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Role of Nonagglutinating Antibody in the Protracted Immunity of Vaccinated Mice to *Pseudomonas aeruginosa* Infection

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Effective immunization against infection with *Pseudomonas aeruginosa* is difficult to evaluate because agglutinin levels decline rapidly. Because fractionation of hyperimmune sera often yields more specific antibody than can be accounted for by direct agglutination tests, an immunoglobulin-specific assay based on antiglobulin augmentation was used to characterize antibody responses of C3H/HeJ mice vaccinated with *P. aeruginosa* type 2 lipopolysaccharide. Nonagglutinating antibodies, initially detected at 2 weeks post-primary vaccination, were predominantly immunoglobulin G after 5 weeks, and they remained elevated at levels usually 32-fold higher than the direct titer throughout the 4-month study period. The sequential production of immunoglobulin M, then immunoglobulin G, followed that found in orthodox immunological responses. Sera that contained nonagglutinating antibodies but not direct agglutinins (14 to 16 weeks) enhanced phagocytosis of *P. aeruginosa* type 2 by macrophages from unimmunized mice and passively immunized mice against lethal challenge doses; bactericidal activity of these sera was not demonstrated in the presence or absence of complement. When challenged with 1, 10, and 100 50% lethal doses at 16 weeks, survival rates of actively immunized mice were significantly higher than those of unvaccinated mice ($P < 0.001$). Thus, at a time when no direct agglutinins were detectable, the augmented system detected nonagglutinating antibodies that could confer protracted resistance in vaccinated mice to pseudomonas infection.

Pseudomonas aeruginosa is a frequent cause of infectious morbidity and mortality in patients with underlying diseases that cause severe impairment of host defense mechanisms (8, 14) such as neoplastic diseases (13, 25), renal transplantations (1), and burns (2, 3). Because this bacterium has been found to be unusually recalcitrant to antimicrobial agents in vivo, infections with *P. aeruginosa* have been difficult to control and/or eradicate in these patients. Therefore, prevention of infections, as well as control of established infections, has assumed major importance in the management of patients from these susceptible populations.

Immune mechanisms have come under scrutiny as potentially useful in the prevention and control of pseudomonas infections, and numerous studies into the basis of pseudomonas immunity have been performed. Antibodies have been found to play an important role in immunity to *P. aeruginosa* infections (5-7, 24, 26). The protective capability of anti-pseudomonas antibody against infection with *P. aeruginosa* has been assessed to be one of a type-specific opsonin that facilitates optimal phagocytosis and killing of *P. aeruginosa* in the presence of functioning granulocytes (5-7, 20, 24, 26). Bjornsen and Michael (5-7) have also shown that immunoglobulin (Ig)G had a greater role than IgM in in vivo protection of mice from pseudomonas infection. Direct bactericidal action of antibodies plus complement on *P. aeruginosa* has not been demonstrated (5, 6). It is not known if cell-mediated immunity plays a role in resistance to *P. aeruginosa*.

Because humoral antibodies appear to be important in resistance to pseudomonas infection, it is not surprising that active immunization with pseudomonas vaccines in humans should prove to be useful in the management of infections in susceptible patient populations. A heptavalent lipopolysaccharide pseudomonas vaccine has been developed by Hanessian et al. (16) that is based upon the seven immunotypes described by Fisher et al. (12); this vaccine has been used to actively immunize burn patients and cancer patients as a possible means of preventing or improving the outcome of pseudomonas infec-

tions in these patients. Such immunization would appear to be effective in burn patients (3, 4, 11), but equivocal results have been obtained with vaccination of cancer patients (15, 22, 23, 27). Haghbin et al. (15) reported that the response to vaccination was not entirely predictable because the hemagglutinin responses varied considerably from one individual to another and from one immunotype to another; also, a substantial portion of the antibodies produced were susceptible to 2-mercaptoethanol and thus were regarded as IgM. They also noted that the degree of antibody response in an individual did not always correlate with resistance to infection; one patient who developed a bacteremia and expired had a homologous hemagglutination antibody titer of 256 (without 2-ME) and <4 (with 2-ME), while another patient who recovered from a bacteremia had an HA titer of 512 without 2-ME inactivation and <4 with inactivation. Young and Meyer (27) also noted that serum antibody (hemagglutination antibody) levels had been low in a few vaccinated leukemic patients who had later become infected with *P. aeruginosa* and had ultimately recovered from their infections. The antibody responses were usually of short duration, and repeated booster doses did not necessarily elicit increases in antibody levels (15, 27). Also, the inability to predict protective IgG antibody production after vaccination was found to be a serious problem in the studies by Pennington et al. (22, 23).

The lack of correlation between passive hemagglutinin levels and the opsonins that enhanced phagocytosis in sera from vaccinated humans (20, 24) or the ability of such sera to protect mice against lethal challenges of *P. aeruginosa* (9, 10, 19, 20) presented an intriguing problem that was further complicated by serum fractionation data. Such separation of hyperimmune sera yielded much more IgG-specific antibody than could be accounted for by the passive hemagglutination test (18, 19, 22, 23). Consequently, it seemed possible that some of the unexplained problems in evaluation of the immune response to vaccination could be due to the utilization of methods that are relatively insensitive for the detection of all immunoglobulin classes in whole serum.

The current study was undertaken to: (i) evaluate the protracted resistance to *P. aeruginosa* infection in mice after primary vaccination, (ii) determine by a highly specific immunoglobulin assay the presence and sequence of immunoglobulin classes after primary and secondary stimulation by vaccine, and (iii) evaluate the role of nonagglutinating antibodies in the resistance to *P. aeruginosa* infection.

MATERIALS AND METHODS

Animals. C3H/HeJ female mice that had the same birthdate were obtained from Jackson Laboratories, Bar Harbor, Me. The mice were housed in groups of 10 per cage and provided with standard pellet diet and water ad libitum. Mice were used in the experiments at the indicated ages.

Antigens and antisera. A strain of *P. aeruginosa* serotype 2 (PA2) and a lipopolysaccharide antigen (LP-PA2) that had been extracted from PA2 (16) were kindly donated by M. Fisher (Parke, Davis, & Co., Detroit, Mich.). A whole cell antigen of PA2 (W-PA2) was prepared from growth that had been harvested in phosphate-buffered saline (PBS), pH 7.2, and 18-h Mueller-Hinton agar plate cultures and heated at 121°C for 1.5 h. Mouse sera that contained agglutinins to PA2 were obtained from 10 mice that had been immunized with W-PA2 and exsanguinated on day 10 post-immunization; the pooled sera had a PA2 agglutinin titer of 1:80.

Immunization and serological procedures. At 11 weeks of age mice (20 to 25 g) were immunized by a single intraperitoneal injection of 100 μg of LP-PA2 in 0.5 ml of PBS. Booster dosages of LP-PA2 were given when the PA2 bacterial agglutinin titer in the immunized mice had fallen to undetectable levels. Half of the remaining mice were given single booster doses of 10 μg of LP-PA2/0.5 ml of PBS, and the other half were given a single booster dose of 1 μg of LP-PA2/0.5 ml of PBS. Nonimmunized mice were used as control animals. Forty immunized and 20 nonimmunized mice were maintained for test serology bleedings. Mice were bled from the retro-orbital plexus weekly with calibrated capillary pipettes. The blood was expressed into saline containing 2 units of heparin per 100 ml so that a 1:10 dilution of plasma was obtained. The agglutinin titer to PA2 of the plasma was determined by the bacterial agglutination test. At the time of the booster dosages, two groups of 20 immunized mice each were given either a 10- or 1-μg booster.

Bacterial agglutination test. Twofold serial dilutions (1:10 to 1:5,120) of the plasma in PBS were prepared in tubes, and 50 μl of each dilution was pipetted into a microagglutination tray. An equal volume of W-PA2 that had been adjusted to an optical density of 0.2 at 540 nm was added to each dilution. The tray was sealed to prevent evaporation, left at room temperature for 4 h, and placed at 4°C overnight. The agglutinin titer was considered to be the highest dilution in which agglutination still occurred. As controls, plasma incubated without W-PA2, and W-PA2 in normal mouse serum gave negative results, whereas W-PA2 in twofold serial dilutions of the hyperimmune LP-PA2 serum gave positive results.

Antimouse immunoglobulin fractions and preparations. Antisera that were specific for the heavy chains of mouse immunoglobulins were obtained as follows: total immunoglobulins produced in goats (Ig) (Cappel Laboratories, Downington, Pa.); IgG_1 and IgG_2 produced in goats (Meloy Laboratories, Springfield, Va.); and IgM produced in rabbits (Miles Laboratories, Elkhardt, Ind.). Each antiglobulin fraction was absorbed with 1/5 volume of packed W-PA2 cells for 4 h at 37°C and centrifuged at 3,000 rpm for

30 min, and then the absorption procedure was repeated. After the second absorption, the fractions were clarified by centrifugation and filtration and were found to contain no PA2 agglutinins by the bacterial agglutination test as described above. Four-milliliter portions of the absorbed anti-immunoglobulin fractions were stored at −70°C until used.

Optimal dilutions of anti-mouse immunoglobulin fractions for the indirect agglutination of mouse immunoglobulin-coated PA2 cells. A subagglutinating dilution (1:160) of anti-PA2 mouse serum was incubated with an equal volume of W-PA2 for 6 h in a 50°C waterbath and placed at 4°C overnight. The coated cells were sedimented and washed three times with PBS by centrifugation; then, the cells were resuspended with PBS to the original volume. Twofold serial dilutions (1:2.5 to 1:80) of the anti-immunoglobulin fractions were prepared in PBS in a microagglutinating tray, and an equal volume (50 μl) of the agglutinin-coated W-PA2 cells was added to each dilution. The tray was incubated for 4 h at room temperature and placed at 4°C overnight. The optimal dilution for the indirect agglutination of immunoglobulin-coated cells was considered to be one dilution less than the highest dilution in which agglutination occurred. The optimal dilutions of Ig, IgG_1, IgG_2, and IgM used in the study were 1:5, 1:5, 1:2.5, and 1:5, respectively.

Indirect agglutination test. Equal volumes of appropriately diluted W-PA2 were added to each of the original plasma dilutions that gave no agglutination in the bacterial agglutination test. The tubes were incubated for 6 h in a 50°C waterbath and placed at 4°C overnight. The W-PA2 cells were sedimented and washed three times in PBS by centrifugation and were then resuspended in PBS to the original volume of the antigen. Four wells of a microagglutination tray were filled with 50-μl volumes of the washed cells from each dilution. Equal volumes of the optimal dilutions of each anti-mouse immunoglobulin fraction were added to one set of washed cell dilutions. The tray was incubated for 4 h at room temperature and placed at 4°C overnight. The titer of PA2 agglutinins that coated the bacterial cells but could not initiate agglutination of the cells corresponded to the highest dilution in which agglutination occurred in the presence of anti-mouse immunoglobulin fractions. As a control, W-PA2 was incubated with normal mouse serum that contained no PA2 agglutinins and tested as above; results were negative.

Mouse challenge experiments. A litmus milk culture of PA2 was used to inoculate a Mueller-Hinton agar plate that was then incubated for 18 h at 37°C. The resulting growth was harvested in PBS, washed three times, and resuspended in PBS. The suspension was adjusted with PBS so that a 1:10 dilution had an optical density of 0.3 to 540 nm. The suspension was further diluted until a cell concentration was reached that had been previously shown to kill 50% nonimmunized mice in 7 days (2.8×10^3 organisms per ml). The final concentration was suspended in a 4% hog gastric mucin adjuvant (12) and was given intraperitoneally in a 1-ml volume per mouse. Equal numbers of immunized and nonimmunized mice were used in each challenge experiment. The number of viable bacteria per ml in the final concentration was determined by colony-forming units. Final survivor values were taken 7 days postchallenge.

Passive protection tests in mice. Mice were passively immunized by an intraperitoneal injection of 0.2 ml of serum; 4 h later, they were challenged with 1 ml of PA2 suspension diluted in PBS to an equivalent of about 1, 10, or 100 50% lethal doses (LD_{50}) of PA2 per animal, as indicated in the previous experiment. As controls, nonimmunized mice also received corresponding challenges. Final survivor values were taken 7 days postchallenge.

Bactericidal activity of serum. A culture of PA2 was grown in Mueller-Hinton broth at 37°C overnight. The growth was harvested by centrifugation and resuspended in broth so that a 1:10 dilution had an optical density of 0.3 at 540 nm. The suspension was then diluted until a cell concentration of 10^4 organisms per ml was reached (previously determined by actual colony count). Volumes of 0.1 ml of the broth culture were placed in four sterile tubes (12 by 75 mm). Sera collected from mice 16 weeks after primary vaccination or from mice 1 week after secondary vaccination were added in 0.2-ml volumes to each of two tubes. Guinea pig serum (Gibco) that had been previously absorbed with PA2 diluted 1:3 in broth was used as a source of complement and added in 0.2-ml volumes to two tubes, one of which also contained 0.2 ml of mouse serum. Mueller-Hinton broth as the diluent was added to the remaining tubes to give a final volume of 0.5 ml. Mouse sera from unvaccinated mice and from a commercial source were used as controls. The bactericidal activity of each serum after heating at 56°C for 30 min was also tested. The tubes were shaken gently and incubated for 2 h in a 37°C waterbath with frequent shaking. Afterwards, 5 ml of cold broth was added to each tube as a constant dilution factor. All tests were performed in duplicate and on two separate occasions. Viable colony counts were determined by the pour plate method, and the average of the four runs was determined.

Preparation of macrophage monolayers. Mice were exsanguinated after cervical dislocation and 3 ml of Hanks balanced salt solution (HBSS) with sodium bicarbonate containing 100 U of heparin per ml were introduced into the peritoneal cavity of each animal. After gentle massage of the abdomen, the peritoneal exudates were removed with heparinized blunt-tip pipettes, pooled, and placed in a sterile glass tube. The cell suspensions were centrifuged at $200 \times g$ for 10 min in a refrigerated centrifuge. After washing in HBSS again, the cells were resuspended in HBSS. Cell viability was determined to be 95% with trypan blue exclusion tests, and a cell count was performed on a Coulter counter (Coulter Electronics, Inc., Hialeah, Fla.). Suspensions of 3×10^6 to 4×10^6 viable macrophages were added to (30 by 15 mm) plastic dishes (Falcon Plastics) which contained 2 ml of Eagles medium enriched with 0.3% (vol/vol) of L-glutamine. The cells were allowed to settle and adhere for 2 h at 37°C in moist air and 5% CO_2.

Opsonophagocytic index of serum. PA2 was grown in Mueller-Hinton broth at 37°C overnight. Bacteria were harvested by centrifugation, washed twice in HBSS, and resuspended in HBSS to give an

adjusted concentration of 10^6 organisms per ml. Equal volumes of PA2 and test sera were incubated at 37°C for 10 min. The supernatant fluids from the previously prepared monolayers were decanted, and the monolayers were washed with 10% heat-inactivated fetal calf serum in HBSS. The monolayers were then recovered with 1.9 ml of HBSS. The bacterial-serum mixture in 0.1-ml portions was added to each monolayer, and the dishes were incubated at 37°C in 5% CO_2 moist air for 60 min. After decanting the supernatant fluids and washing the monolayers twice, dishes were then air-dried and stained with Lucke pyronin stain (21). The total number of intracellular bacteria in 100 macrophages was counted. The phagocytic and opsonophagocytic indexes for each serum were determined by the following formulae: phagocytic index = total number of bacteria in 100 macrophages/100 and the opsonophagocytic index = phagocytic index with test serum from immunized mice/phagocytic index with serum from non-immunized mice.

Statistical analysis. The probability of 0.05 ($P = 0.05$) was chosen as the level of statistical significance. A difference was judged as statistically significant only when the calculated chi-square equalled or exceeded the tabulated chi-square at a probability of 0.05.

RESULTS

Optimal antibody response to vaccination with LP-PA2. Four concentrations of LP-PA2 of 100, 10, 1, and 0.1 μg were used as primary vaccination doses for four groups of 10 mice each. A secondary vaccination dose of 10 μg was given to each mouse 12 weeks later. Mice were bled on days 0, 3, 7, 10, 14, 21, 35, and weekly thereafter. Sera from the specific day's bleedings were pooled and examined for agglutinins against PA2. Results of these titrations are shown in Fig. 1. The lower concentrations of 1 and 0.1 μg/mouse elicited no detectable antibody in their respective groups after primary vaccination. However, the antibody titers in the groups that had received 10 μg/mouse and 100 μg/mouse rose rapidly. The agglutinin titer in the 10-μg group peaked on day 7 at 1:40 and fell to undemonstrable levels by day 10. The peak titer for the 100-μg group at days 10 and 14 was fourfold higher, and the decline in titer was more gradual, as an undetectable agglutinin level was not reached until week 11 after primary vaccination.

After the secondary vaccination of the mice at week 12, the response kinetics of the 10- and 100-μg groups were similar to those obtained after the primary vaccination; the titer of the 10-μg group peaked at 1:40 1 week after booster and that of the 100-μg group peaked at 1:160 2 weeks after secondary vaccination. Although the titers declined at about the same rate in both groups, the decreases were much more gradual than after primary vaccination and, 6 weeks after secondary vaccination, the mice in both groups still had detectable antibody levels. In the two groups that had no detectable agglutinin levels after primary vaccination, there were demonstrable antibody titers in mice from both groups after secondary vaccination. The antibody response in the 0.1-μg group appeared to be basically similar to the primary response to the 10-μg vaccination, but was maintained through 6 weeks after secondary vaccination. The agglutinin peak level of the 1-μg group was twofold higher than the response elicited in the 10-μg group, but the level was the same for both groups 6 weeks later, 1:10.

Thus, the response to vaccination appeared to be dose related, and smaller vaccine doses prob-

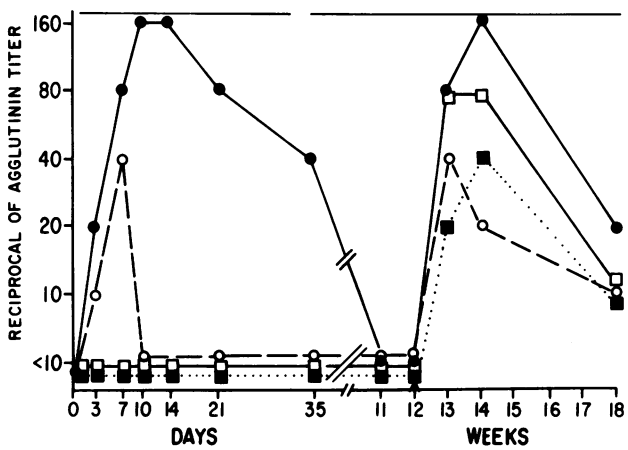

FIG. 1. *Relationship of primary and secondary immunization with LP-PA2 and the magnitude of the antibody response in mice. Pooled sera from 10 mice similarly treated were used for each determination. Vaccine dose per mouse: 100 μg (●); 10 μg (○); 1 μg (□); 0.1 μg (■); 10-μg booster (↑).*

ably primed recipients to respond to a greater degree when given a booster dose. However, since the vaccine dosage of 100 μg/mouse gave a higher and more substained response than the other concentrations, it was used as the immunizing dosage in all subsequent experiments.

Protracted resistance to PA2 infection in vaccinated C3H mice. Mice that had been vaccinated with 100 μg of LP-PA2 and mice that were unvaccinated were subjected to challenge doses of PA2 at various weeks after primary and secondary immunization (Table 1). The mice in both groups were challenged on the specific days with 1 LD_{50} (2.8×10^3 microorganisms) in most instances and with 10 and 100 LD_{50} on one occasion. Immunity provided by primary vaccination was still evident at 16 weeks post-immunization (90% survival rate), although agglutinin titers had declined to undetectable levels; agglutinin titers had never exceeded 1:80 (peaked at week 2), a relatively low level when compared to the levels obtained by active immunization with other bacterial vaccines. Not only was the immunity long-lasting, but it was also very efficient in that no more than 11% of the mice ever succumbed upon challenge with PA2. Survival rates among the vaccinated mice were significantly higher than among the unvaccinated controls. In fact, vaccinated mice were able to survive challenge doses of 1 and 10 LD_{50} at a time when the agglutinin levels against the challenge bacterium were not detectable in sera from these mice.

A booster vaccination dose of 10 μg was given to one group of mice. One week later, this group was challenged with 1, 10, and 100 LD_{50}. There was no appreciable difference between the survival rates of the secondary vaccinated mice and those of the primary vaccinated mice who received the same challenge doses; for the mice who received 100 LD_{50}, 79% of them survived. Survival rates of unimmunized mice never exceeded 40%, and the percent of survivors decreased as the challenge doses increased.

Passive protection against PA2 infection with serum from immunized mice. Pooled sera obtained from mice 15 weeks after primary vaccination were used to immunize unvaccinated mice passively before challenge with PA2 (Table 2). As previously mentioned, there were no detectable agglutinins in these pooled sera. Each mouse was given 0.2 ml of serum (diluted 1:10) intraperitoneally 4 h before intraperitoneal challenge with 1, 10, or 100 LD_{50}. As a control, the mice were given 0.2 ml of undiluted normal mouse serum (no agglutinins detected by direct and indirect test) 4 h before the challenge doses. The sera from the vaccinated mice protected nonimmunized mice against fatal PA2 infection, while normal serum possessed little protective activity. Moreover, when the challenge dose was increased 10- to 100-fold, protection by immune serum was also demonstrable. Thus, depending on the challenge dose, treatment with normal serum produced survival rates of 16 to 45%, while immune sera produced statistically higher survival rates of 69 to 90%.

Serum bactericidal activity. Table 3 shows the average number of PA2 growing in the presence of serum and/or complement after incubation at 37°C for 2 h. There was no evidence that serum, whether immune or nonimmune, exerted any bactericidal activity, as the total number of viable organisms in either serum was not appreciably different from controls containing broth only; nor did complement alone affect

TABLE 1. *Resistance of C3H mice to PA2 challenge at various times after vaccination*

Weeks post-immunization	Agglutinin titer	Challenge dose (LD_{50})	% Survivors among C3H mice[a]		P value
			Not immunized	Immunized	
2	80	1	25	89	<0.001
5	40	1	33	94	<0.01
15	<10	1	45	95	<0.02
16	<10	1	40[b]	90	<0.02
		10	25[b]	90	<0.001
16[c]	160[d]	1	40[b]	100	<0.01
		10	25[b]	85	<0.001
		100	16	79	<0.001

[a] There were 20 mice in each challenge group. A total of 320 mice were used.

[b] Nonimmunized mice were a control group for immunized groups that received no booster dose or a 10-μg booster dose.

[c] Mice were given 10 μg/mouse as a booster dose at week 15.

[d] Titer at 1 week post-secondary vaccination.

TABLE 2. *Passive immunization of C3H mice with sera from immunized mice*[a]

Challenge dose (LD_{50})	% Survivors[b]	
	Immunized	Controls
1	90	45
10	80	25
100	69	16

[a] Sera from mice at 15 weeks post-primary vaccination; agglutinin titer of sera was <1:10. Each mouse was passively immunized with 0.2 ml of sera diluted 1:10 4 h before challenge. Controls were injected with 0.2 ml of undiluted normal mouse sera 4 h before challenge.

[b] There were 20 mice in each challenge group.

TABLE 3. *Bactericidal activity of sera from immunized mice against PA2*

Serum	Direct agglutinin titer	Avg no. of PA2			
		Complement[b] + serum + organism	Serum + organism	Organism + complement	Organism + diluent
14 weeks[b]					
Immunized	<10	2.5×10^4	2.31×10^4	2.06×10^4	2.7×10^4
15 weeks					
Immunized	<10	2×10^4	2.53×10^4	2.34×10^4	3.1×10^4
18 weeks					
No booster	<10	2.8×10^4	2.6×10^4	2.6×10^4	2.46×10^4
10-μg booster	160	2.78×10^4	2.49×10^4	2.07×10^4	2.9×10^4
1-μg booster	80	2.81×10^4	2.6×10^4	2.43×10^4	2.75×10^4
Normal mouse sera	<10	3×10^4	2.7×10^4	2.9×10^4	2.56×10^4
Nonimmunized	<10	2.4×10^4	1.9×10^4	2.2×10^4	2.6×10^4

[a] Absorbed guinea pig complement contained 86 $C'H_{50}$ units/ml.
[b] Time after immunization at which serum was drawn.

the viable count. Assays with complement in combination with heat-inactivated, normal, or immune sera also were not bactericidal for PA2.

Phagocytic activity of macrophages from unvaccinated mice in immune sera. As mice 16 weeks after primary vaccination and 1 week after secondary vaccination had already been shown to resist lethal challenge, sera from the corresponding groups were examined for opsonic activity in the presence of macrophages from nonimmunized mice, and then this activity was compared with the activity of sera from nonimmunized mice (Table 4). It was apparent that sera from vaccinated mice greatly enhanced the phagocytosis of PA2. Even in serum from primary vaccinated mice that contained no detectable agglutinins, more organisms (4.62) were ingested by the macrophages than when the organisms had been incubated in serum from nonimmunized mice (2.56). Sera from mice that had received 1- and 10-μg booster doses were even more efficient in opsonizing PA2; the average numbers of ingested bacteria for sera with agglutinin titers of 1:80 and 1:160 was 5.61 and 6.52, respectively, as compared to 2.56 for sera from nonimmunized mice. Whereas normal opsonophagocytic indexes ranged from 0.8 to 1.2 when the phagocytic index of test serum is compared to the phagocytic index of control serum, the index for the primary vaccination serum was higher than normal, 1.81, and those of the 1- and 10-μg secondary vaccination sera were as high as 2.19 and 2.55, respectively. However, if the phagocytic index of the primary vaccination serum was compared to the phagocytic indexes of the 1- and 10-μg booster serum, the opsonophagocytic index between primary and 1-μg booster sera was within the normal range, but the index between primary and 10-μg sera was slightly above the norm of 1.4. Thus, opsonins in sera from vaccinated mice, regardless of the detectable agglutinin levels, enhanced the phagocytosis of PA2, although secondary vaccination with the higher dose increased the opsonic efficiency of the sera to a slight degree.

TABLE 4. *Effect of immunization on the phagocytosis of PA2*

Serum[a]	Phagocytic index[b]	Opsonophagocytic index[c]
No booster	4.62	1.81
1-μg booster	4.62	2.19
10-μg booster	4.62	2.55
Control	2.56	

[a] Sera drawn at 16 weeks post-primary vaccination.
[b] Phagocytic index determined at 60 min.
[c] Opsonophagocytic index = Phagocytic index from immunized mice/Phagocytic index from nonimmunized control mice.

Presence of nonagglutinating antibodies in sera from immunized mice. As the agglutinin levels in sera from immunized mice did not appear to correlate entirely with the ability of the mice to resist infection with PA2, the likelihood of an antibody that was protective being present but undetectable by the ordinary agglutination test became a distinct possibility. To determine whether nonagglutinating antibodies were present in the sera of immunized mice, an indirect agglutination method similar to the Coombs test was used. After the ordinary agglutination reaction took place, the bacterial suspensions in tubes that exhibited negative reactions were centrifuged and washed twice in saline to remove any unbound antibodies, and the agglutinogen in each tube was resuspended in diluent to its original volume. Anti-mouse immunoglobulin serum that was specific for total immunoglobulin fractions and that had been absorbed previously with heat-killed PA2 cells

was then added to each tube. Indirect agglutinin titers were 4 to 32 times higher than the titers in the ordinary agglutination (Fig. 2). A marked rise of indirect agglutinin titers was noted as the direct agglutinin titers began to fall in the sera obtained at 5 weeks after primary vaccination. Indirect agglutinin titers of 1:160 were observed at 14 and 15 weeks after primary vaccination, times at which there were no demonstrable direct agglutinins. The level of the indirect agglutinin response in mice who received booster doses of 10 and 1 μg/mouse did not appear to be dose related because both groups had titers of 1:2,560 1 week after secondary immunization. Neither did the direct response appear dose related because the titers differed by only one tube (1:160 for the 10-μg group and 1:80 for the 1-μg group).

When sera from mice who had not been immunized were tested, there were no detectable antibodies by either the direct or indirect method. Thus, immunization with LP-PA2 not only initiated antibody production that was specific for PA2, but, by utilizing the indirect antibody method, it was proven that these antibodies were maintained at a fairly high level for extended periods of time.

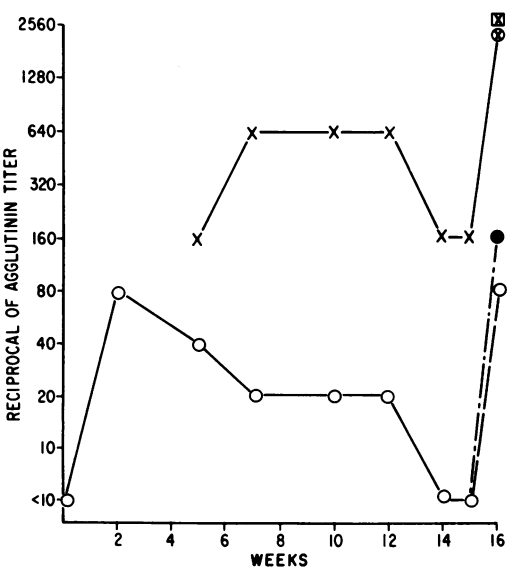

FIG. 2. *Agglutinin titers of C3H mouse sera to PA2 detected by direct agglutination and by indirect agglutination augmented by anti-mouse immunoglobulins. Symbols:* O——O, *Direct agglutinin titer;* O- -O, *direct agglutinin titer, 1-μg booster;* ●- -●, *direct agglutinin titer, 10-μg booster;* ×——×, *titer augmented with anti-mouse immunoglobulin;* ⊠, *10-μg booster augmented titer;* ⊗, *1-μg booster augmented titer.*

Immunoglobulin class of indirect agglutinins in vaccinated mice. Agglutinogens in the negative tubes were prepared as described above. Anti-mouse immunoglobulin sera that were specific for the heavy chains of IgM, IgG_1, and IgG_2 were added to an aliquot of each washed agglutinogen. The results are shown in Fig. 3. For the first 2 weeks, direct agglutinins were the only antibodies detected. By week 2, the direct agglutinin levels were beginning to decline and indirect agglutinins were detectable. IgM was the predominant class of indirect agglutinins at week 5, but while the IgM titers were usually higher than the direct titers at the various test times, the decline in IgM titers was similar to the decline seen in the direct agglutinin titers; the IgM indirect response to the two booster doses also closely paralleled the direct response to the secondary vaccinations. In contrast, IgG_1 indirect agglutinin titers, not demonstrable until week 5, also increased and, by week 7, had reached a titer of 1:640. Although the titers did decline eventually, but never below 1:80, the secondary vaccinations elicited rapid rises in titers that were 16 times higher than the direct agglutinin titers. IgG_2 indirect agglutinins were first detected at week 7 after primary vaccination and never fell below 1:160 (weeks 14 and 15), and the responses to secondary vaccinations with 10 and 1 μg were 16 and 8 times higher than the direct titers, respectively. It would then appear that IgG was the main immunoglobulin class in the nonagglutinating antibodies and that IgG_1 was involved earlier and declined earlier than IgG_2, while both subclasses responded rapidly and strongly upon secondary vaccination regardless of the dosage.

DISCUSSION

The use of a specific immunoglobulin assay to measure anti-pseudomonas antibodies of the IgM and IgG classes in sera from vaccinated mice was useful in the characterization of the primary and secondary responses to a lipopolysaccharide vaccine prepared from *P. aeruginosa*. Although the pattern of the antibody response to primary vaccination as detected by the direct agglutination test agreed with data previously reported (9, 18, 19, 22, 23, 27); namely, a rapid rise in antibody titer that peaked at 2 weeks postvaccination and a decline in titer to low levels at 1 month to indetectable levels at 4 months, an entirely different pattern evolved with the more sensitive test. At 5 weeks after primary vaccination when the direct titer had begun to decline, the indirect method detected specific antibody levels that were fourfold higher than the direct titer and, rather than declining,

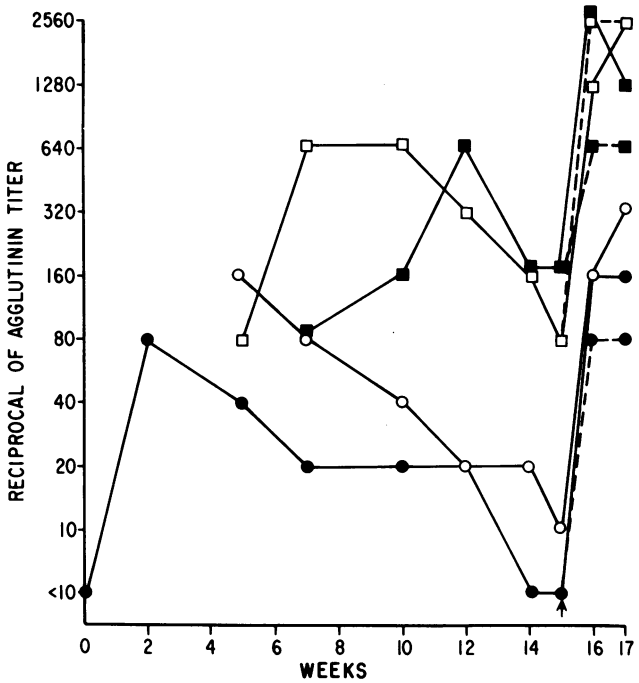

FIG. 3. *Immunoglobulin class of antibodies to PA2 in immunized C3H mice as detected by an indirect agglutination test. There was no IgM indirect titer response to the microgram booster dose. Symbols:* ●, *Direct agglutinin titer;* ○, *anti-IgM;* □, *anti-IgG*$_1$; ■, *anti-IgG*$_2$; ↑, *booster dose;* ——, *10 μg/mouse;* - - -, *1 μg/mouse.*

the antibody levels in the serum were increasing. After the initial rise, the antibody levels were always 32-fold higher than the direct titer, even when direct antibody levels were undetectable (14 weeks postvaccination). IgG was the predominant immunoglobulin class in the augmented titer. The augmented IgM titer was eightfold higher than the direct titer, but the slope of each was similar in that both declined as the time after vaccination lengthened.

After secondary stimulation by vaccine, the IgG activity increased sooner and reached titers eightfold higher than those noted in the primary response, whereas the direct agglutinin titer of the secondary response was the same as the titer of the primary response. The augmented IgM titer again differed only slightly from the direct titer. Surprisingly, the smaller booster dose was as effective in eliciting the IgG response as a larger dose. This finding is important because smaller booster doses can be used and the unpleasant side effects that accompany large vaccine doses could be avoided. Thus, with the immunoglobulin assay, the sequential production of IgM followed by IgG indicated that immunization with *P. aeruginosa* vaccine elicited an orthodox primary and secondary immunological response to vaccination.

The indirect antibody titers were also associated with significant protection in vivo and in vitro. Sera in which no direct agglutinins were detected but did contain indirect antibodies enhanced the phagocytosis of *P. aeruginosa* by nonimmune macrophages. Mice that had been actively immunized or passively immunized with the above-described sera were also protected against lethal challenge with *P. aeruginosa*. These findings imply that there are antibodies present in the sera of vaccinated animals that are incapable of causing agglutination, but can opsonize bacterial cells in order that *P. aeruginosa* can be effectively phagocytosed and killed. These non-agglutinating antibodies are maintained at high levels long after primary vaccination and are probably responsible for the resistance of immunized mice to *P. aeruginosa* infection at a time when the direct agglutinins are quite low. Thus, the commonly held belief that subjects with low direct antibody levels are insufficiently protected against pseudomonas infection is not necessarily true, and antibody levels should be determined by indirect methods before making such an assumption.

The potential usefulness of a *P. aeruginosa* vaccine in cancer patients, especially leukemic patients, has been hampered by insensitive

means of assessing the IgG antibody response to vaccination. With an augmentation immunoglobulin assay, the antibody response elicited by vaccination can be adequately monitored in these patients, and their immune status to *P. aeruginosa* can be determined with a greater degree of predictability.

LITERATURE CITED

1. **Anderson, R. J., L. A. Schafer, D. B. Olin, and J. C. Eickhoff.** 1973. Septicemia in renal transplantation recipients. Arch. Surg. **106**:692-694.
2. **Alexander, J. W.** 1967. Serum and leukocyte lysosomal enzymes: derangements following severe thermal injury. Arch. Surg. **95**:482-491.
3. **Alexander, J. W., M. W. Fisher, and B. G. MacMillan.** 1971. Immunological control of pseudomonas infection in burn patients: a clinical evaluation. Arch. Surg. **102**:31-35.
4. **Alexander, J. W., M. W. Fisher, B. G. MacMillan, and W. A. Altermeier.** 1969. Prevention of invasive pseudomonas infection in burns with a new vaccine. Arch. Surg. **99**:249-256.
5. **Bjornson, A. B., and J. G. Michael.** 1970. Biological activities of rabbit immunoglobulin M and immunoglobulin G antibodies to *Pseudomonas aeruginosa*. Infect. Immun. **2**:453-461.
6. **Bjornson, A. B., and J. G. Michael.** 1971. Contribution of humoral and cellular factors to the resistance to experimental infection by *Pseudomonas aeruginosa* in mice. I. Interaction between immunoglobulins, heat-labile serum factors, and phagocytic cells in the killing of bacteria. Infect. Immun. **4**:462-467.
7. **Bjornson, A. B., and J. G. Michael.** 1972. Contribution of humoral and cellular factors to the resistance to experimental infection by *Pseudomonas aeruginosa* in mice. II. Opsonic, agglutinative, and protective capacities of immunoglobulin G anti-*Pseudomonas* antibodies. Infect. Immun. **5**:775-782.
8. **Bryant, R. E., A. F. Hood, C. E. Hood, and M. G. Koenig.** 1971. Factors affecting mortality of gram negative bacteremias. Arch. Intern. Med. **127**:120-128.
9. **Crowder, J. G., M. W. Fisher, and A. White.** 1972. Type-specific immunity in pseudomonas diseases. J. Clin. Med. **79**:47-54.
10. **Davis, S. D.** 1975. Efficacy of modified human immune serum globulin in the treatment of experimental murine infections with seven immunotypes of *Pseudomonas aeruginosa*. J. Infect. Dis. **134**:717-721.
11. **Feller, I., and C. Pierson.** 1968. Pseudomonas vaccine and hyperimmune plasma for burn patients. Arch. Surg. **97**:225-229.
12. **Fisher, M. W., H. B. Devlin, and F. J. Gnabasik.** 1969. New immunotype schema for *Pseudomonas aeruginosa* based on protective antigens. J. Bacteriol. **98**:835-836.
13. **Fishman, L. S., and D. Armstrong.** 1974. *Pseudomonas aeruginosa* bacteremia in patients with neoplastic disease. Cancer **30**:764-773.
14. **Freid, M. A., and K. L. Vosti.** 1968. Importance of underlying disease in patients with gram negative bacteremias. Arch. Intern. Med. **121**:418-423.
15. **Haghbin, M., D. Armstrong, and M. L. Murphy.** 1973. Controlled prospective trial of *Pseudomonas aeruginosa* vaccine in children with acute leukemia. Cancer **32**:761-766.
16. **Hanessian, S., W. Regan, D. Watson, and T. H. Haskell.** 1971. Isolation and characterization of antigenic components of a new heptavalent pseudomonas vaccine. Nature (London) New Biol. **229**:209-210.
17. **Harvath, L., and B. R. Anderson.** 1976. Evaluation of type-specific and non-type-specific pseudomonas vaccine for treatment of pseudomonas sepsis during granulocytopenia. Infect. Immun. **13**:1139-1143.
18. **Homma, J. Y., H. Shionoya, H. Yamada, and Y. Kawake.** 1971. Production of antibody against *Pseudomonas aeruginosa* and its serological typing. Jpn. J. Exp. Med. **41**:89-94.
19. **Jones, R. J., M. Hall, and C. R. Ricketts.** 1972. Passive protective properties of serum fractions from mice inoculated with an anti-pseudomonas vaccine. Immunology **23**:889-895.
20. **Jones, R. J., and E. A. Roe.** 1975. Protective properties and haemagglutinins in serum from humans and in serum from mice injected with a new polyvalent pseudomonas vaccine. Br. J. Exp. Pathol. **56**:34-43.
21. **Lucke, B., M. Strumia, S. Mudd, M. McCutcheon, and E. Mudd.** 1933. On comparative phagocytic activity of macrophages and polymorphonuclear leucocytes. J. Immunol. **24**:455-462.
22. **Pennington, J. E.** 1974. Preliminary investigations of *Pseudomonas aeruginosa* vaccine in patients with leukemia and cystic fibrosis. J. Infect. Dis. **130**(Suppl):S159-S162.
23. **Pennington, J. E., H. Y. Reynolds, R. E. Wood, R. A. Robinson, and A. S. Levine.** 1975. Use of *Pseudomonas aeruginosa* vaccine in acute leukemia and cystic fibrosis patients. Am. J. Med. **58**:629-636.
24. **Roe, E. A., and R. J. Jones.** 1974. Intracellular killing of different strains of *Pseudomonas aeruginosa* by human leucocytes. Br. J. Exp. Pathol. **55**:336-343.
25. **Whitecar, J. P., Jr., M. Luna, and G. P. Bodey.** 1970. Pseudomonas bacteremia in patients with malignant diseases. Am. J. Sci. **260**:216-223.
26. **Young, L. S.** 1972. Human immunity to *Pseudomonas aeruginosa*. II. Relationship between heat-stable opsonins and type-specific pseudomonas lipopolysaccharide. J. Infect. Dis. **126**:257-277.
27. **Young, L. S., and R. D. Meyer.** 1973. *Pseudomonas aeruginosa* vaccine in cancer patients. Ann. Intern. Med. **79**:518-527.