizes of challenge doses into mice passively protected as previously described. Table III shows results obtained when mice were given various numbers of cells after injection of antisera prepared against

Table III. Passive protection in relation to the size of the challenge dose.[a]

Immunizing Agent	Titer of Antiserum		Challenge Dose (LD 50)	Percent Survival
	PHA[b]	BA		
Cell Wall	1/204	1/32	20	77
			50	60
			100	0
Heat-stable lipopoly-saccharide	1/102	1/64	10	90
			20	58
	1/52	1/32	20	80
			100	8

[a] Mice were passively immunized with 0.2 ml immune rabbit serum diluted in saline, then challenged with viable PA-7 suspended in 10 percent TSB-saline. Deaths were recorded for a 72 hour period after challenge.

[b] Original serum titer was divided by the serum dilution performed. Cell wall antiserum was diluted 1/20, heat-stable lipopoly-saccharide sera diluted 1/10.

cell walls or heat-stable lipopolysaccharide. As expected, the degree of protection afforded by a given antiserum was inversely related to the size of the challenge dose. These data demonstrate that all of the four types of antisera can afford protection against moderately large challenges (20 - 50 LD 50) of PA-7, but are of little use against very severe challenges (100 LD 50).

It was also of some interest to determine whether the immune rabbit serum prepared against PA-7 antigens could provide protection for mice against challenge by two other strains of Pseudomonas aeruginosa. Bacterial agglutination tests with the formalin-killed vaccines of strains PA-7, PA-1 and PA-479 as antigens, showed that none of the four major antigenic preparations used stimulated the production of antibodies which agglutinated PA-1 or PA-479 vaccines. Moreover, passive protection studies in which antiserum directed against the cell wall preparation was used to protect the mice, showed that antiserum dilutions which protected 60 - 75 percent of the mice challenged with PA-7 protected less than 20 percent of the mice challenged with PA-1 or PA-479. Fisher and his coworkers (1969) have shown a lack of correlation between agglutination and protection among different strains of Pseudomonas aeruginosa; however, our results seem to agree more closely with the findings of Bass and McCoy (1971), who showed that cross-agglutination and cross-protection were analogous.

b) Fractionated Serum

IgM and IgG serum fractions purified as described in the Materials

and Methods were also used in passive protection studies with mice. The results presented in Tables IV and V (IgM and IgG fractions respectively) demonstrate that both immunoglobulin types provide passive protection against infection by PA-7. When the passive hemagglutination titers of the fractions were considered, neither of the two fractions from cell wall or protein-lipopolysaccharide appeared to be more efficient than the other; the data obtained from sera prepared against the heat-stable lipopolysaccharide and formalin-killed vaccine, however, indicate that IgG globulins in these sera provide better protection than do the IgM globulins. Bjornson and Michael (1970), working with immunoglobulin fractions obtained from the serum of rabbits immunized with purified muco-polysaccharide from the slime layer, also showed that the IgG fraction offered superior protection to mice against infection by Pseudomonas aeruginosa.

Because of the well-known superiority of IgM globulin in participating in agglutination-type reactions, the increased efficiency of protection afforded by the IgG fraction of immune serum may simply reflect a much larger number of IgG molecules than there are IgM molecules in a given volume of their respective fractions. The precipitin results obtained with IgM and IgG serum fractions from a sample of anti-cell wall serum (day 44) support this hypothesis, since there was found to be substantially greater amount of IgG than IgM antibody, on a molar ratio, in the two fractions, which

Table IV. Passive protection of mice provided by the IgM-containing fraction of immune rabbit serum.[a]

Immunizing Agent	Titer of IgM Fraction		Dilution of Fraction	Challenge Dose (LD 50)	Percent Survival
	PHA	2-MePHA[b]			
Cell Walls	1/64	-	1/1	20	60
			1/10	20	60
Protein-lipopoly-saccharide	1/16	-	1/2	20	60
			1/10	20	20
Heat-stable lipopoly-saccharide	1/512	1/32	1/2	20	10
			1/10	20	10
			1/50	20	0
Formalin-killed vaccine	1/256	1/64	1/2	10	60
			1/10	10	60

[a] Mice were injected intraperitoneally with 0.2 ml of the IgM-containing fraction of immune rabbit serum diluted in sterile, pyrogen-free saline. Four hours later, viable PA-7 cells suspended in 0.2 ml of 10 percent TSB-saline were injected intraperitoneally, Deaths were recorded for a 72 hour period post-challenge.

[b] Aliquots of the serum fractions were incubated with equal volumes of 0.2 M 2-mercaptoethanol immediately before use in the passive hemagglutination test (PHA) as described in the Materials and Methods.

Table V. Passive protection of mice provided by the IgG-containing fraction of immune rabbit serum.[a]

Immunizing Agent	Titer of IgG Fraction		Dilution of Fraction	Challenge Dose (LD 50)	Percent Survival
	PHA	2-MePHA[b]			
Cell walls	1/256	1/256	1/2	20	83
			1/10	20	70
			1/30	20	50
	1/1024	1/1024	1/10	20	100
			1/25	20	67
			1/50	20	25
Protein lipopoly-saccharide	1/512	1/512	1/10	20	67
			1/50	20	0
Heat-stable lipopoly-saccharide	1/32	1/32	1/2	20	44
			1/10	20	56
Formalin-killed vaccine	1/32	1/32	1/2	20	78
			1/10	20	78

[a] Mice were injected intraperitoneally with 0.2 ml of the IgG- containing fractions diluted in sterile, pyrogen-free saline. Four hours later, the animals received the challenge organisms suspended in 0.2 ml of 10 percent TSB-saline. Deaths were recorded for a 72-hour period after the challenge injection.

[b] Aliquots of the serum fractions were incubated with equal volumes of 0.2 M 2-mercaptoethanol immediately before use in the passive hemagglutination test (PHA) as described in the Materials and Methods.

afforded similar degrees of protection to mice against a Pseudomonas aeruginosa infection. Figure 12 shows the precipitin curves obtained when heat-stable lipopolysaccharide was precipitated by these serum fractions. At the zone of equivalence, 66 µg of IgG antibody was precipitated while only 16 µg of IgM antibody was precipitated at the equivalence point; since the molecular weight of the IgM globulins is approximately 6 times that of IgG molecules, the above values show that the IgG serum fraction contains 15 - 20 times as many immunoglobulin molecules per unit volume, as does the IgM fraction. Thus it seems reasonable to suppose that a larger number of immunoglobulins in the IgG fraction may be responsible for the enhanced protective effect noted generally for the IgG fractions.

V. Clearance Studies

Preliminary clearance tests were performed on mice which had survived (for 72 hours) an intraperitoneal challenge of viable cells numbering less than, or equal to, the LD50. The spleen, liver, peritoneal fluid, and heart blood were assayed for the presence of viable PA-7. Previous studies have shown that the majority of bacterial cells injected intraperitoneally into mice are sequestered and cleared by the liver and spleen (Benecerraf et al. 1959). Organisms isolated from the peritoneal fluid in our studies were considered to indicate persistence and probably multiplication of the injected bacteria, while Pseudomonas aeruginosa

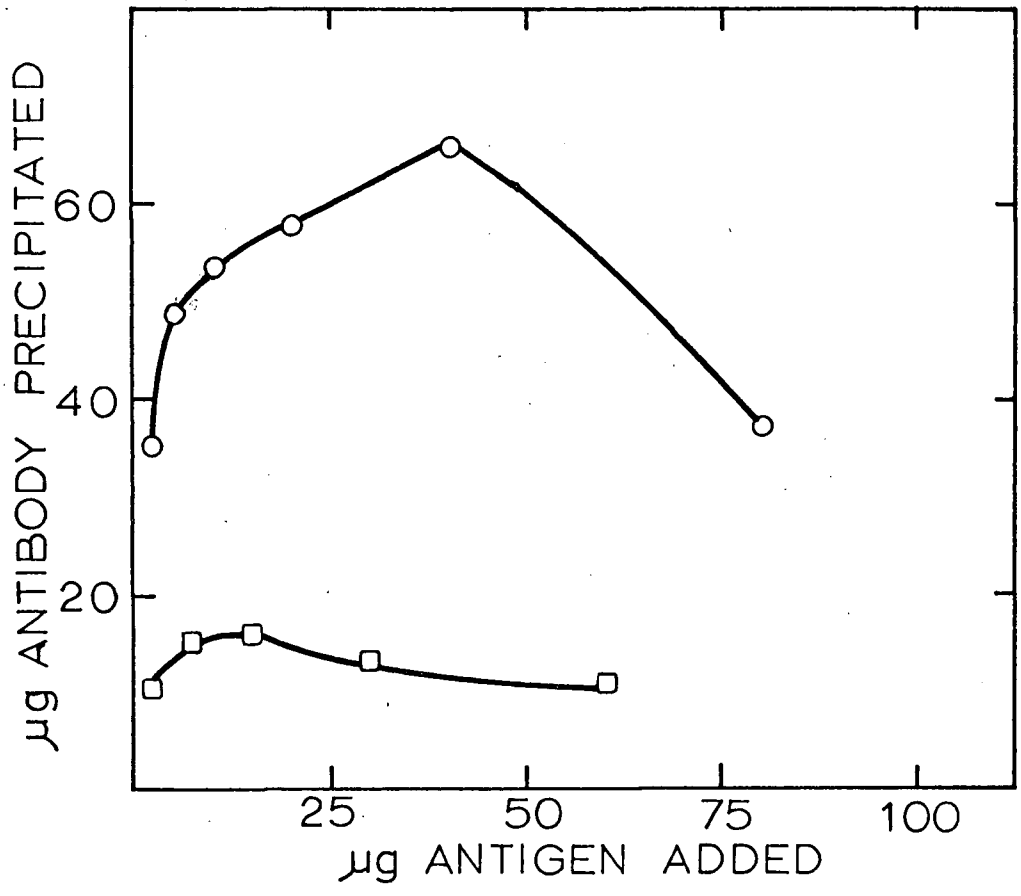


Figure 12. Precipitation analysis of purified IgG and IgM serum fractions.

Serum prepared against the cell wall preparation was fractionated on Sephadex G-200 and DEAE-cellulose as described in the Materials and Methods to obtain purified immunoglobulin fractions. These fractions were used in the precipitation tests as described in the Materials and Methods.
Symbols: O—O, IgG; □—□, IgM.

in the heart blood was thought to indicate inadequate clearance of the injected organisms, allowing for seeding of the bacteria from the peritoneal fluid, and possibly from the spleen and liver, with generalized spread throughout the body. Most of these control mice were found to be cleared of injected cells by 3 - 5 days after the challenge.

The passive protection data presented earlier had shown that most deaths in the immune serum-protected groups occurred within the first three day post-challenge period; it was thus considered of interest to determine whether or not the challenge organisms were cleared from the survivors of this 72 hour period. Table VI presents the results obtained when mice passively protected by immune rabbit sera were sacrificed at different time intervals after the challenge injection, and their organs assayed for PA-7. Although there is a certain amount of variation in the clearance patterns among different animals within the same group, definite trends were seen. Experiments involving cell wall antisera were interesting because of the faster rate of clearance of organisms from animals protected by serum taken later in the response (day 44), as opposed to that observed in mice protected by antiserum taken at an earlier date (day 11). Figure 8 showed that no 2-mercaptoethanol resistant antibody was detectable in serum obtained on day 11, whereas approximately 50 percent of the passive hemagglutinating antibody was resistant to the action of 2-mercaptoethanol

Table VI. Clearance of challenge organisms from mice passive protected by immune rabbit serum.[a]

Immunizing Agent	Antiserum Dilution	Challenge Dose (LD 50)	Days Post Challenge	Clearance[b] Organ			
				Spleen	Liver	PF	HB
Cell Wall (serum from day 11)	1/2	20	3	0/5	0/5	3/5	5/5
			6	4/4	4/4	1/4	3/4
	1/20	20	3	0/4	0/4	2/4	4/4
			6	4/4	4/4	3/4	4/4
Call Wall (serum from day 44)	1/2	20	3	4/4	4/4	4/4	4/4
	1/10	20	3	4/4	4/4	4/4	4/4
Protein-lipo-polysaccharide	1/2	20	5	3/5	2/5	4/5	5/5
			8	2/5	2/5	3/5	4/5
Formalin-killed vaccine	1/1	20	7	4/4	4/4	4/4	4/4
	1/2	20	7	0/4	0/4	ND[c]	ND
	1/2	10	7	4/4	4/4	ND	ND

[a] Organs and fluids were removed from survivors of the 72-hour test period in the passive protection tests, and qualitatively assayed for the presence of PA-7 cells.

[b] Reported as the number of the particular organ free of viable PA-7, over the number of mice assayed.

[c] N.D. - not determined

Abbreviations: PF - peritoneal fluid; HB - heart blood

in the serum obtained on day 44. It is unclear why the later antiserum promoted an increased rate of clearance, particularly since Bjornson and Michael (1970) have reported that IgM molecules appear to be at least as efficient as IgG globulins in an opsonophagocytosis assay. This data does correlate well, however, with passive protection studies involving the purified immunoglobulin fractions (Tables IV and V) in which the IgG fractions were found generally to offer better protection.

Data from experiments in which antiserum prepared against the formalin-killed vaccine was used as the source of protecting antibodies (Table VI) would appear to indicate that both the size of the challenge dose and the antiserum dilution affect the rate of clearance of the injected bacterial cells, suggesting that there is an optimal ratio of organisms to antibody molecules within the body of the mouse for maximum clearance.

The reason for the poor clearance of PA-7 from mice protected by serum prepared against the protein-lipopolysaccharide fraction, in spite of the good protective ability of this serum, is not known. It is possible that this serum is lacking in specific opsonins, and simply exerts a delaying action on the lethal activity of the injected bacteria; an effect of this sort was noted by Laborde and de Fajardo (1969) when some of a group of mice which had been protected by immune rabbit serum prepared against whole cell vaccines died 10 - 20 days after challenge with Pseudomonas aeruginosa.

The poorer clearing potential of this serum may also have been due to a lack of bactericidal activity. Bjornson and Michael (1970) and Muschel et al. (1969) have reported that some strains of Pseudomonas aeruginosa are sensitive to a complement dependent bactericidal activity in normal and immune serum. However, using the methods outlined by these workers, we were unable to demonstrate significant bactericidal activity, even with undiluted whole serum (prepared against the cell wall fraction). Thus, we were not able to test the hypothesis proposed above, and thus unable to offer an explanation for the lack of clearance of injected organisms in the mice protected by serum prepared against the protein-lipopolysaccharide.

GENERAL DISCUSSION

Four antigenic fractions, namely the heat-stable lipopolysaccharide, protein-lipopolysaccharide, and cell wall preparations, and the formalin-killed vaccine, were prepared from a strain of Pseudomonas aeruginosa and compared, not only in terms of their ability to stimulate serum antibodies, but also in terms of their capacity to elicit the production of protective antiserum. All four antigens were found to be immunogenic in rabbits, although differences in the level and duration of the response elicited were noted. In addition, antisera prepared in rabbits against all four fractions were found to be protective for mice challenged with the homologous strain.

Studies by Jones and his coworkers (1971) have demonstrated that antiserum taken from mice three days after immunization with a fraction from the culture filtrate of Pseudomonas aeruginosa were able to protect mice against challenge by the homologous strain. No agglutinating antibody activity was demonstrable in this serum, although the protective activity could be removed from the serum by absorption with bacterial cells of the homologous strain. It seems likely then, that the protection afforded by the early mouse serum was due to antibody, but that the titer was too low to be detected by the bacterial agglutination test. In fact,

because the protective factor was associated with the macroglobulin serum fraction, Jones and coworkers proposed that early IgM production was responsible for the protective effect. Bass and McCoy (1971) also demonstrated that the protective ability of a serum was directly related to its anti-*Pseudomonas* antibody content. The results presented in this study show a direct relationship between the antibody content of a serum, and its protective ability; moreover, this antibody activity can be most easily and sensitively measured by means of the passive hemagglutination test.

There is some question as to the mechanism by which immune serum exerts its protective effect and also as to which of the two major immunoglobulin classes is the more important in this regard. IgM globulin molecules have been reported to be more efficient, on a molar basis, in *in vitro* tests such as opsonophagocytosis, complement-dependent bactericidal reactions, and agglutination-type reactions (Pike and Chandler, 1969; Bjornson and Michael, 1970), and thus might be expected to be more efficient in promoting the elimination of injected organisms by the reticulo-endothelial system. However, at the same time, the IgG antibodies appear to be better able to protect mice against experimental bacterial infections (Dolby and Dolby, 1969; Bjornson and Michael, 1970). One explanation which has been offered for this anomaly is that the smaller IgG molecules are able to diffuse more rapidly than IgM globulins, and thus enter the circulation much more quickly

to help counteract the spread of organisms from the infection site, (Bjornson and Michael, 1970). Our results lend support to this hypothesis, since they indicated that serum containing a high proportion of 2-mercaptoethanol resistant antibody activity was more effective in eliminating injected organisms than was serum taken earlier in the response and containing mainly IgM antibody.

Landy and his coworkers (1965), working with Salmonella enteritidis antigens in rabbits, have reported that, whereas a single injection of antigen stimulated serum antibody production, a hyperimmunizing series of injections was necessary to stimulate an IgG response. The data presented in this report also shows that IgG antibodies make up a significant portion of the antibody response to the cell wall antigenic fraction (and seemingly to the other antigenic preparations as well) only late in the response to the series of injections; recall injections, however, stimulated a much more rapid rise in the IgG titer.

On the basis of the results obtained by Bjornson and Michael (1970), and upon consideration of our data which suggests that, for two of the four types of antisera at least, IgG globulins may be more protective, it would appear that antigens capable of stimulating the production of IgG antibody may be desirable for further studies. All four of our antigenic fractions could thus be considered to be useful antigens, since all were found to stimulate the production of both IgM and IgG antibodies in rabbit serum;

in addition, all four preparations are relatively non-toxic for experimental animals.

The cell wall fraction might be considered to be the most complete antigen, rendering it useful for comparative purposes with the soluble antigenic preparations. In fact, immunodiffusion analysis has indicated that there is at least one common antigen in all of these preparations. That this antigen may be identifiable with the heat-stable lipopolysaccharide fraction which coats onto sheep red blood cells is suggested by the ability of all four anti-serum types to react with this antigen. It is as yet unclear, though, whether or not this common antigen is protective, and also, what, if any, is its relationship to the protective antigen of Pseudomonas aeruginosa which can be isolated from the slime layers (Alms and Bass, 1967).

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