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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

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Techniques have been described for the purification of mouse polymorphonuclear leukocytes (PMN) and macrophages and for determining their bactericidal activity against *Pseudomonas aeruginosa* in a rotating suspension. Requirements for human anti-*Pseudomonas* opsonins and heat-labile mouse serum factors have been determined. In the macrophage system, although heat-labile mouse serum factors had enhancing properties, both immunoglobulin G (IgG) and immunoglobulin M (IgM) opsonins alone sufficed to induce bactericidal activity. In contrast, both opsonins and heat-labile mouse serum factors were required for bactericidal activity by the mouse PMN. In the absence of heat-labile mouse serum factors, IgG and IgM opsonins contributed to the bactericidal activity of mouse macrophages to the same extent. However, in the presence of these factors, much less IgG than IgM antibody was required to achieve maximum bactericidal activity by these cells. Similarly, IgG opsonins were found to be more efficient than IgM opsonins in enhancing the killing of *P. aeruginosa* by mouse PMN.

Rabbit immunoglobulin G (IgG) and immunoglobulin M (IgM) antibodies to *Pseudomonas aeruginosa* have been shown to differ in biological acitivities in vitro and in vivo. Although increased effectiveness of IgM antibody over IgG, on a molar basis, was demonstrated in vitro, IgG antibody was shown to be superior to IgM in mouse protective activity (1). This paradox remained unexplained in our previous publication. As both humoral and cellular factors are essential to the resistance to microbial infection, it seemed important to characterize the bactericidal capacity of mouse macrophages and polymorphonuclear leukocytes (PMN) and to measure the contribution of anti-*Pseudomonas* IgG and IgM opsonins and normal mouse serum factors to the cellular bactericidal process. It was also hoped that a more accurate definition of the function and capacities of the humoral and cellular factors might suggest an explanation of the differences in efficiency of protection between IgG and IgM.

The ability of mouse peritoneal macrophages to phagocytize and kill a variety of intracellular and extracellular microorganisms has been studied in great detail (3-5, 13). Most of these investigations have been concerned with cellular immunity in the resistance to infection, and qualitative comparisons have been made between the bactericidal activities of normal and "immune" macrophages (13). Most investigations have employed techniques in which macrophages attached to glass or grown in tissue culture have been utilized. Until recently, little was known about the capacity of mouse macrophages to kill intracellularly compared with that of other phagocytic cells. Quantitative studies, in which mouse macrophages kept in suspension by a rotating magnet were used, suggested that *P. aeruginosa* is killed efficiently by these cells and that the killing mechanism is dependent upon an intact respiratory chain (14). PMN from a variety of species have been shown to be capable of phagocytosis and intracellular killing of microorganisms. But the bactericidal activity of the mouse PMN has not been investigated, primarily because no bactericidal assay has been developed that utilizes the mouse PMN in suspension.

The present report describes techniques for the

purification of mouse PMN and macrophages and for determining their bactericidal activity in rotating suspensions. Mouse PMN and macrophages were compared for bactericidal activity against *P. aeruginosa*. Antibody requirements and dependence on mouse heat-labile serum factors for the bactericidal activity of these two cell populations were also established.

MATERIALS AND METHODS

Organism. *P. aeruginosa* P4 was isolated from a cystic fibrosis patient at the Boston Children's Hospital. It has been identified as a serotype 2 strain of the immunotype schema of Fisher, Devlin, and Gnabasik (8).

Immunoglobulin preparations and antigens. Antisera rich in IgM and IgG were prepared by immunizing human volunteers with a *P. aeruginosa* polyvalent vaccine consisting of seven serotypes. IgG was prepared by the fractionation method of Cohn. The sera, IgG, and the antigens were kindly provided by Myron W. Fisher, Parke, Davis & Co., Detroit, Mich. IgM was separated from the antisera by gel filtration (Sephadex G-200) by the method of Flodin and Killander (9). By immunoelectrophoretic analysis (16), IgM preparations were found to contain other high-molecular-weight serum proteins such as alpha-2 macroglobulin and beta lipoprotein, but they were free of IgG. Preparations of IgG contained small amounts of albumin but were free of IgM and IgA.

Mice. Ten-week-old CF_1 female mice (Carworth Farms, Kalamazoo, Mich.) were used.

Passive hemagglutination. Dilutions of immunoglobulin preparations were added to sheep red blood cells coated with serotype 2 antigen. Hemagglutination titers were determined by the microtiter method.

Preparation of macrophage suspensions. Mice were exsanguinated by cervical dislocation, and 3 ml of Hanks balanced salt solution with sodium bicarbonate (Microbiological Associates, Inc., Bethesda, Md.) containing 100 units of heparin per ml was immediately introduced into the peritoneal cavity of each animal. After gentle massage of the abdomen, peritoneal exudates were removed with siliconized Pasteur pipettes, pooled, and placed in a polystyrene tube (17 by 100 mm, no. 2001; Falcon Plastics, Los Angeles, Calif.). The cell suspension was centrifuged at 200 × *g* for 12 min in a refrigerated centrifuge (model PR-2; International Equipment Co., Boston, Mass.). The supernatant fluid was removed, and the cells were suspended in the Hanks solution and washed again. Cell suspensions contained 85 to 90% macrophages, 10 to 15% lymphocytes, and 0 to 3% PMN.

Preparation of PMN suspensions. Mice were injected intraperitoneally with 3 ml of Brain Heart Infusion Broth (Difco). After 18 to 24 hr, mice were exsanguinated by cervical dislocation, and peritoneal exudates were prepared as described for macrophage suspensions. These exudates contained PMN, lymphocytes, and macrophages. The peritoneal exudates from three mice were pooled, placed in a tissue culture flask (25 cm_2, no. 3012; Falcon Plastics), and incubated at 37 C to allow attachment of the macrophages to the plastic. The attached cells consisted of approximately 80% macrophages and 20% PMN. After 30 min of incubation, the unattached cells were removed to a polystyrene tube (17 by 100 mm) and centrifuged at 200 × *g* for 12 min. The cells were washed once with Hanks solution and suspended to the desired volume. This cell population consisted of 60 to 70% PMN, 25 to 35% lymphocytes, and 5 to 10% macrophages.

Cell viability, white blood cell counts, and differentials. Cells were counted in a hemacytometer, and it was found that 95% or more excluded trypan blue dye. Differential counts were performed by using wet-mount preparations consisting of one drop of cell suspension and one drop of Weiler's crystal violet in 0.1 M citric acid.

Bactericidal assay. Bacteria were grown in 10 ml of Brain Heart Infusion Broth at 37 C overnight. The bacteria were washed once in Hanks solution and resuspended in the original volume. A 0.1-ml amount of an appropriate culture dilution, 0.2 ml of immunoglobulin preparation, 0.2 ml of normal mouse serum, and 0.5 ml of leukocyte suspension were added to a polypropylene tube (12 × 75 mm, no. 2005; Falcon Plastics). Hanks solution was substituted for the immunoglobulin preparation and leukocyte suspension in the controls. A ratio of 25 to 35 macrophages or PMN per bacterium was maintained in all experiments. (Lower ratios between the phagocytes and bacteria did not result in an efficient killing of bacteria.) The tubes were tumbled end over end (9 rev/min) on a Multipurpose rotator (model 150V; Scientific Industries, Inc., Springfield, Mass.) at 37 C for 3 hr. Shorter periods of incubation (30 min to 2 hr) were explored, but maximal bactericidal effect was achieved only after incubation for 3 hr. The samples were removed, diluted in distilled water to disrupt the leukocytes, and plated on Brain Heart Infusion Agar. Plates were incubated at 37 C overnight, and the colonies were counted. Samples taken from control tubes at the beginning and end of the incubation showed that no growth of bacteria occurred in the test tubes during the incubation period.

RESULTS

Bactericidal activity of mouse peritoneal macrophages against P. aeruginosa. Passive hemagglutination is a rapid, sensitive, reproducible method for determining the presence of anti-*Pseudomonas* antibodies. Therefore IgG and IgM antibody was measured by this technique with sheep red blood cells coated with serotype 2 antigen. Both IgG and IgM preparations were diluted with Hanks balanced salt solution to contain hemagglutinating titers of 1:1,024. A single preparation of each class of antibody was used throughout this investigation.

To measure the contribution of purified immunoglobulins to the bactericidal activity of mouse peritoneal macrophages, bactericidal assays were developed as described above. Initial

TABLE 1. *Contribution of purified immunoglobulins to bactericidal activity of mouse peritoneal macrophages*

Immunoglobulin class	Dilution of immunoglobulin added to assay	No. of bacteria per ml recovered after 3-hr incubation[d]	Reduction in bacterial counts from control[d]
Reaction mixtures[a] with macrophages			
IgM[b]	1×10^{0}	4.25	0.98
	1×10^{-1}	3.95	1.28
	1×10^{-2}	4.18	1.05
	1×10^{-3}	4.40	0.83
	2×10^{-3}	4.60	0.63
	4×10^{-3}	4.83	0.40
	8×10^{-3}	5.10	0.13
IgG[b]	1×10^{0}	4.42	0.81
	1×10^{-1}	4.22	1.01
	1×10^{-2}	3.96	1.27
	2×10^{-2}	4.09	1.14
	4×10^{-2}	4.21	1.02
	8×10^{-2}	4.15	1.08
	1×10^{-3}	4.25	0.98
	1×10^{-4}	3.89	1.34
	2×10^{-4}	4.23	1.00
	4×10^{-4}	4.59	0.64
	8×10^{-4}	4.59	0.64
Control[c]		5.23	
Reaction mixtures without macrophages			
IgM	1×10^{0}	5.32	
IgG	1×10^{0}	4.95	
Hanks solution only		5.36	

[a] Reaction mixtures consisted of immunoglobulin, macrophages, bacteria, and heated mouse serum.

[b] Undiluted IgG and IgM had agglutinin titers of 1:1,024.

[c] Control consisted of Hanks solution, macrophages, bacteria, and heated mouse serum.

[d] Values expressed in $\log_{10}$. Number of bacteria in each test at 0 time was 1.75×10^{5} cells/ml (expressed in $\log_{10}$, 5.24).

experiments indicated that 20% heat-inactivated (56 C for 30 min) mouse serum had to be added to the mixtures to achieve any degree of bactericidal activity with mouse peritoneal macrophages.

Mouse peritoneal macrophages were bactericidal only upon addition of either IgG or IgM opsonins plus heated mouse serum (Table 1). The end point of bactericidal activity was arbitrarily set at 0.6 $\log_{10}$ reduction from the control. Based on equivalent titers of hemagglutinating antibodies, 20 times less IgG than IgM antibody was required to reach this end point. As reported in our previous publication (1), IgM is a more efficient (about 20- to 40-fold) agglutinating antibody than IgG. As fewer IgM agglutinins are required for the reaction, it may be assumed that both classes of opsonins enhanced the bactericidal activity of mouse peritoneal macrophages against *P. aeruginosa* approximately to the same extent.

Effect of mouse heat-labile serum factors on bactericidal activity of mouse peritoneal macrophages. Heat-labile serum factors have been shown to enhance the phagocytosis and intracellular killing of opsonized bacteria (10). Therefore in later experiments fresh mouse serum was substituted for the heated serum in the macrophage bactericidal assay.

No bactericidal activity was observed when only IgG or IgM antibody and fresh mouse serum were added to the bacteria (Table 2). In the absence of opsonins, mouse peritoneal macrophages

TABLE 2. *Effect of heat-labile mouse serum factors on the bactericidal activity of mouse peritoneal macrophages*

Immunoglobulin class	Dilution of immunoglobulin added to assay	No. of bacteria per ml recovered after 3-hr incubation[d]	Reduction in bacterial counts from control[d]
Reaction mixture[a] with macrophage			
IgM	10^{-2}	3.90	0.95
	10^{-3}	4.34	0.51
	10^{-4}	4.60	0.25
	10^{-5}	4.70	0.15
IgG	10^{-4}	3.27	1.58
	10^{-5}	3.31	1.54
	10^{-6}	4.24	0.61
	10^{-7}	4.34	0.51
Control[b]		4.85	
Reaction mixture without macrophage			
IgM[c]	10^{0}	5.28	
IgG[c]	10^{0}	5.23	
Hanks solution only		5.28	

[a] Reaction mixtures consisted of immunoglobulin, macrophages, bacteria, and fresh mouse serum.

[b] Control consisted of Hanks solution, macrophages, bacteria, and fresh mouse serum.

[c] Undiluted IgG and IgM had agglutinin titers of 1:1,024.

[d] Values expressed in $\log_{10}$. Number of bacteria in each test at 0 time was 1.41×10^{5} cells/ml (expressed in $\log_{10}$, 5.15).

TABLE 3. *Effect of IgG and IgM opsonins and heat-labile serum factors on bactericidal activity of mouse polymorphonuclear leukocytes*

Treatment of mouse serum	Reaction mixture[a]	No. of bacteria per ml recovered after 3-hr incubation[b]	Reduction in bacterial counts from control[b]
56 C, 30 min	IgG + Hanks + bacteria (control)	4.52	
	Hanks + cells + bacteria	4.72	−0.20
	IgG + cells + bacteria	4.28	0.24
Untreated	IgG + Hanks + bacteria (control)	4.83	
	Hanks + cells + bacteria	4.62	0.21
	IgG + cells + bacteria	3.48	1.35
56 C, 30 min	IgM + Hanks + bacteria (control)	4.70	
	Hanks + cells + bacteria	4.66	0.04
	IgM + cells + bacteria	4.72	−0.02
Untreated	IgM + Hanks + bacteria (control)	4.72	
	Hanks + cells + bacteria	4.73	−0.01
	IgM + cells + bacteria	3.49	1.23

[a] IgG and IgM had agglutinin titers of 1:10.

[b] Values expressed in $\log_{10}$. Number of bacteria in each test at 0 time was 2.7×10^4 cells/ml (expressed in $\log_{10}$, 4.43).

reduced bacterial counts to some extent. However, the killing was minimal, and, because of possible experimental error, the validity of this finding could not be concluded. Addition of fresh mouse serum did not increase the bactericidal effect promoted by IgM opsonins over that observed with heated mouse serum (Table 1 and 2). However, in the presence of fresh serum, far fewer IgG opsonins were required to obtain appreciable bactericidal activity by macrophages. Therefore, based on equivalent agglutinating titers of the antibodies, in the presence of heat-labile serum factors, 1,000 times fewer IgG than IgM opsonins were required to reach an equivalent end point of bactericidal activity.

Bactericidal activity of mouse PMN. The assay described above was developed to determine the contribution of humoral factors to the bactericidal activity of the mouse PMN. Cell suspensions prepared from the peritoneal exudates of mice stimulated with Brain Heart Infusion Broth contained 60 to 70% PMN, 25 to 35% lymphocytes, and 5 to 10% macrophages. A ratio of 25 to 35 PMN per bacterium was maintained in all experiments. It was determined that the contaminating macrophages in the cell suspensions exerted no bactericidal activity.

Unlike macrophages, mouse PMN, in the presence of either IgG or IgM opsonins and heat-inactivated mouse serum, were incapable of killing the test strain of *P. aeruginosa* (Table 3). These results suggested that heat-labile serum factors were required for PMN bactericidal activity. Therefore, fresh mouse serum was substituted for the heat-inactivated mouse serum in the bactericidal assays. The results showed that the mouse PMN will function only in the presence of both opsonins and heat-labile mouse serum factors. Subsequently, IgG and IgM opsonins were compared for contribution to the bactericidal activity of the mouse PMN against *P. aeruginosa* (Table 4). Based on equivalent agglutinating titers of the antibodies, IgG opsonins were found to be 10,000 times more efficient than IgM opsonins in promoting the bactericidal activity of the mouse PMN.

It was of interest to compare the bactericidal capacities of the mouse PMN and macrophage in the presence of IgG or IgM opsonins and heat-labile mouse serum factors (Table 2 and 4). The capacities of mouse PMN and mouse macrophage to kill bacteria when opsonized with IgM antibody were about equal. The capacity of the mouse PMN to kill bacteria opsonized with IgG antibody was 10 times greater than that of the mouse macrophage.

DISCUSSION

Although numerous modifications of technique have been described for studying the bactericidal activity of PMN and macrophages from rabbit and guinea pig (6, 2, 18), the mouse systems provide an exceptionally useful tool, as the mouse is used extensively for in vivo experiments in infection and immunity.

In this study, the requirements for antibody and heat-labile mouse serum factors in intracellular killing have been determined. Although IgG and IgM opsonins alone sufficed to induce the bactericidal activity, such activity was enhanced by heat-labile mouse serum factors. In contrast, both opsonin and heat-labile mouse serum factors were required for the bactericidal activity by the mouse PMN. These observations were made with human immunoglobulins and may differ in a homologous

TABLE 4. *Contribution of purified immunoglobulins to bactericidal activity of mouse polymorphonuclear leukocytes (PMN)*

Immunoglobulin class	Dilution of immunoglobulin added to assay	No. of bacteria per ml recovered after 3-hr incubation[d]	Reduction in bacterial counts from the control[d]
Reaction mixture with PMN[a]			
IgM	10^0	3.17	1.31
	10^{-1}	3.41	1.07
	10^{-2}	3.24	1.24
	10^{-3}	3.86	0.62
IgG	10^{-1}	2.83	1.65
	10^{-2}	2.89	1.59
	10^{-3}	3.00	1.48
	10^{-4}	2.84	1.64
	10^{-5}	3.00	1.48
	10^{-6}	2.76	1.72
	10^{-7}	3.82	0.66
Control[b]		4.48	
Reaction mixture without PMN			
IgM[c]	10^0	4.60	
IgG[c]	10^0	4.20	
Hanks solution only		4.65	

[a] Reaction mixtures consisted of immunoglobulin, PMN, bacteria, and fresh mouse serum.

[b] Control consisted of Hanks solution, PMN, bacteria, and fresh mouse serum.

[c] Undiluted IgG and IgM had agglutinin titers of 1:1,024.

[d] Values expressed in $\log_{10}$. Number of bacteria in each test at 0 time was 2.5×10^4 cells/ml (expressed in $\log_{10}$, 4.40).

system in which mouse opsonins are used. This is being investigated at present. Although the complement from CF_1 mice was nonhemolytic and nonbactericidal, it is probable that the heat-labile mouse serum factors that enhanced bactericidal activities of mouse PMN and macrophages were components of complement. It has been shown recently that components of complement 1 through 4 are required for optimal phagocytosis and intracellular killing of bacteria by utilizing human PMN (10).

The opsonizing effects of IgG and IgM antibodies on the bactericidal activities of mouse macrophages and PMN were compared. In the absence of heat-labile mouse serum factors, IgG and IgM opsonins contributed to the bactericidal activity of mouse macrophages to the same extent. However, in the presence of fresh mouse serum, much less IgG than IgM antibody was required to achieve maximal bactericidal activity by these cells. Del Guercio et al. reported that guinea pig IgG and IgM anti-*Salmonella typhimurium* antibodies in the absence of complement were equally cytophilic, on a molar basis, for guinea pig macrophages (7).

In this regard, our results differ from those of Lay and Nussenzweig (12) and Rabinovitch (15), who reported that mouse complement was required for the attachment and ingestion of erythrocytes opsonized with anti-erythrocyte IgM antibodies by mouse macrophages. In our system, macrophage bactericidal activity was observed with IgM opsonins in the absence of complement. Moreover, mouse complement did not enhance the bactericidal activity. It is possible that cellular recognition of antibacterial IgM opsonins is different from that of anti-erythrocyte IgM opsonins.

IgG opsonins were found to be more efficient than IgM opsonins in enhancing the killing of *P. aeruginosa* by mouse PMN. Maximum bactericidal activity by these cells required 10,000 times less IgG opsonin than IgM opsonin. These results agree with those of Smith et al., who compared the opsonic activities of rabbit IgG and IgM anti-*Proteus* antibodies and found that IgG antibody was superior to IgM antibody in enhancing the intracellular killing of *Proteus mirabilis* by rabbit PMN (17).

The mechanisms by which mouse PMN and macrophages kill bacteria intracellularly probably differ. One investigator suggested recently that the killing mechanism of the mouse peritoneal macrophage is dependent on an intact respiratory chain (14). The bactericidal mechanism of the PMN seems to be dependent on the presence of a peroxidase (11). Although results presented in this report do not clarify which phagocytic cell type is more important in providing protection against *P. aeruginosa* infections, they do suggest that both cell types are involved. Our results do not clarify the possible multiple role of antibodies and heat-labile factors in phagocytosis and in intracellular killing. Our experiments were designed to measure only the final effect, that of microbial elimination.

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