INFECTION AND IMMUNITY, Dec. 1987, p. 3204–3206
0019-9567/87/123204-03$02.00/0

Vol. 55, No. 12

NOTES

Opsonophagocytosis of *Pseudomonas aeruginosa* Treated with Antiflagellar Serum

TINA R. ANDERSON AND THOMAS C. MONTIE*

Department of Microbiology, University of Tennessee, Knoxville, Tennessee 37996-0845

Received 22 June 1987/Accepted 13 August 1987

Hyperimmune rabbit flagellar antisera were shown to enhance opsonophagocytosis of *Pseudomonas aeruginosa* by mouse peritoneal polymorphonuclear leukocytes. This response was specific for flagellar immunoglobulin G as indicated by cross-opsonization experiments.

Phagocytosis of bacteria by polymorphonuclear leukocytes (PMNs) is enhanced by antibodies to bacterial cell envelope components. These antibodies constitute one important class of opsonins (9). With gram-negative bacteria, including *Pseudomonas aeruginosa*, complement is opsonic (5) but seems more critical in the opsonization of rough strains than it is in the opsonization of smooth clinical isolates (5, 14). In experiments with *P. aeruginosa*, the evidence favors a role for PMNs in efficient protection with cell-surface-derived antibodies (2, 4, 15). Studies to date have concentrated on polysaccharide components, including lipopolysaccharide and mucoid exopolysaccharide (2, 5, 10, 11, 14), as opsonin-generating antigens. Recently, interest has turned to evaluating some *P. aeruginosa* proteins which generate antibodies as opsonins (6, 12). Stimulation of nonopsonic phagocytosis, particularly in cystic fibrosis isolates, may involve the presence of pili and other attachment factors (13).

Our studies have focused on protection derived from flagellum-stimulated antibody. It has been shown that the major types of flagellins are a homologous b type (53 kilodaltons) and a heterologous a type (45 to 52 kilodaltons), with possible subtypes a_0, a_1, a_2, a_3, and a_4 (1). Both types of flagellum elicit immunoglobulin G antibodies in rabbits, and these antibodies provide flagellum-specific protection in two burned-mouse models (D. Drake and T. C. Montie, Can. J. Microbiol., in press). Recent enzyme-linked immunosorbent assay data show that the a-type strains tested, including strains with different subtypes, show a dominant cross-reacting common epitope (T. C. Montie and T. R. Anderson, submitted for publication).

It has been suggested, from passive immunization experiments with flagellar antisera, that treatment with cyclophosphamide of mice already immunosuppressed by burn injury severely reduces the protective effectiveness of hyperimmune antisera (Drake and Montie, in press). Depression of PMN levels therefore apparently contributes to loss of protection, and this information suggests that flagellar antibody may contribute to opsonization of serum-resistant, virulent challenge strains. To test this hypothesis, we isolated mouse peritoneal PMNs and examined the opsonizing capacity of hyperimmune serum. The results of these studies are reported here.

* Corresponding author.

Six smooth strains of *P. aeruginosa* were used. Their serotypes were determined according to the International Antigenic Typing System system. Flagellar-antigen type was designated by the method of Ansorg (3), by M_rs of the flagellin (1), and by enzyme-linked immunosorbent assay (Montie and Anderson, submitted). Strain PAO1 (b type) and strain AK1152, an ethyl methanesulfonate Fla^- mutant of PAO1 used for removing antibodies to somatic antigens, were from A. Kroprinski, Queen's University at Kingston, Ontario, Canada. Strains SBI-N and 1210 (a types) and M-2 (b type) were provided by I. A. Holder, Shriners Burn Institute, Cincinnati, Ohio. Strain 170018 (a type) was obtained from B. Lanyi, National Institute of Hygiene, Budapest, Hungary. Stocks were maintained at 4°C as dilute suspensions in brain heart infusion broth. Bacteria were grown in brain heart infusion broth at 37°C for 15 h, harvested by centrifugation at 4,100 × *g* for 15 min at 25°C, washed twice with 0.85% saline, and suspended at 10^8 bacteria per ml in Hanks balanced salt solution with 0.1% gelatin, unless otherwise noted.

Fetal calf serum was purchased from HyClone Laboratories, Logan, Utah. Rabbits were prebled for normal rabbit serum. Antisera to crude flagellar antigens from strains M-2, 1210, and 170018 were raised in female New Zealand rabbits as previously described (1; Drake and Montie, in press). To remove antibodies to somatic antigens, serum samples were adsorbed on Formalin-killed and lyophilized *P. aeruginosa* AK1152 (Fla^-) or heat-treated (100°C, 1 h) strain M-2 cell suspensions. Adsorbed antisera were shown to consist predominantly of immunoglobulin G antibodies and were free of reactivity to lipopolysaccharide O antigen (Drake and Montie, in press). Antisera typically had a titer by enzyme-linked immunosorbent assay of >1/128,000 against homologous flagella. Rabbit serum was frozen in portions at −70°C.

For preparation of PMNs, female CF1 mice were injected intraperitoneally with 2 ml of 10% Proteose Peptone (Difco Laboratories, Detroit, Mich.) 18 h before harvest to elicit PMN production. Animals were sacrificed by CO_2 asphyxiation, injected intraperitoneally with 4 to 5 ml of Hanks balanced salt solution, and massaged, and the intraperitoneal fluid was removed. Cells were pelleted by centrifugation at 110 × *g* for 10 min at 25°C and suspended at a final concentration of 10^7 PMN per ml in RPMI medium (GIBCO Laboratories, Grand Island, N.Y.) with 5% fetal calf serum. Viability, as measured by trypan blue exclusion, was typi-

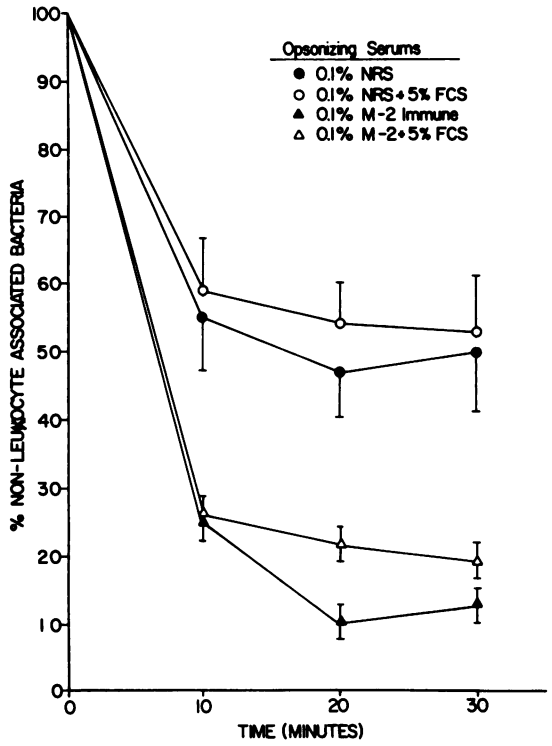

FIG. 1. Opsonizing capacity of antiflagellar rabbit serum. NRS, Normal or preimmune rabbit serum; FCS, fetal calf serum.

cally 95%. Cytocentrifuge smears, prepared by centrifuging preparations at 700 rpm for 5 min in a Shandon cytocentrifuge and staining with Wright stain, indicated 73% PMNs and 27% macrophages. Bacteria were opsonized by the addition of 0.1% serum and incubation for 30 min at 37°C on a rotary shaker at 175 rpm. Enumeration of bacteria prior to and after opsonization showed that bacteria were not lysed by serum. Equal volumes of opsonized bacteria in Hanks balanced salt solution with 0.1% gelatin and PMNs in RPMI medium with 5% fetal calf serum were mixed at time zero to give a ratio of 10 bacteria to 1 PMN. Prior to mixing, a sample of bacteria was taken, serially diluted, and plated to determine the number of bacteria at time zero. Cultures were incubated at 37°C on a rotary shaker at 175 rpm. At various times, portions were mixed with an equal volume of ice-cold Hanks balanced salt solution and centrifuged at 130 × *g* for 7 min at 25°C. The supernatant was diluted appropriately with 0.85% saline and plated on brain heart infusion agar plates for bacterial enumeration. Plates were incubated overnight at 37°C, and bacterial colonies were counted.

Experiments were performed to compare the opsonizing capacity of flagellar hyperimmune serum with that of normal rabbit serum (Fig. 1). The maximum number of cell-associated bacteria was recorded after 10 to 20 min. The hyperimmune serum showed greater opsonizing activity, since 75% of bacteria treated with antiflagellar serum was removed by PMNs, compared with 45% of bacteria opsonized by normal rabbit serum. The results were essentially the same whether or not fetal calf serum was present during opsonization. Similar results were obtained in three replicate experiments. These data are consistent with results showing that flagellar antigen or antibody is highly protective in a burned-mouse model (7, 8; Drake and Montie, in press). The opsonizing properties of hyperimmune serum would also explain the lack of invasion of burn wound bacteria into the blood and organ systems of passively immunized animals (Drake and Montie, in press).

TABLE 1. Opsonic activity related to homology between flagellar antigen and antiserum type[a]

Expt	Bacterial strain (type)	Opsonizing serum (type)	% Leukocyte associated
1	PAO1 (b)	Normal rabbit serum	5
		M-2 (b)	89
		170018 (a_0, a_3, a_4)	25
2	SBI-N (a)	Normal rabbit serum	25
		M-2 (b)	22
		1210 (a_0, a_1, a_2)	76

[a] Incubation times for experiments 1 and 2 were 10 and 15 min, respectively. Analyses by Student *t* test of the mean value differences between M-2 and 170018 (experiment 1) and between M-2 and 1210 (experiment 2) gave significant *t* values beyond the 0.001 level.

To ascertain the specificity of flagellar antibody in enhancing phagocytosis, antisera specific for each of the two flagellar types, a and b, were compared (Table 1). In experiment 1, b-type strain PAO1 was mixed with either an a-type or a b-type serum for opsonization. Opsonic activity for strain PAO1 treated with b-type flagellar antiserum was over twice that obtained for bacteria treated with an a-type-specific serum (heterologous). Analogous data were obtained with a-type bacteria (strain SBI-N) treated with an a-type-specific antiserum.

Although *P. aeruginosa* may be isolated in the O-antigen-deficient form from infected patients with cystic fibrosis or in patients exhibiting chronic infections, bacteremic isolates are generally serum-resistant, smooth strains containing O antigen. The focus of these experiments, therefore, was on opsonophagocytosis of bacteremic type strains. Initial experiments involved high concentrations of antiserum in the opsonizing system. However, bacteria aggregated, and results were therefore difficult to interpret. When antiserum was diluted 1,000-fold, opsonization was retained without aggregation interference.

These experiments constitute the first report of the opsonizing capability of flagellar antisera. That the activity observed was due to flagellar antibody was reinforced by the data in Table 1, which show that serum (a or b type) will opsonize only the bacterium of the corresponding flagellar type. It is notable that the antiserum to strain 1210 flagellar immunotype (a_0, a_1, a_2) opsonized the incompletely characterized a-type strain SBI-N. Recently, the above-mentioned results have been confirmed by a radiolabeled assay. Results from preliminary opsonophagocytosis experiments with [^{3}H]thymidine-labeled bacteria showed that bacteria opsonized with homologous antiserum bound to phagocytes sevenfold more than did normal rabbit serum controls (T. C. Montie, T. R. Anderson, and H. Sellin, manuscript in preparation). The opsonization results are in complete agreement with data demonstrating 100% protection against topical challenge of the human burn isolate, strain SBI-N, by strain 1210 flagellum-specific antiserum as well as antiserum to flagella from a second clinical a-type isolate, strain 7191 (Montie et al., in preparation). Neither of these a-type strains, SBI-N and 7191, has been completely characterized with respect to a subtype, but both demonstrate strong cross-reactivity in enzyme-linked immunosorbent assay (Montie and Anderson, submitted). These data are consistent with the proposal that only two antigens are needed for a potential vaccine, i.e., one a type and one b type.

This work was supported by a contract from the U.S. Army Medical and Development Command.

We thank Lee Coker, Steve Foster, and Robert N. Moore for their very helpful aid and advice on the isolation of mouse PMNs and Helen Sellin for her helpful discussions.

LITERATURE CITED

1. **Allison, J. S., M. Dawson, D. Drake, and T. C. Montie.** 1985. Electrophoretic separation and molecular weight characterization of *Pseudomonas aeruginosa* H-antigen flagellins. Infect. Immun. **49**:770–774.
2. **Ames, P., D. DesJardins, and G. B. Pier.** 1985. Opsonophagocytic killing activity of rabbit antibody to *Pseudomonas aeruginosa* mucoid exopolysaccharide. Infect. Immun. **49**:281–285.
3. **Ansorg, R.** 1978. Flagella specific H antigenic schema of *Pseudomonas aeruginosa*. Zentralbl. Bacteriol. Parsitenkd. Infektionskr. Hyg. Abt. 1 Orig. Reihe A **242**:228–238.
4. **Cryz, S. J., Jr., E. Fürer, and R. Germanier.** 1983. Simple model for the study of *Pseudomonas aeruginosa* infections in leukopenic mice. Infect. Immun. **39**:1067–1071.
5. **Engels, W., J. Endert, M. A. F. Kamps, and C. P. A. van Boven.** 1985. Role of lipopolysaccharide in opsonization and phagocytosis of *Pseudomonas aeruginosa*. Infect. Immun. **49**:182–189.
6. **Hancock, R. E. W., L. M. Mutharia, and E. C. A. Mouat.** 1985. Therapeutic potential of monoclonal antibodies against *Pseudomonas aeruginosa* protein F. Eur. J. Clin. Microbiol. **4**:224–227.
7. **Holder, I. A., and J. Naglich.** 1986. Experimental studies of the pathogenesis of infections due to *Pseudomonas aeruginosa*: immunization using divalent flagella preparation. J. Trauma **26**:118–122.
8. **Holder, I. A., R. Wheeler, and T. C. Montie.** 1982. Flagellar preparations from *Pseudomonas aeruginosa*: animal protection studies. Infect. Immun. **35**:276–280.
9. **Horowitz, M. W.** 1982. Phagocytosis of microorganisms. Rev. Infect. Dis. **4**:104–123.
10. **Pier, G., and D. M. Thomas.** 1983. Characterization of the human immune response to a polysaccharide vaccine from *Pseudomonas aeruginosa*. J. Infect. Dis. **148**:206–213.
11. **Pier, G. B.** 1982. Safety and immunogenicity of high molecular weight polysaccharide vaccine from immunotype 1 *Pseudomonas aeruginosa*. J. Clin. Invest. **69**:303–308.
12. **Pollock, M.** 1983. Antibody activity against *Pseudomonas aeruginosa* in immune globulin prepared for intravenous USP in humans. J. Infect. Dis. **147**:1090–1098.
13. **Speert, D. P., B. A. Loh, D. A. Cabral, and I. E. Salit.** 1986. Nonopsonic phagocytosis of nonmucoid *Pseudomonas aeruginosa* by human neutrophils and monocyte-derived macrophages is correlated with bacterial piliation and hydrophobicity. Infect. Immun. **53**:207–212.
14. **Vreede, R. W., J. H. Marcelis, and J. Verhoef.** 1986. Antibodies raised against rough mutants of *Escherichia coli* and *Salmonella* strains are opsonic only in the presence of complement. Infect. Immun. **52**:892–896.
15. **Young, L. S.** 1972. Human immunity to *Pseudomonas aeruginosa*. II. Relationship between heat-stable opsonins and type-specific lipopolysaccharides. J. Infect. Dis. **126**:277–287.