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Biological Activities of Rabbit Immunoglobulin M and Immunoglobulin G Antibodies to *Pseudomonas aeruginosa*

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Immunoglobulin (Ig)M and IgG antibodies, prepared in the rabbit against the protective antigen of *Pseudomonas aeruginosa* P4, were compared as to their biological activities in vitro and in vivo. In vitro biological activities of these antibodies were determined by passive hemagglutination, bactericidal, and opsonophagocytic tests. Increased effectiveness of IgM over IgG on a molar basis was demonstrated in all of these tests. However, in mouse protection tests, in which the purified globulins were injected intraperitoneally 4 hr prior to challenge with *P. aeruginosa* suspended in hog gastric mucin, IgM anticapsular antibody was found to be less effective than IgG antibody. The exact mechanism whereby IgG antibody exerts more protective ability than IgM antibody is still unknown. We present evidence to suggest that the difference in activity between the two classes of antibody is due to the ability of the IgG antibody to enter the bloodstream more rapidly than the IgM antibody and also to the ability of IgG to diffuse rapidly through the tissues of the organs.

The renewed interest in antimicrobial immunity has been stimulated by the increasing incidence of infections caused by antibiotic-resistant pathogens. Although bacterial vaccines are available for numerous pathogens, many of them are only of limited protective value (18). Most of these vaccines are composed of whole bacteria since the protective antigens of many pathogens have not been identified. Therefore, the low protective ability of many vaccines may be due to the multiplicity of competing antigens found in the whole cell and to the inability of the immunized individual to produce sufficient amounts of protective antibody. Our investigation was concerned with the biological activities of the classes of antibody produced by immunization with *Pseudomonas aeruginosa*. It has been shown that antibody produced against this substance provides protection in experimental animals against a challenge with virulent *Pseudomonas*.

MATERIALS AND METHODS

Preparation of the antigen. *P. aeruginosa* P4 was isolated from a cystic fibrosis patient in the Boston Children's Hospital. The organism was grown on Trypticase Soy Agar and 1% dextrose at 37 C overnight. The bacteria were washed from agar plates with sterile saline and separated by centrifugation. The sedimented bacteria were removed, and the supernatant was precipitated with 95% ethanol. The precipitate was dissolved in distilled water and, after the addition of 10^{-3} M $MgSO_4$ and 10 μg of deoxyribonuclease per ml, the preparation was incubated at 37 C for 1 hr. Precipitation with 95% ethanol was repeated three more times. The preparation was redissolved in distilled water and then lyophilized. The capsular antigen was found to consist of a mucopolysaccharide containing mannuronic and guluronic acids as determined by paper chromatography.

Immunization with the purified mucopolysaccharide. Five-pound New Zealand albino rabbits (ca. 2.27 kg) were immunized with the purified mucopolysaccharide. Rabbits were injected intravenously with 1 mg of mucopolysaccharide three times weekly over a period of several months. Serial bleedings by cardiac puncture were done at various time intervals to collect sera rich in different classes of antibody.

Qualitative analysis of the antisera. Mercaptoethanol sensitivity was used as an indicator of immunoglobulin (Ig)M by using a modification of the original Deutsch method (6). Equal volumes of antiserum and 0.2 M 2-mercaptoethanol were mixed and allowed to stand at 4 C for 1 hr. Each sample was dialyzed for 2 hr against 0.02 M iodoacetic acid in phosphate-buffered saline (*p*H 7.2) and then in fresh saline overnight. The antisera were tested for antibody activity before and after mercaptoethanol treatment by passive hemagglutination of sheep red blood cells coated with the mucopolysaccharide.

Purification of the globulins. Diethylaminoethyl

(DEAE) cellulose chromatography was used for the purification of IgG from whole serum by using the method described by Fahey et al. (8). The antisera rich in IgM were separated by gel filtration with Sephadex G-200 by using the method of Flodin and Killander (13). Later fractionation of IgG was also accomplished by this method. Eluates were collected in 4- to 5-ml portions by a fraction collector operating on time. Each portion collected was measured for absorbance at 280 nm in a Hitashi Perkin Elmer Coleman III spectrophotometer to determine protein content. Protein rich peaks were concentrated by negative pressure dialysis.

Absorption and elution of the antibodies from the antisera. An attempt was made to quantitate the amount of antibody in each of the purified antibody fractions by absorbing the antibodies to heat killed bacteria and subsequently eluting them with weak acid (30, 31). Bacterial cells were washed twice with saline, heated at 65 C for 1 hr, and washed once with 0.1 M sodium acetate (*p*H 3.9). After two more washings with saline, 3 ml of rabbit antiserum was added to 12 ml of bacteria (10^9 cells/ml). The mixture was incubated at 37 C for 90 min and then at 4 C with gentle stirring for 18 hr. The bacteria were then centrifuged at 10,000 × *g* for 1 hr and washed twice with saline to remove any unabsorbed antibody. Sodium acetate (3 ml) was added to the cells, and the mixture was gently stirred at 37 C for 90 min. The suspension was then centrifuged, and the supernatant fluid was removed, concentrated to 0.5 ml by negative pressure dialysis, and dialyzed against saline overnight. The supernatant fluid was tested for the presence of antibody by passive hemagglutination of sheep red blood cells coated with the mucopolysaccharide.

Precipitin analysis. Quantitation of the amount of antibody in each of the purified preparations was determined by precipitin analysis. Increasing concentrations of the mucopolysaccharide were added to 0.1 ml of purified immunoglobulin fractions. After incubation for 1 hr at 37 C and 24 hr at 4 C, the precipitates were centrifuged; the supernatant fluids were tested for the presence of antibody by passive hemagglutination of sheep red blood cells coated with mucopolysaccharide. The protein precipitates were washed three times with cold saline to remove any adherent antibody and were dissolved in 0.1 ml of 0.1 N NaOH. Protein was measured by the Lowry method (22) by using a standard crystalline bovine serum albumin curve. The amount of antibody in each of the purified fractions was determined by subtracting the amount of protein in the added antigen from the total protein precipitated at the zone of equivalence.

In vitro tests. (i) Immunoelectrophoresis was carried out by a modification of the standard method of Scheidegger (33) in 2% Noble agar made in 0.05 M Veronal buffer, *p*H 8.2. (ii) "Immunoplates" prepared by Hyland Laboratories (Los Angeles, Calif.) were used to measure precipitation between the antisera and mucopolysaccharide. (iii) Passive hemagglutination involved exposure of various dilutions of the antisera and immunoglobulin preparations to sheep red blood cells coated with the mucopolysaccharide (27). The mucopolysaccharide (1 mg/ml) was boiled for 0.5 hr and added to 10% sheep red blood cells. This preparation was incubated for 1 hr at 37 C. The sensitized red blood cells were then washed three times with saline. A 0.1-ml amount of antisera was added to an equal volume of the sensitized red blood cells and incubated for 1 hr at 37 C. The titer was two times the reciprocal of the highest dilution showing macroscopic hemagglutination. (iv) Bactericidal activity was determined by mixing dilutions of the antibodies with guinea pig complement and a known inoculum of bacteria (24). A 0.1-ml amount of inoculum containing approximately 10^3 organisms in saline, 0.1 ml of antibody, and 0.3 ml of a 1:5 dilution of commercially available guinea pig complement were mixed and incubated at 37 C for 3.5 hr. The contents of each tube were removed to a sterile petri dish and mixed with 10 ml of Trypticase Soy Agar. After incubation at 37 C for 24 to 48 hr, the colonies were enumerated to determine bactericidal activity. A complement control in which 0.1 ml of saline was substituted for the antiserum was included in each experiment. (v) The opsonophagocytic test (31) was performed by injecting opsonized bacteria intraperitoneally into mice. A bacterial culture dilution was mixed with various concentrations of antibody fractions and incubated for 15 min. The opsonized bacteria (0.5 ml) were injected into each mouse. After 30 min, the peritoneum was opened and the bacteria were washed out with 1 ml of Trypticase Soy Broth. The washings were mixed with Trypticase Soy Agar in a sterile petri dish and incubated for 24 to 48 hr at 37 C. The bacterial colonies were then enumerated to determine opsonophagocytic activity. Saline was substituted for the antibody preparation in the controls.

In vivo tests: bacteria. Mice were challenged with bacteria grown for 4 hr in Trypticase Soy Broth. The virulence of *P. aeruginosa* P4 was enhanced by suspending the bacteria in 5% Wilson's hog gastric mucin.

Animals. Ten-week-old CF female mice (Carworth Farms, Kalamazoo, Mich.) were used throughout our studies.

Mouse protection. The protective capacity of the antibodies was tested by injecting mice intraperitoneally with 0.25 ml of purified immunoglobulin fractions 4 hr prior to intraperitoneal challenge with *P. aeruginosa* P4 suspended in mucin. Mortality was observed after 72 hr.

RESULTS

Purification of the globulins. Preparation of both classes of immunoglobulin was required for comparison of their in vitro and in vivo biological activities. It was essential to separate IgM and IgG into fractions, each free of the other immunoglobulin. As described above, DEAE cellulose chromatography was used for the purification of IgG, and gel filtration with Sephadex G-200 was used for the separation of IgM from IgG.

By immunoelectrophoretic analysis, IgM preparations were found to contain other high-molecular-weight serum proteins such as alpha-2-

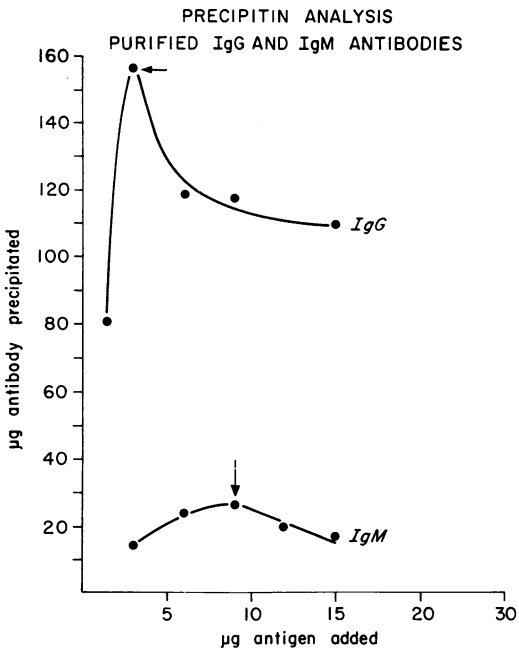

FIG. 1. *Precipitin analysis of purified IgG and IgM measuring anticapsular antibodies to Pseudomonas aeruginosa. At the zone of equivalence, 1 mg of IgM preparation contained 26 μg of anticapsular antibody, and 3 mg of IgG preparation contained 157 μg of anticapsular antibody.*

macroglobulin and beta lipoprotein but were free of IgG. Preparations of IgG were free of all other serum proteins.

Quantitation of antibody in the purified preparation. As described by other investigators for antibodies to other gram-negative bacteria (31), it was our intention to quantitate the amount of antibody in each of the purified preparations by the absorption elution procedure described above. Under our experimental conditions, the technique was unsuccessful since no antibody could be demonstrated in the eluates. The fact that we had obtained both classes of precipitating antibodies by our immunization procedures led us to quantitate antibody by precipitin analysis. Although it is known that IgM is less effective than IgG in precipitation reactions, our results indicated that precipitin analysis could provide an adequate basis for comparison of in vitro and in vivo biological activities.

At the zone of equivalence, 1 mg of the IgM preparation contained 26 μg of anticapsular antibody (Fig. 1). It was also shown that, at the zone of equivalence, 3 mg of IgG preparation contained 157 μg of anticapsular antibody. Antibody was demonstrable in the supernatant fluids only in the zone of antibody excess.

TABLE 1. *Hemagglutinating activity of purified immunoglobulins*[a]

Immunoglobulin	Amt (μg) of prepn required for minimal degree of macroscopic hemagglutination
IgM	0.0325
IgG	0.196

[a] Molar ratio of hemagglutinating activity: IgM/IgG = 40/1.

Our data indicated that the purified preparations obtained from the different antisera could be analyzed for antibody concentration by precipitin analysis. Preparations with equivalent hemagglutinating titers were found to contain similar antibody concentrations.

In vitro biological activities of purified IgM and IgG antibodies. Our primary concern was to determine which class of antibody was more efficient on a weight basis. In vitro tests included passive hemagglutination, the bactericidal reaction, and the opsonophagocytic test. Purified preparations of IgM used for all in vitro tests contained 26 μg of antibody per 0.1 ml and those of IgG contained 157 μg of antibody per 0.1 ml of test solutions.

Passive hemagglutination tests involved exposure of dilutions of the antibodies to sheep red blood cells coated with the mucopolysaccharide. The amount of antibody required for a minimal degree of macroscopic hemagglutination is expressed in Table 1. On a weight basis, approximately six times as much IgG as IgM antibody was required for similar hemagglutinating activity. The molar ratio of hemagglutinating activity of IgM to IgG was about 1:40, indicating the in vitro effectiveness of IgM over IgG.

Bactericidal activity was determined by mixing various dilutions of the antibodies with guinea pig complement and a known inoculum of bacteria (Table 2). A concentration of 6.5×10^{-3} μg of IgM antibody was sufficient to cause 65% killing, whereas no bactericidal activity was observed with as much as 39.25 μg of IgG antibody. The molar ratio of the bactericidal activities of IgM to IgG was, therefore, greater than 1:250,000, proving the superior activity of the IgM antibody in this system.

The opsonophagocytic test was performed by injecting opsonized bacteria intraperitoneally into mice. The elimination of viable bacteria from the peritoneal cavity was taken as evidence of opsonophagocytic activity. After 30 min of incubation, the peritoneum was opened and the bacteria were washed out and counted. Approximately 5,000 bacterial cells were injected into each mouse. About one-fifth of the original

TABLE 2. *Bactericidal activity of purified immunoglobulins*[a]

Immunoglobulin antibodies	No. of surviving bacteria 210 min after exposure to bactericidal mixture	Killing
μg		%
IgM		
0	454	0
6.5×10^{-1}	7	99
6.5×10^{-2}	50	89
6.5×10^{-3}	160	65
6.5×10^{-4}	440	2
6.5×10^{-5}	450	0
IgG		
0	454	0
39.25	496	0
3.925	502	0
3.925×10^{-1}	444	0
3.925×10^{-2}	520	0
3.925×10^{-3}	510	0
3.925×10^{-4}	500	0

[a] Molar ratio of bactericidal activity: IgM/IgG = 250,000/1.

TABLE 3. *Opsonophagocytosis by purified immunoglobulins*[a]

Immunoglobulin antibodies	No. of bacteria recovered from mouse peritoneum after 30 min[b]	Bacteria opsonophagocytized
μg		%
IgM		
0	1,350	0
2.6×10^{-3}	19	99
2.6×10^{-4}	129	91
2.6×10^{-5}	154	88
2.6×10^{-6}	990	23
IgG		
0	1,224	0
7.85×10^{-1}	30	98
7.85×10^{-2}	120	90
7.85×10^{-3}	1,398	0

[a] Molar ratio of opsonophagocytic activity: IgM/IgG = 5,000/1. Number of bacteria injected into peritoneal cavity: 5×10^3.

[b] Each value expresses an average from a group of two mice.

number of injected bacteria were recovered from control mice after 30 min (Table 3). It was found that 2.6×10^{-5} μg of IgM antibody was sufficient for the opsonophagocytosis of 90% of the recoverable organisms, whereas 7.85×10^{-2} μg of IgG was required for similar activity. The molar ratio of opsonophagocytic activity of IgM to IgG was about 1:5,000, again showing the effectiveness of IgM over IgG. To ascertain that the peritoneal killing of bacteria was accomplished by the phagocytes and not by complement or other humoral factors, fresh mouse serum or peritoneal fluid free of cells was mixed with IgG or IgM antibodies and an inoculum of viable *P. aeruginosa*. By using the previously described bactericidal test technique, the effect of the mouse serum and peritoneal fluid on the bacteria was determined. In all tests, no bactericidal effect of mouse serum or peritoneal fluid on our strain of *P. aeruginosa* was detected.

Our results clearly indicated the in vitro superiority of IgM antibody over IgG antibody as measured by passive hemagglutination, bactericidal, and opsonophagocytic tests. This is in agreement with other investigators who have examined the in vitro biological activities of antibodies to other gram-negative bacteria. Results such as these would indicate that the IgM antibody would operate in vivo by enhancing the killing of the infecting organism and its opsonophagocytosis and that it would confer greater immunity to the host than the IgG antibody when passively transferred.

In vivo biological activities of purified IgM and IgG antibodies. Our primary concern was to compare the protective abilities of IgM and IgG antibodies when these antibodies were passively transferred to mice infected with *P. aeruginosa* P4. Since injection of the antisera by the intraperitoneal route has been found to be more effective in providing protection than by the intravenous route, the purified preparations were tested for protective ability by intraperitoneal administration.

Purified IgM preparations used in protection tests contained 30.5 μg of antibodies per 0.1 ml, and those of IgG contained approximately 126 μg of antibodies per 0.1 ml. Antibodies were injected 4 hr prior to challenge with strain P4 suspended in hog gastric mucin. Mortality was observed after 72 hr. Table 4 shows the relationship between control animals injected with saline prior to challenge and those given anticapsular IgM antibody. Survival of animals given anticapsular IgM antibody did not differ significantly from the controls, and no protection was engendered by as much as 75 μg of purified IgM antibody. Table 5 shows the protective effect afforded by anticapsular IgG antibodies. All animals survived if they were given 78.75 or 157.5 μg of IgG antibody 4 hr prior to the bacterial challenge. A 31.5-μg amount of anticapsular IgG antibody was required to protect 60% of the animals from death.

Thus, the experiments with purified globulin preparations showed that IgG anticapsular antibodies were more protective than IgM anti-

TABLE 4. *Protective effect of purified IgM in mice challenged with Pseudomonas aeruginosa*

Amt of antibody injected per mouse	No. of bacteria in challenging dose	Mortality after 72 hr	Survival
μg			%
0	1.5×10^6	12/14	14
	1.5×10^5	27/35	22
	7.5×10^4	6/10	40
	3.0×10^4	5/10	50
75	1.5×10^6	3/4	25
	1.5×10^5	13/20	35
	7.5×10^4	6/8	25
	3.0×10^4	4/8	50
37.5	1.5×10^6	4/4	0
	1.5×10^5	13/20	35
	7.5×10^4	5/8	37.5
	3.0×10^4	5/8	37.5

TABLE 5. *Protective effect of purified IgG in mice challenged with Pseudomonas aeruginosa*

Amt of antibody injected per mouse	No. of bacteria in challenging dose	Mortality after 72 hr	Survival
μg			%
0	5×10^5	10/10	0
	5×10^4	5/10	50
	5×10^3	2/10	80
157.5	5×10^5	0/5	100
	5×10^4	0/5	100
78.75	5×10^5	0/5	100
	5×10^4	0/5	100
31.5	5×10^5	4/10	60
	5×10^4	2/10	80

bodies on a weight basis. These results were quite surprising since IgM antibody was found to be far more effective than the IgG antibody in all in vitro tests studied.

Virulence of opsonized bacteria. Since IgM antibody had been shown to be less efficient in mouse-protective activity, it was important to determine if opsonization of the bacteria with this class of antibody prior to its injection would increase survival of the animals. Increased survival would indicate that, during the time in which antibody resided in the peritoneum prior to challenge with bacteria, some mechanism was operative which decreased the opsonizing ability of the IgM antibody. The results in Table 6 showed that opsonization of bacteria with either 15 μg of IgM antibody or 63 μg of IgG antibody caused survival of 60% of mice.

These results indicated that opsonization of

TABLE 6. *Mouse virulence of opsonized Pseudomonas aeruginosa bacteria*

Substance used for opsonization	Amt of antibody used for opsonization	No. of bacteria in challenging dose	Mortality after 72 hr	Survival
	μg			%
Saline	0	1.1×10^6	5/5	0
		1.1×10^5	5/5	0
IgM	15.0	1.1×10^6	5/5	0
		1.1×10^5	2/5	60
IgG	63.0	1.1×10^6	5/5	0
		1.1×10^5	2/5	60

bacteria with IgM and IgG antibodies prior to intraperitoneal injection decreased their virulence. Likewise, it was shown that mucin did not significantly inhibit phagocytosis of opsonized bacteria, as mucin was mixed with the opsonized bacteria prior to its injection. These results also suggested that protection conferred by the passive administration of antibodies cannot always be equated with the administration of preopsonized bacteria.

Ability of the purified antibodies to promote opsonophagocytosis. To explain the low efficiency of IgM protection in vivo, we postulated that during its residence in the peritoneum this antibody may in some way lose its opsonizing ability. Macroglobulin antibodies are known to be cytotropic and may become attached to various tissue cells, thereby making the antibody ineffective as an opsonizer. It was decided to inject the purified globulins intraperitoneally into mice and examine their ability to promote opsonophagocytosis over various periods of time. Antibody preparations were identical to those used for mouse protection tests. IgG antibody (78.5 μg) and IgM antibody (37.5 μg) were injected intraperitoneally into each mouse. At 4, 8, 17, and 23 hr after the antibody injection, 5,000 organisms were introduced into the peritoneum. After 30 min, the bacteria were washed out and counted. The results of these experiments are shown in Table 7. During the test period, the number of bacteria opsonophagocytized by IgM and IgG antibodies was relatively constant, indicating that both antibodies were still effective in opsonophagocytosis after residence in the peritoneum for 23 hr. Moreover, 37.5 μg of IgM antibody was as effective as 78.5 μg of IgG antibody in this process. From these results, it was concluded that the low efficiency of IgM protection in vivo could not be explained by its loss of opsonizing ability.

Presence of the purified globulins in the blood

TABLE 7. *Persistence of immunoglobulins in mouse peritoneum capable of promoting opsonophagocytosis*[a]

Time after antibody injection	Substance injected	No. of bacteria recovered from mouse peritoneum after 30 min[b]	Per cent opsonophagocytized
hr			
4	Saline	944	0
	IgM[c]	5	99
	IgG[d]	10	98
8	Saline	1,020	0
	IgM	107	90
	IgG	5	99
17	Saline	1,062	0
	IgM	191	82
	IgG	116	89
23	Saline	1,278	0
	IgM	245	80
	IgG	79	93

[a] Number of bacteria injected into a mouse peritoneum: 5×10^8.

[b] Each value expresses an average from a group of two mice.

[c] Amount of IgM antibodies injected per mouse: 37.5 μg.

[d] Amount of IgG antibodies injected per mouse: 78.5 μg.

circulation of mice. An attempt was made to determine if both IgM and IgG antibodies were capable of entering the bloodstream of mice and to determine the length of time each persisted there. It was possible that macroglobulin might be limited to the peritoneum and be incapable of intravascular entrance because of its size or other unknown reasons. IgG antibody is of smaller size; therefore, it would be expected to enter the bloodstream and diffuse through the tissues of the organs. If this were the case, animals injected with IgM antibody in mouse-protection tests would be then defenseless if a sufficient number of organisms entered the bloodstream from the peritoneum. It was also possible that rabbit IgM protein might be eliminated more rapidly by the host than IgG because of its greater lability.

Purified preparations of IgM containing 37.5 μg of antibody and of IgG containing 78.75 μg of antibody were injected intraperitoneally into mice. At various time intervals after antibody injection, the animals were exsanguinated. Normal mouse serum contained no hemagglutinating activity. The hemagglutinating titers of the original purified preparations injected were known to be 400 for IgM and 200 for IgG; therefore, the amount of antibody detectable in the mouse bloodstream could be calculated by multiplying the ratio of the titer of the mouse serum to the titer of the original preparation by the number of μg of antibody in the original preparation. The blood volume of the experimental mouse was considered to be 3 ml.

TABLE 8. *Persistence of anti-Pseudomonas antibodies in the bloodstream of mice*

Time after intraperitoneal injection	Titer of hemagglutinins in mouse serum[a]	Amt (μg) of antibody in mouse bloodstream	Amt of antibody recovered (per cent of total)
hr			
IgM[b]			
2	20	5.61	15
4	20	5.61	15
8	80	22.5	60
12	80	22.5	60
17	40	11.25	30
24	20	5.61	15
IgG[c]			
4	20	23.4	30
12	20	23.4	30

[a] Each value expresses an average from a group of two mice.

[b] Amount of IgM antibodies injected per mouse: 37.5 μg.

[c] Amount of IgG antibodies injected per mouse: 78.75 μg.

The results of these experiments are shown in Table 8. Fifteen per cent of the injected IgM antibody was detected in the mouse circulation after 2 hr. The maximum amount of IgM antibody was detected between 8 and 12 hr after injection. After 12 hr, IgM antibody began to decline, and by 24 hr only 15% of the injected antibody was detected in the circulation. Only 15% of the IgM antibody had entered the circulation 4 hr after antibody injection, whereas 30% of the IgG antibody was present there. This indicated that IgG antibody entered the circulation from the peritoneum more quickly than IgM antibody. It was also of interest that at 12 hr the proportion of the recovered IgM antibody in the bloodstream was greater than that of IgG antibody (60% versus 30%). It is possible that the IgG antibody once present in the bloodstream is capable of diffusing through the tissues of the organs, thereby lowering its concentration in the blood. Contrary to this, IgM antibody may be unable to diffuse through the tissues due to its larger size or other reasons.

In these experiments, we showed that both IgM and IgG antibodies entered the circulation from the peritoneum and remained there in sufficient amounts over a 24-hr period. Our results suggested the possibility that the IgM

TABLE 9. *Enumeration of Pseudomonas aeruginosa cells in mouse organs after passive administration of immunoglobulins*

Immunoglobulin	Time after intraperitoneal injection[a]	No. of viable bacteria per organ[b]				
		Spleen	Liver	Kidney	Peritoneum	Blood
	hr					
Controls	3	5×10^3	4.6×10^5	3.5×10^3	4×10^5	1.2×10^3
	12	1×10^8	4×10^9	8.4×10^6	2.2×10^9	3.7×10^5
	22	4×10^9	3.6×10^9	1.6×10^7	2.5×10^9	6.6×10^5
IgG injected[c]	3	4×10^2	5×10^4	1.5×10^3	10^3	45
	12	1×10^2	2.2×10^3	2.9×10^4	10^2	3×10^3
	22	3×10^4	2.8×10^5	3.8×10^4	1.2×10^4	3×10^2
IgM injected[c]	3	3×10^3	1.6×10^5	2.7×10^3	2.8×10^4	1.1×10^2
	12	6.5×10^5	1.5×10^7	2.4×10^5	10^5	2.8×10^3
	22	6×10^8	1.4×10^9	4.5×10^7	4.2×10^6	8×10^4

[a] Amt of bacteria injected: 7.5×10^4.
[b] Each value expresses an average from a group of 2 mice.
[c] Amt of immunoglobulin administered per mouse: IgG, 37.5 μg; IgM, 75 μg.

antibody may leave the peritoneum at a slower rate than the IgG antibody and that the IgM antibody, once in circulation, cannot reach the tissues of the organs due to its extravascular properties. The finding that the IgM antibody was less effective in mouse protection tests cannot be explained by the inability of this antibody to enter the circulation or by its rapid degradation.

Enumeration of bacteria in mouse organs. To determine the effect of the protective antibodies on the distribution of *P. aeruginosa* in the various organs of the mouse, the number of bacteria in the blood, peritoneum, spleen, liver, and kidney was counted at various times after intraperitoneal injection of bacteria. Antibodies were injected intraperitoneally 4 hr prior to bacterial challenge. As shown in Table 9, there was a substantial decrease in the number of bacteria in all organs of animals pretreated with IgG. However, in mice injected with IgM, a reduction in bacterial counts in comparison to the controls was observed only in the peritoneum and blood circulation, indicating that IgM antibody is less efficient or slower in its ability to diffuse into various organs of the body.

DISCUSSION

IgM antibacterial antibodies have been shown to be more effective than those of the IgG class as measured by several parameters in vitro (5, 23, 29, 31, 32, 34) but the protective ability of antibacterial IgM and IgG antibodies in vivo has not been previously investigated. *P. aeruginosa* has a well-defined protective antigen that stimulates production of both classes of antibodies and, therefore, can serve as an excellent model for the comparison of the in vitro and in vivo biological activities of purified IgM and IgG antibodies.

In vitro biological activities of purified IgM and IgG antibodies to *P. aeruginosa* were determined by passive hemagglutination, bactericidal, and opsonophagocytic tests. On a weight basis, IgM antibody was superior to IgG antibody in all of these tests. These observations indicated that the IgM antibody would operate in vivo by enhancing the killing of the infecting organism and that it would confer greater immunity to the host when passively transferred. However, in mouse-protection tests, in which the purified globulins were injected intraperitoneally 4 hr prior to challenge with *P. aeruginosa*, IgM anticapsular antibody was found to be less effective on a weight basis than IgG.

The possible causes of the lower efficiency of the IgM protection in vivo were examined. At first, it was postulated that residence of the IgM antibody in the peritoneum might in some way decrease its opsonizing ability, but IgM was found to be effective as an opsonizer in the peritoneum for 24 hr after its injection.

It was suggested that the size of the macroglobulin antibodies might limit them to the peritoneum and make them incapable of intravascular entrance. It was also possible that rabbit IgM protein might be eliminated more rapidly than IgG by the host due to its greater lability. Our results showed that both IgM and IgG antibodies entered the circulation from the peritoneum and remained there in sufficient amounts over a 24-hr period. There is still the possibility

that IgM antibody may leave the peritoneum at a slower rate than IgG antibody and that IgM antibody, once in circulation, can not reach the organs rapidly enough to provide protection.

Since it has been suggested that complement may be required for opsonic activity of IgM antibody (16; W. Lay and V. Nussenzweing, Fed. Proc. 27:621, 1968), it was important to determine if the mucin which was used in mouse-protection tests to enhance the virulence of *P. aeruginosa* was to some degree anticomplementary. Keefer and Spink (20) observed no anticomplementary action when 0.5 ml of defibrinated human blood was added to 0.1 ml of 5% suspension of mucin. Olitzki (28), however, demonstrated that mucin and agar lowered the titer of guinea pig complement. The effect of mucin on mouse complement is difficult to assay since mouse blood contains only a minimal amount of hemolytic complement. Olitzki has shown that neither the blood nor the peritoneal fluid of the mouse could be used as a good source of hemolytic complement (28). In our experiments, we were unsuccessful in demonstrating hemolysis with complement obtained from CF_1 mice. However, we showed that mucin did not inhibit the phagocytosis of bacteria opsonized with either IgM or IgG antibodies. In experiments in which the virulence of opsonized bacteria was examined, mucin was mixed with the opsonized bacteria prior to injection. If complement were required for phagocytosis of bacteria opsonized with IgM antibody, and if mucin were anticomplementary, then survival of animals given bacteria opsonized with IgM antibody should have been decreased. These animals survived, indicating that mucin did not inhibit phagocytosis of bacteria opsonized with IgM antibody.

The exact mechanism whereby IgG antibody exerts more protective ability than IgM antibody is still unknown. We suggest that the difference in activity between the two classes of antibodies is due to the ability of the IgG to diffuse through the tissues of the organs. Our observations indicate that in mouse-protection tests, at the time of challenge, sufficient IgG antibody is present in circulation and in the tissues to prevent extensive multiplication of the organism. Although opsonophagocytosis in the peritoneum of animals pretreated with IgM antibody is operative, some organisms do invade the bloodstream, multiply, and proliferate in the blood and organs, resulting in death of the animal. It is suggested that IgM antibody enters the bloodstream too slowly to combat the fulminating infection in progress and that the IgM antibody does not enter extravascular spaces easily, having much difficulty in diffusing through the body tissues. In most of our experiments the immunoglobulins were injected intraperitoneally, but in some unreported experiments IgM and IgG preparations were given intravenously. The data obtained from these experiments also showed the preferential antimicrobial protection of IgG.

Our results may help to explain in part the apparent low degree of immunity achieved by active immunization with some gram-negative pathogens, such as *Salmonella typhosa*. Vaccination with the enteropathogens generally induces formation of circulating antibodies of the IgM type (4). Recent studies of the immunity to meningococcus showed that the protective antibodies to this pathogen are of the IgG type (17). This is also in agreement with recent observations of *Bordetella pertussis* vaccine which was found to stimulate the production of IgG antibodies after immunization (7). Therefore, it seems that successful immunization procedures are those in which the principal antibody produced is IgG, whereas those which result in the production of IgM antibodies exclusively are perhaps only of equivocal benefit.

P. aeruginosa, the opportunist which invades host tissues and establishes infection most readily when the host is already weakened by other conditions such as burns, malignancy, cystic fibrosis, or diabetes mellitus, is resistant to most antibiotics (21). Therefore, immunotherapy is again becoming an important part of the treatment of *Pseudomonas* infections. The finding that normal human gamma globulin was protective in experimental infection and that specific antibodies were involved was the first evidence to suggest that *P. aeruginosa* infections were susceptible to immunological control (12, 15, 26). Protective activity has been demonstrated in antisera from animals immunized with heat-killed bacteria (19, 25), in sera or plasma from human volunteers immunized with a similar vaccine (10), and in plasma from burn-infected patients (9). Successful active immunization has been demonstrated with whole cell vaccines in humans (10) and with various antigenic extracts of the organisms in rats (1) and in mice (2, 3). The highest degree of immunity has been shown to be achieved by immunizing with the protective antigen extracted from a strain of *P. aeruginosa* identical to the strain used for challenge. A polyvalent vaccine has been prepared recently by Fisher (11). The vaccine consists of the protective antigens from seven different serotypes of *P. aeruginosa*.

Knowledge of the protective abilities of the different classes of immunoglobulin is necessary if active and passive immunization are to be attempted. Our finding that IgG anticapsular

antibody is more protective than IgM antibody supports the feasibility of both procedures. Stable IgG fractions of hyperimmune serum can be prepared with relative ease, whereas the IgM fraction is more difficult to purify and preserve.

It is important to emphasize that no claim is made to deny the protective ability of IgM against bacterial infections in vivo. All of our data, however, indicate that in the past perhaps too much emphasis has been given to in vitro tests, which as shown in our study do not always correlate with that which happens in the in vivo system.

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LITERATURE CITED

1. Alexander, W., W. Brown, H. Walker, A. D. Mason, and J. A. Montcrief. 1966. Studies on the isolation of an infection-protective antigen from *Pseudomonas aeruginosa*. Surg. Gynecol. Obstet. Int. Abstr. Surg. 123:965–977.
2. Alms, T. H., and J. A. Bass. 1967. Immunization against *Pseudomonas aeruginosa*. I. Induction of protection by an alcohol precipitated fraction from the slime layer. J. Infec. Dis. 117:249–256.
3. Alms, T. H., and J. A. Bass. 1967. Immunization against *Pseudomonas aeruginosa*. II. Purification and characterization of the protective factor from the alcohol precipitated fraction. J. Infec. Dis. 117:257–264.
4. Altemeier, W. A., J. A. Bellanti, and E. L. Buescher. 1969. The IgM response of children to *Salmonella typhosa* vaccine. I. Measurements of anti-typhoid concentrations and their proportions of total serum IgM globulins. J. Immunol. 103:917–923.
5. Del Guercio, P., G. Tolone, F. Braga Andrade, G. Biozzi, and R. A. Binaghi. 1969. Opsonic, cytophilic, and agglutinating activity of guinea pig IgG and IgM anti-*Salmonella* antibodies. Immunology 16:361–371.
6. Deutsch, H., and J. Morton. 1957. Dissociation of human serum macroglobulin. Science 125:600–601.
7. Dolby, J., and D. Dolby. 1969. The antibody activities of 19S and 7S fractions from rabbit antisera to *Bordetella pertussis*. Immunology 16:737–747.
8. Fahey, J. L., P. F. McCoy, and M. Goulian. 1958. Chromatography of serum proteins in normal and pathologic sera: the distribution of protein bound carbohydrate and cholesterol, siderophilin, thyroxin-binding protein, B-12 binding protein, alkaline and acid phosphatases, radioiodinated albumin and myeloma proteins. J. Clin. Invest. 37:272–284.
9. Feingold, D. S., and F. Oski. 1965. *Pseudomonas* infection. Arch. Intern. Med. 116:326–328.
10. Feller, I., and I. Kamei. 1964. *Pseudomonas* vaccine and hyperimmune plasma in the treatment of burned patients. Surg. Forum 15:46–47.
11. Fisher, M. W., H. B. Devlin, and F. J. Grabasik. 1969. New immunotype schema of *Pseudomonas aeruginosa* based on protective antigens. J. Bacteriol. 98:835–836.
12. Fisher, M. W., and M. C. Manning. 1958. Studies on the immunotherapy of bacterial infections. I. The comparative effectiveness of human gamma globulin against various bacterial species in mice. J. Immunol. 81:29–31.
13. Flodin, P., and J. Killander. 1962. Fractionation of human serum proteins by gel filtration. Biochim. Biophys. Acta 63:403–410.
14. Fox, J. E., and E. J. L. Lowbury. 1953. Immunity fo *Pseudomonas pyocyanea* in man. J. Pathol. Bacteriol. 65:519–542.
15. Gaines, S., and M. Landy. 1955. Prevalence of antibody to Pseudomonas in normal human sera. J. Bacteriol. 69:628–633.
16. Gerlings-Petersen, B., and K. Pondman. 1965. Relationship between complement the heterogeneity of antibody and phagocytosis. Bibl. Haematol. 23:829–833.
17. Gotschlich, E. C., I. Goldschneider, and M. S. Artenstein. 1969. Human immunity to the meningococcus. IV. Immunogenicity of group A and group C meningococcal polysaccharides in human volunteers. J. Exp. Med. 129:1367–1384.
18. Gregg, M. B. (ed.). 1969. Morbidity and mortality. Collected recommendations of the Public Health Service Advisory Committee on Immunization Practices, vol. 18.
19. Jones, R., D. Jackson, and E. J. L. Lowbury. 1966. Antiserum and antibiotic in the prophylaxis of burns against *Pseudomonas aeruginosa*. Brit. J. Plast. Surg. 19:43–57.
20. Keefer, C., and W. Spink. 1938. Studies on gonococcal infection. IV. The effect of mucin on the bacteriolytic power of whole blood and immune serum. J. Clin. Invest. 17:23–30.
21. Liu, P. V., Y. Abe, and J. L. Bates. 1961. The roles of various fractions of *Pseudomonas aeruginosa* in its pathogenesis. J. Infec. Dis. 108:218–228.
22. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with Folin phenol reagent. J. Biol. Chem. 193:265–275.
23. Michael, J. G., and F. S. Rosen. 1963. Association of "natural" antibodies to Gram negative bacteria with gamma-1-macroglobulins. J. Exp. Med. 118:619–626.
24. Michael, J. G., J. Whitby, and M. Landy. 1962. Studies on natural antibodies to Gram negative bacteria. J. Exp. Med. 115:131–146.
25. Millican, R. C., and J. Rust. 1960. Efficacy of rabbit *Pseudomonas* antiserum in experimental *Pseudomonas aeruginosa* infection. J. Infec. Dis. 107:389–394.
26. Millican, R., J. Rust, and S. M. Rosenthal. 1957. Gamma globulin factors protective against infections from Pseudomonas and other organisms. Science 126:509–511.
27. Neter, E., L. F. Bertram, and C. E. Arbesman. 1952. Demonstration of *Escherichia coli* 055 and 011 antigens by means of hemagglutination test. Proc. Soc. Exp. Biol. Med. 79: 255–257.
28. Olitzki, L. 1948. Mucin as a resistance lowering substance. Bacteriol. Rev. 12:149–172.
29. Pike, R. M., M. L. Schulze, and C. H. Chandler. 1966. Agglutinating and precipitating capacity of rabbit anti-*Salmonella typhosa* γG and γM antibodies during prolonged immunization. J. Bacteriol. 92:880–886.
30. Robbins, J. B. 1965. Studies on the interaction of immunoglobulins towards protein antigens with biological activity, p. 241–251. *In* J. Sterzl (ed.), Molecular and cellular basis of antibody formation. Academic Press Inc., New York.
31. Robbins, J. B., K. Kenny, and E. Suter. 1965. Isolation and biological activities of rabbit IgM and IgG anti-*Salmonella typhimurium* antibodies. J. Exp. Med. 122:385–402.
32. Rowley, D., and K. J. Turner. 1966. Number of molecules of antibody required to promote phagocytosis of one bacterium. Nature (London) 210:496–498.
33. Scheidegger, J. J. 1955. Une micro methode de l'immunoelectrophorese. Int. Arch. Allergy Appl. Immunol. 7:103–110.
34. Smith, J. W., J. A. Barnett, R. P. May, and J. P. Sanford. 1967. Comparison of the opsonic activity of IgG and IgM anti-*Proteus* globulins. J. Immunol. 98:336–343.